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Effect of mevalonate analogues upon cholesterol biosynthesis

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SUMMARY

Three analogues of mevalonic acid have been found to inhibit cholesterol biosynthesis in liver homogenates in the following order of efficacy: 3-methyl-3-hydroxy pentanoic acid > Δ^2-3-methyl pentenoic acid > Δ^3-3-methyl pentenoic acid. The hydroxy acid produces half the maximum inhibition at a concentration of 5 μmoles/ml of homogenate. This corresponds to a ratio of 100 μmoles of inhibitor to 1 μmole of substrate.

The presence of cholesterol-containing plaques in atherosclerotic lesions has stimulated studies of compounds that inhibit the biosynthesis of the sterol. Blohm and MacKenzie (1) have reported that the oral administration of Triparanol (MER-29), whose site of action has recently been determined (2), lowers both serum and tissue levels of cholesterol.

Major efforts in the search for compounds which antagonize cholesterol biosynthesis have been undertaken as a result of work showing that isoprenoids are intermediates in cholesterol formation. Thus, when β,β'-dimethyl acrylyl-CoA was considered at one time to be a major isoprenoid intermediate, analogues of this compound were tested both *in vivo* (3, 4) and *in vitro* (5) for their effect upon cholesterol biosynthesis. The demonstration that mevalonic acid (6) is an important precursor of isoprenoid units had led to the testing of analogues of this compound.

Stewart and Woolley (7), Tamura *et al.* (8), and Wright (9) have screened a variety of compounds as mevalonate antagonists in certain lactobacilli which require the acid for growth.

Using rat liver homogenates, Singer *et al.* (10) have shown that fluoromevalonic acid inhibits the formation of cholesterol from both C^{14}-labeled acetate and mevalonic acid.

Mentzer *et al.* (3) have reported that liver cholesterol levels in rats are lowered by 30% after the oral administration of Δ^3-3-methyl pentenoic acid while the Δ^2 isomer is ineffective. These compounds are structurally similar to β,β'-dimethylacrylic acid.

* Deceased August 20, 1960.

Gey *et al.* (5) have found that both the Δ^3 compound and Δ^4-3-methyl-3-hydroxy-pentenoic acid reduce the incorporation of C^{14}-acetate into cholesterol in rat liver homogenate.

Supniewski *et al.* (4) studied the effect of the Δ^3 compound in pigeons maintained on an atherosclerogenic diet. The intramuscular administration of this material reduced aortic lesions which had developed during cholesterol feeding. Given together with the atherosclerogenic diet, the compound prevented the formation of sclerotic nodules.

The reportedly greater effectiveness of the Δ^3 acid compared to the Δ^2 acid and the structural similarity of the Δ^3 acid to mevalonic acid were considerations which led to the present work. These efforts involved a study of the inhibitory effects upon cholesterol biosynthesis from mevalonic acid-2-C^{14} *in vitro* by three compounds: the Δ^2 acid, Δ^3 acid, and 3-methyl-3-hydroxy-pentanoic acid. We have found that the hydroxy acid is the most effective compound.

METHODS

Materials. Digitonin was obtained from Fisher Scientific Co.; ATP and DPN were purchased from Sigma Chemical Co. The dibenzylethylenediamine salt of mevalonic acid-2-C^{14} was obtained from Isotope Specialties Co. The compounds tested for inhibitory properties were prepared by synthetic procedures to be described.

Methods. The amine salt of C^{14}-mevalonate was converted to an aqueous solution of potassium mevalonate as follows: An aqueous solution of the amine

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salt was adjusted to pH 10 with 2 N KOH. The free amine was extracted into ether, and the aqueous solution was adjusted to pH 7 with 0.1 N HCl. The solution was diluted to a final concentration of 0.2 μmole of mevalonate/0.1 ml. The number of counts per 0.1 ml was 5.4×10^4 cpm. Since only the L-isomer of the mevalonate forms cholesterol, the effective concentration is 0.1 μmole/0.1 ml containing 2.7×10^4 cpm. One-tenth milliliter of this solution was routinely added to each sample of liver homogenate.

A solution was prepared consisting of 22.5 g of KOH and 20 mg of cholesterol dissolved in 100 ml of 90% ethanol. This was used for the hydrolysis of cholesterol esters and for subsequent isolation of the total cholesterol. The phosphate buffer used was that of Bucher (11) with sucrose omitted.

Preparation of Biological Material. The general procedures of Bucher and McGarrahan (12) were followed. Sprague-Dawley rats were killed by cervical dislocation, their livers perfused *in situ* with cold buffer, excised, and homogenized. The homogenates were centrifuged at 0° for 10 minutes at 600 × *g*. The supernatant was used for incubations.

