Factors Influencing Incorporation of Mevalonic Acid into Cholesterol by Rat Liver Homogenates.* (23436)

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A number of studies have established unequivocally that acetate is a precursor of cholesterol. Several other compounds, including pyruvic, acetoacetic, dimethyl acrylic, hydroxy methyl glutaric acids, farnesol, farnesinic acid, and squalene have been suggested as intermediates in the biogenesis of the sterols. Recently the discovery(1), isolation(2), characterization and synthesis(3,4) of a new compound, mevalonic acid (beta-methyl-beta, delta-dihydroxy-valeric acid), which is converted to cholesterol by rat liver homogenates (5) in relatively high yield was announced. Several isotope labeling experiments have now established a mechanism by which the "head to tail" condensation of mevalonic acid units to yield dihydroisoprene chains may be visualized(6,7,8,9). During the course of studies on incorporation of mevalonic acid into cholesterol by rat liver homogenates, a number of conditions and compounds have been observed which influence the extent of the conversion. These findings may be useful to those concerned with sterol biosynthesis.

Methods and materials. The biosynthetic experiments involving conversion of mevalonic acid (MVA) to cholesterol were carried out with liver homogenates by the procedures of Bucher(10,11) as modified by Rabinowitz and Gurin(12) and by Tavormina *et al.*(5,6). Unless specified otherwise, livers from young rats (either sex, less than 150 g in weight, preferably 70-80 g) were rapidly removed following death by decapitation. The livers were cooled in a beaker of ice water. After 5 min the livers were suspended in a small amount of homogenizing buffer and cut into small pieces with scissors. Buffer was then added to a total volume (liver and buffer) equal to 3 times the volume of liver. The mixture was then ground with a loose-fitting Potter-Elvehjem homogenizer (2-3 excursions of the plunger). As is the case with acetate incorporation, incorporation of MVA into cholesterol is also markedly influenced by the clearance between the plunger and bowl of the homogenizer and, as pointed out by Bucher, with acetate, for high incorporation a homogenizer especially ground with a clearance of 0.5 mm should be used. Combined homogenates were centrifuged in a clinical centrifuge at 1500 rpm for 3 minutes and the supernatant (including an intermediate layer) decanted. Aliquots of this suspension were used as the enzyme source. The incubations were carried out in 125 ml Erlenmeyer flasks containing 5 ml of homogenate, 1 mg of ATP, 1 mg of DPN, 1 mg 2-C^{14}-MVA calculated as the DL dibenzylethylenediamine (DBED) salt from which the dibenzylethylenediamine had been removed by extraction with ether from alkaline solution, 1-2 ml of test compound or water, all in a total volume of 7-8 ml. Two preparations of 2-C^{14}-MVA have been used, one (Prep A) containing 4,000 cpm/mg, the other (Prep B), 15,500 cpm/mg. The flasks were then aerated in a stream of oxygen for 25 seconds, stoppered, and incubated for 4½ hours at 37° in a reciprocating water bath (50 oscillations per minute). Operations prior to incubation were carried out at 5°C. Where microbiological assays for MVA in the enzyme experiments were carried out, 0.1 ml aliquots were removed at the end of the experiment, diluted to 10 ml and heated at 100° for 10 min. to yield inactivated preparations. The term "mevalonic acid, % utilization" used in the tables refers to the per cent of added mevalonic acid "used up" during the enzyme reaction as determined by microbiological assay. The incubation mixtures were saponified, the cholesterol extracted with petroleum ether, precipitated with digitonin, the precipitate washed successively with 85% ethanol, acetone-ether(1:2), and finally with ether. The precipitates were suspended in petroleum

* This investigation was supported by a research grant from the National Science Foundation.

TABLE I. Effect of Age of Rats and Aeration Conditions on Incorporation of Mevalonic Acid into Cholesterol.

Exp.	Size of rats, g	Aeration	Cholesterol, cpm/mg C	Mevalonic acid, % utilization
1	46	Oxygenated and stoppered	1500	100
	46	Open to air	552	100
	339	Oxygenated and stoppered	912	100
	339	Open to air	469	71
2	65	Oxygenated and stoppered	1947	100
	113	*Idem*	376	100
	210	"	699	100
	400	"	159	68

The experiments carried out with a "loose" homogenizer. The 1 mg of MVA (calculated as the DL DBED salt), used as labeled precursor, contained 15,500 cpm (Prep. B).

ether-acetone-ethanol (1:1:1) and plated. Counts were obtained with a gas-flow counter. The activity of the isolated cholesterol is expressed in terms of counts per minute per mg of cholesterol carbon corrected to infinite thinness with a self absorption curve prepared from cholesterol digitonide. Duplicate flasks were used for each experimental condition: variation between duplicates was usually less than 5%.

Microbiological determinations of MVA were carried out with *Lactobacillus acidophilus* (*bifidus*) ATCC 4963 essentially as described by Skeggs *et al.*(1). MVA as the standard was used in the form of the DL DBED salt. Incubation was at 37° for 18 to 24 hours.

The farnesol, squalene, and hydroxy methyl glutaric acid used were commercial preparations. Three samples of farnesinic acid were studied: #1†, prepared from geranylacetone by the Reformatsky reaction; #2‡, and #3§, prepared from nerolidol. Emulsions of farnesol, farnesinic acid, or squalene were prepared in 1% gelatin by dispersion with a tight-fitting homogenizer. Solutions of farnesinic acid were prepared by suspending the compound in water and adding the minimum amount of dilute sodium hydroxide required to affect solution. Hydroxy methyl glutaric acid was dissolved in water and the solution neutralized with dilute sodium hydroxide.

Results. Effect of age. Bloch *et al.*(13) first observed a correlation of activity with age in the incorporation of acetate into cholesterol by rat liver slices. Subsequent investigators in the field have usually specified the use of "young rats," although the use of "adult rats" has sometimes been described. The present biosynthetic experiments were carried out with rats varying in size from 46 g to 400 g. The liver from very young rats is much more active synthetically than that from older rats (Table I). Similarly, microbiological assays for unmetabolized MVA indicated that this factor is completely utilized by homogenates from younger rats, but utilization is not complete with homogenates from older rats.

The present studies indicate that the "dividing line" between high and low incorporation may be about 100 g. Since the same correlation of age with synthetic activity exists with MVA as with acetate, it is suggested that if there is a limiting reaction influenced by age rather than by a general decline in metabolism, this reaction must be between MVA and cholesterol rather than between acetate and MVA.

Effect of method of killing. It has been suggested(14) that rats killed by dislocation of the cervical vertebrae may yield homoge-

† We are indebted to Drs. W. A. Bolhofer and J. M. Sprague, Merck Sharp and Dohme Laboratories, West Point, Pa., for this compound.

‡ We are indebted to Dr. Gilbert Stork, Department of Chemistry, Columbia University, New York City, for this compound.

§ We are indebted to Drs. Frank Dituri and Samuel Gurin, Department of Physiological Chemistry, University of Pennsylvania, Philadelphia, for this compound.

TABLE II. Effect of Method of Killing and Aeration Conditions on the Incorporation of Mevalonic Acid into Cholesterol.

Method of killing	Aeration	Cholesterol, cpm/mg C
Dislocation of vertebrae	Oxygenated and stoppered	1445
Idem	Open to air	474
Decapitation	Oxygenated and stoppered	2035
"	Open to air	746

The experiment was carried out with a "loose" homogenizer. The 1 mg of MVA (calculated as the DL DBED salt), used as labeled precursor, contained 15,500 cpm (prep. B).

nates more active than those killed by decapitation. However, under the present conditions rats killed by decapitation yield homogenates at least equal in activity (Table II).

Effect of cofactors. Rabinowitz and Gurin have demonstrated(12) that with acetate as a labeled precursor, ATP (or AMP), DPN, and coenzyme A are required for synthesis of cholesterol by *fractionated* rat liver homogenates. Amdur *et al.* have shown(7) a requirement for ATP, DPN (or TPN or their reduced forms), but not for coenzyme A for synthesis of squalene from MVA by a yeast enzyme preparation. In the present studies with whole homogenates no requirement for added ATP could be demonstrated, although DPN is stimulatory (Table III).

Effect of substrate level. Various levels of labeled substrate from 0.1 mg to 1.0 mg MVA per flask have been tested (Table IV). With an active enzyme preparation derived from the livers of very young rats by the use of a "loose" homogenizer, the incorporation of MVA into cholesterol is essentially a straight line function of the amount of added substrate. With a less active preparation from older rats prepared with a "tight" homogenizer, incorporation is approximately the same at the lowest level, but the extent of incorporation rapidly falls off with increasing substrate level. Despite the fact that with an active enzyme preparation MVA utilization is invariably complete as determined by microbiological assay of the digests at the conclusion of an experiment, it is estimated that under the most favorable conditions studied probably no more than 25-35% of added DL MVA appears in cholesterol.

Effect of oxygenation. Oxygen is required for biosynthesis of cholesterol with the usual enzyme preparations. Under anaerobic conditions the synthesis stops at squalene(15). It was found (Tables I and II) that cholesterol synthesis is much more extensive in flasks where the homogenate was swirled in a stream of oxygen and then stoppered than in

TABLE III. Effect of Cofactors on Incorporation of Mevalonic Acid into Cholesterol.