Each incubation mixture contained 2 ml of homogenate, to which was added 1 mg of ATP, 2 mg of DPN, 0.1 μmole of 1-mevalonate-2-C^{14}, and specified concentrations of inhibitory compounds. The homogenate with added cofactors was allowed to come to 38° in a Dubnoff shaker. Substrate and inhibitors were then added, the samples gassed with 95% O_2-N_2 for 1 minute, and then incubated for specified times.

Isolation of Cholesterol. The reaction was terminated by pouring the sample into an 18 mm × 150 mm Pyrex test tube containing 4 ml of KOH reagent. Cholesterol was isolated according to a procedure of Frantz and Bucher (13).

Each sample was heated overnight in a sealed tube at 85° in order to saponify esterified sterol. Cholesterol was removed from the hydrolysis mixture by three extractions into 10 ml of ether. The extracts were evaporated in 40-ml centrifuge tubes under a stream of nitrogen at 60° and the residues were triturated in a bath at 60° with 2 ml of alcohol-acetone 1/1 (v/v). The tubes were centrifuged, and the whole supernatant was transferred to 12-ml centrifuge tubes. To each sample 1 ml of 0.5% digitonin in 50% ethanol solution was added, and the mixture was chilled overnight. The resulting digitonide was plated on 2.4-cm circles of Whatman No. 42 filter paper and then washed with cold 50% ethanol solution. Each sample was dried, weighed, and then counted with a model M-5 Nuclear Chicago gas flow counter. All counting data were corrected to "infinite thinness."

Synthesis of Analogues of Mevalonic Acid. Figure 1 outlines the synthesis of the analogues of mevalonic acid. The general procedures of Kon and Linstead (14) were followed.

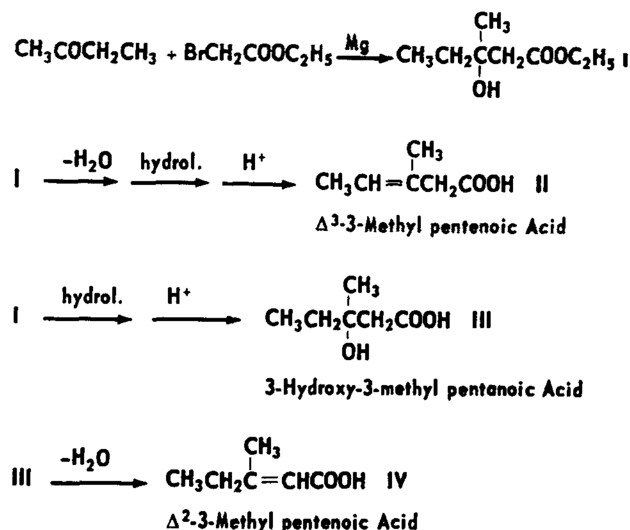

FIG. 1. Outline of synthesis of analogues of mevalonic acid. Compounds *II*, *III*, and *IV* were tested *in vitro*.

A Reformatsky reaction between methyl-ethyl ketone and ethyl bromoacetate was performed in the presence of magnesium with benzene as solvent. The resulting hydroxy ester was dehydrated by heating in the presence of $KHSO_4$. This procedure gave the unsaturated ester, which in turn was hydrolyzed in base to give the Δ^3 acid. Some hydroxy ester was hydrolyzed in alcoholic KOH to give the hydroxy acid. The latter compound was converted to the Δ^2 acid by heating it in the presence of acetic anhydride.

Analytical values and physical constants for the analogues are given below:

Δ^3-3-Methyl pentenoic acid

Anal. Calcd. for $C_6H_{10}O_2$: C, 63.11; H, 8.84. Found: C, 62.87; H, 8.66.

Constants: b.p., 55°–56.5° (0.03 mm Hg). n_D^{20}, 1.4471 (reported 1.4469 in Ref. 14). R_f, 0.71, in the chromatographic system (15): *n*-amyl alcohol-water-formic acid, 20:12:1, descending on Whatman No. 1 paper.

Δ^2-3-Methyl pentenoic acid

Anal. Calcd. for $C_6H_{10}O_2$: C, 63.11; H, 8.84. Found: C, 62.90; H, 9.02.

Constants: b.p., 67°–69° (0.03 mm Hg) m.p., 46°–48° (reported 48°–49° for the *trans* acid in Ref. 14). R_f, 0.63, in the *n*-amyl alcohol system.

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Δ^3-*Hydroxy-3-methyl pentonic acid*

Anal. Calcd. for $C_6H_{12}O_3$: C, 57.62; H, 9.16. Found: C, 53.46; H, 8.93.

Constants: b.p., 93°–95° (0.03 mm Hg). n_D^{20}, 1.4442. R_f, 0.41, in the *n*-amyl alcohol system.