Exp.	Homogenizer	Substrate activity, cpm	System	Cholesterol, cpm/mg C
1	Tight	4,000 (Prep. A)	Complete	300
			Less ATP	312
			" DPN	236
			" ATP and DPN	232
2	Loose	15,500 (Prep. B)	Complete	1831
			Less ATP	1756

TABLE IV. Effect of Substrate Level on Incorporation of Mevalonic Acid into Cholesterol.

Exp.	Homogenizer	Size of rats, g	Substrate Level, mg	Substrate Activity, cpm	Cholesterol, cpm/mg C
1	Tight	150	.1 Prep. A	400	64
			.2	800	95
			.5	2,000	134
			1.0	4,000	144
2	Loose	70	.1 Prep. B	1,550	308
			.2	3,100	603
			.5	7,750	1365
			1.0	15,500	2395

TABLE V. Effect of Various Analogs on Incorporation of Mevalonic Acid into Cholesterol.

Exp.	Homogenizer	Substrate activity, cpm	Analog	Cholesterol cpm/mg C	Cholesterol % reduction	Mevalonic acid, % utilization
1	Tight	4,000 (Prep. A)	None	171		94
			Farnesol, 4 mg (emulsion)	20	88	85
			Farnesinic acid #1, 4 mg (")	0	100	64
			Squalene, 4 mg (")	133	22	90
2	"	*Idem*	None	485		98
			Farnesol, 4 mg (")	68	86	98
			Farnesinic acid #1, 4 mg (")	0	100	94
			Squalene, 4 mg (")	384	21	98
3	"	"	None	80		58
			Farnesinic acid #1, 1 mg (solution)	3	96	36
			Hydroxy methyl glutaric acid, 1 mg (")	80	0	58
4	"	15,000 (Prep. B)	None	209		
			Farnesinic acid #1, 2 mg (")	10	95	
			" " #2, 2 " (")	13	94	
			" " #3, 2 " (")	11	95	
5	Loose	*Idem*	None	2000		
			Farnesinic acid #1, 1 mg (")	1352	32	
			" " #2, 1 " (")	1349	32	
			" " #3, 1 " (")	1022	48	

flasks that were not oxygenated but were left open to the air. Whether or not the results obtained are a function of oxygen tension alone has not been determined.

Effect of various postulated cholesterol intermediates. Bloch(16) observed that when squalene, 7-dehydrocholesterol, Δ 7-cholestenol, or cholesterol itself is incubated with rat liver slices along with C^{14}-acetate, the counts found in isolated cholesterol are markedly reduced. On the other hand, incubation with a number of other sterols, farnesol, farnesal, farnesinic acid or nerolidol did not influence the cholesterol counts. He interpreted these findings to mean that 7-dehydrocholesterol, Δ 7-cholestenol, and squalene are preferentially incorporated into cholesterol over acetate and are therefore intermediates in cholesterol biogenesis. The negative results obtained with the second group of compounds were interpreted as indicating that these compounds are not intermediates. Using essentially the same technic except that fractionated rat liver homogenates rather than liver slices were employed and the labeled precursor was a biologically-active impurity contained in a preparation of beta-hydroxy-beta-methyl glutaric acid, Dituri *et al.*(17) found that the incubation of labeled precursor with farnesol or farnesinic acid led to the isolation of squalene markedly less active than that found in the absence of either of these compounds. Added carrier farnesinic acid was re-isolated from the incubation mixtures and was found to contain a small but detectable amount of label. No label was found, however, with carrier farnesol. These investigators concluded that farnesol and farnesinic acid are intermediates in the biosynthesis of squalene. Evidence that farnesinic acid is not a precursor of cholesterol has been presented by Sandermann and Stockmann(18), who synthesized C^{14}-labeled farnesinic acid by the Reformatsky reaction from geranylacetone and found that the compound is not a precursor of cholesterol in rat liver. Insufficient data are given, however, for a critical evaluation of the work.

In the present studies, when farnesol, farnesinic acid (all 3 samples), or squalene is incubated with rat liver homogenates along with 2-C^{14}-MVA, the counts found in cholesterol isolated from the digests are markedly reduced over the values found in the cholesterol from control digests. (Table V). Similarly, MVA utilization, as determined by microbiological assay of the factor remaining after the period of synthesis, was reduced by the

analogs. Of the 3 compounds studied, farnesinic acid is the most active. The effect was observed both when farnesinic acid was present in an emulsion and when it was present in true solution as the sodium salt. The effect observed with farnesinic acid was demonstrable both with a "highly active" enzyme preparation and with "less active" preparations. Farnesol and squalene were less active than farnesinic acid. Neither of the latter 2 compounds could be studied in aqueous solution. Hydroxy methyl glutaric acid, at least in comparable amounts, was found to be inactive with respect to both cholesterol biosynthesis and MVA utilization.