RESULTS AND DISCUSSION

Standard Incubation Conditions. Figure 2 illustrates the rate of incorporation of mevalonate-2-C^{14} into cholesterol under standard incubation conditions. The rate of sterol synthesis was linear for 10 minutes, and the system incorporated no more C^{14} into sterol after 15 minutes. A 5-minute incubation period was chosen for activity measurements since the curve was linear and about 5% conversion of mevalonic acid to cholesterol occurred at this time. Thus the effects of the inhibitory compounds were tested during a period of maximal rate of steroid synthesis.

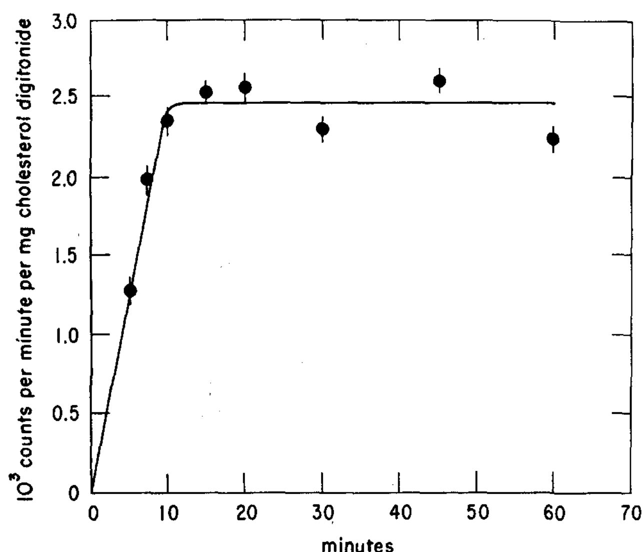

FIG. 2. Rate of incorporation of mevalonate-2-C^{14} into cholesterol in rat liver homogenates. Each point is the average of closely agreeing results of three samples.

Our choice of a 5-minute incubation time requires further comment. Although the reaction stops after 15 minutes in the absence of an inhibitor, it is possible that in the presence of an inhibitor the system might incorporate more C^{14} beyond the 15-minute period. This could occur if the activated mevalonic acid were metabolized solely by way of sterol biosynthesis. This appears to be the case from the work of Tavormina and Gibbs (6), Popják *et al.* (16), and Elwood *et al.* (17). If the inhibitor itself were metabolized, the duration of C^{14} incorporation into sterol could also be extended beyond 15 minutes since, as inhibitor concentration decreased, activated mevalonate would be converted to sterol. Therefore, if the incubation time had been the 2-hour period recommended by Frantz and Bucher (13) and Tavormina and Gibbs (6), the inhibition might have been less than during the 5-minute period of maximal rate of cholesterol synthesis. We have found in preliminary experiments that concentrations of inhibitors which produced 80% inhibition during a 5-minute incubation period caused a 65% to 70% inhibition during a 1-hour incubation time. This difference is probably significant since the counting error in the isotopic assay procedure did not exceed 5%.

Comparison of the Effects of the Three Compounds upon Cholesterol Biosynthesis. Figure 3 shows the effect of the three compounds in reducing the incorporation of isotope from mevalonate-2-C^{14} into cholesterol. The hydroxy acid is the most effective; the Δ^2 acid is next, and the Δ^3 acid is the least effective.

Half-maximal inhibition by the hydroxy acid occurred at a concentration of 5 µmoles/ml of homogenate. The ratio in µmoles of inhibitor to substrate is 100:1. The inhibition is probably not a result of changes in ionic strength since Bucher (11) found that the addition of 100 µmoles of α-ketoglutarate to samples of homogenate had no effect upon the incorporation of isotopic acetate into cholesterol. In the present system the addition of 5 µmoles of compound to 1 ml of homogenate would increase the molar concentration of the buffer by 10% at most.

Comparison of the Effects of Inhibitors in Tissue from the Same Animal. The data described so far were ob-

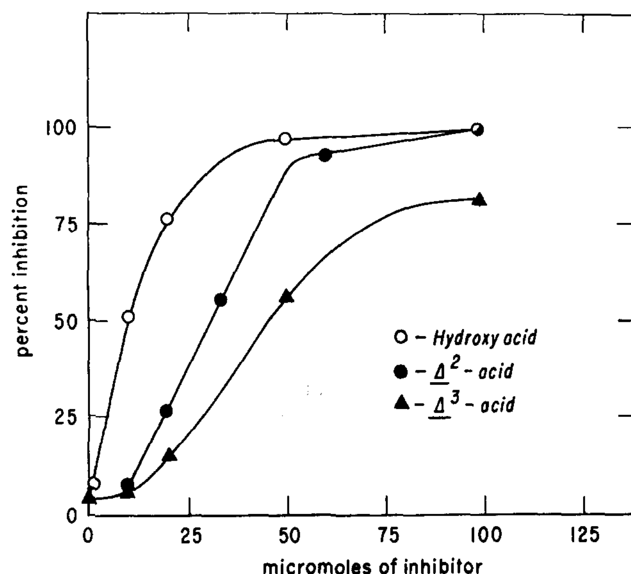

FIG. 3. Comparison of inhibitors. Each of the three acids was tested for its effect upon incorporation of mevalonate-2-C^{14} into cholesterol. Each point is the average of closely agreeing results from three samples.