It is appreciated that a variety of interpretations may be applied to the results obtained with these analogs since a similar effect in influencing the incorporation of mevalonic acid would be expected with a compound that is either a precursor or an antimetabolite. With respect to squalene, independent evidence that squalene is a precursor of cholesterol seems so overwhelming that the effect observed here probably means that squalene competes with MVA for incorporation into cholesterol. The results obtained with squalene, therefore, are probably attributable to a precursor effect and the role of squalene in biogenesis of cholesterol is not questioned. As far as farnesinic acid is concerned, the influence of the compound on acetate incorporation is controversial, but the counts found in squalene from the impurity contained in hydroxy methyl glutaric acid or in cholesterol from MVA in the present work are markedly reduced by the compound. In the absence of labeled analog it is impossible with the liver technic employed to determine whether a metabolite or antimetabolite effect is operative. Separate studies have shown that in a system involving utilization of MVA by *Lactobacillus acidophilus* farnesinic acid is a definite antimetabolite(19). The fate of MVA in the lactobacillus is at present unknown and it is conceivable that farnesinic acid is a metabolite in one system and an antimetabolite in another. Until more conclusive results are obtained, it seems reasonable to question the role of farnesinic acid as an obligatory intermediate in cholesterol biosynthesis. The data obtained with farnesol are more difficult to interpret. While the work described herein was nearing completion, it was reported(20) that the feeding of farnesol to rats failed to influence the cholesterol content of the liver, nor did the prior administration of farnesol influence cholesterol synthesis from administered 1-C^{14}-acetate. On the other hand, the feeding of squalene led to the accumulation of excess cholesterol in the liver and less synthesis of cholesterol from administered acetate. Possibly farnesol is a weak antimetabolite of cholesterol biosynthesis.

Summary. Incorporation of mevalonic acid into cholesterol by rat liver homogenates is favored by (a) use of tissues from very young rats, (b) use of a "loose" homogenizer, and (c) saturation of the homogenate with straight oxygen and stoppering. Incorporation of mevalonic acid is less in the presence of compounds that may be intermediates or antimetabolites of cholesterol biosynthesis. With whole homogenates biosynthesis is not markedly influenced by the method of killing the rats or by additions of ATP.

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Received July 8, 1957. P.S.E.B.M., 1957, v96.

Cataracts Following Mumps Virus in Early Chick Embryos.* (23437)

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Clinical reports dealing with the effect on the human fetus of German measles (rubella) in the mother, stressed the occurrence of cataracts along with heart damage, deafness, mental retardation and other defects. Other viruses have been suspected of being etiologically related to congenital defects and mumps virus has featured prominently among reports of such cases(1,2). Experimental work on the developing chick embryo in this laboratory has shown that infection with Newcastle disease and influenza A viruses resulted in specific damage to the developing lens, the extent depending on method of inoculation, stage of development of the embryo and virus titer of the inoculum(3-7). While these effects were definite and have been amply confirmed, these viral infections in the embryo were lethal within 48 hours. More recently, inoculation of the developing chick embryo with the Enders strain of mumps virus produced effects which were compatible with survival of many of the embryos for 3 to 15 days after inoculation and prominent in the results was the presence of cataracts.

Materials and methods. Virus preparations. The Enders strain of mumps virus, obtained from the American Type Culture Collection, was inoculated in 10^{-3} dilution into the allantoic cavities of 8-day chick embryos. The infectious allantoic fluids, harvested after incubation at 35°C for 7 days, served as stock virus suspension. In order to assure a high titered preparation, the fluid from each egg was harvested separately and tested for hemagglutinating activity at 1-100, 1-500 and 1-1000 dilutions. Much variation was observed, ranging from no hemagglutination to a titer of 1-1000. Fluids showing titers of 1-500 or more were pooled and dispensed in ampoules for storage at −70°C. For inoculating into early chick embryos the suspensions were diluted in buffered saline (pH 7.2) to the required infectivity. In some preparations 500 units of penicillin and 100 μg of streptomycin per ml were added to control bacterial growth. However, earlier mortality was noted when antibiotics were added to the mumps virus inoculum. In most instances, therefore, no antibiotic was added so as to eliminate the possibility that changes in the embryo were the result of a possible synergistic action of antibiotics and virus.

Infectivity titrations. Infectivity titers were determined by inoculating 10-fold dilutions of the virus suspensions into the allantoic cavities of respective groups of 8-day-old embryos. These were tested after 7 days for virus growth as indicated by hemagglutinability of the allantoic fluids. The ID_{50} was calculated by the method of Karber(8). Young embryos to be tested for rise in virus titer were harvested whole, and ground in a Ten Broeck tissue grinder. A portion of tissue was then measured by volume and sufficient

* This investigation was supported by a research grant from the Microbiological Institute, Public Health Service.