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tained by use of tissues from separate animals for each compound. In Figure 4 are compared the inhibitory effects of the three compounds at two different concentrations in tissue from the same animal. The results are essentially similar to those shown in Figure 3.

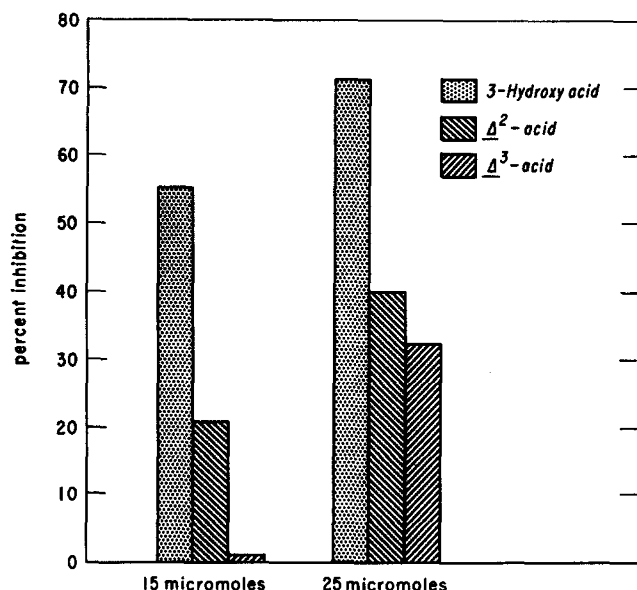

FIG. 4. Comparison of inhibitors in the same homogenate. Aliquots of the same homogenate containing C^{14}-mevalonate were incubated for 5 minutes with two different concentrations of each inhibitor. The height of each bar is the average of closely agreeing results from three samples.

As was mentioned above, the Δ^2 acid is more effective than the Δ^3 acid. This differs from the *in vivo* results of Mentzer *et al.* (3), who reported a converse relationship.

The Hydroxy Acid as a True Inhibitor. It is possible that the hydroxy acid might be converted to mevalonate in the homogenate. The reduced incorporation of isotope in the presence of the acid then could be explained by an increased pool of unlabeled mevalonate. If such a dilution is significant in the present system, it may be possible to decrease further the amount of isotope incorporation into sterol by preincubation of the inhibitor with homogenate before adding the labeled substrate.

To test this possibility, we preincubated hydroxy acid with homogenate. In the results described in Table 1, sample 1 is a control for sample 2, with inhibitor and substrate incubated together starting at zero time. Sample 3 is a control for samples 4 and 5. The latter samples were incubated with inhibitor for 15 and 5 minutes, respectively, before addition of substrate. Cofactors were added twice to these samples: once, together with the hydroxy acid in case any metabolism of this compound depended upon these cofactors;

TABLE 1. RESULTS OF PREINCUBATION STUDIES WITH INHIBITOR

Sample	C^{14}-Mevalonate at 0 Minutes	Cofactors at 0 Minutes	Cofactors at 5 Minutes	Cofactors at 15 Minutes	Hydroxy Acid at 0 Minutes	Hydroxy Acid at 5 Minutes	Hydroxy Acid at 15 Minutes	Sterol	Inhibition
								cpm/mg	*per cent*
1	+	+	–	–	–	–	–	1344	–
2	+	+	–	–	+	–	–	556	59
3	+	+	–	+	–	–	–	1869	–
4	+	+	+	–	–	+	–	894	52
5	+	+	–	+	–	–	+	909	52

The effect of preincubating homogenate with hydroxy acid is compared with the result of incubating samples to which C^{14} substrate and hydroxy acid were added at zero time. Each value is the average of three closely agreeing results.

for the second time, together with substrate in order to take into account the effects of adenosine triphosphatase and diphosphopyridine nucleotidase during the periods of preincubation.

The results indicate that there is no significant difference in the incorporation of isotope between the homogenates with which hydroxy acid has been preincubated and homogenates with which both acid and isotopic substrate has been incubated simultaneously.

Although these results are not conclusive, it appears that the hydroxy acid is stable for 20 minutes and acts as an inhibitor, not a diluent.

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