Inhibition of Cholesterol Biosynthesis in Vitro by 
β-Diethylaminoethyl Diphenylpropylacetate Hydrochloride (SKF 525-A)

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β-Diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525-A) has been shown to inhibit a number of reactions involved in drug metabolism (1), including side-chain oxidation, dealkylation, deamination, hydroxylation, and deesterification. Moreover, recent studies afforded no information concerning the possible mechanism whereby this compound produced its hypocholesterolemic effect. In the experiments reported here, this drug has been found to inhibit the conversion of mevalonate-2-Cl4 to cholesterol. It also interfered with the incorporation of this substrate into other nonsaponifiable lipids, but it did not affect the decarboxylation of mevalonate-1-C14. These data suggest that the site of inhibition lies between isopentenyl pyrophosphate and squalene on the biosynthetic pathway leading to cholesterol.

EXPERIMENTAL PROCEDURE

Animals—Carworth strain male rats weighing 60 to 120 g were used in this study. In agreement with the observations of Wood and Migicovsky (3), it was found that rat liver homogenates prepared from animals on a Purina fox chow diet supplemented with corn oil had a greater capacity to incorporate acetate-1-C14 into cholesterol than did homogenates from the control group. In addition, the presence of corn oil in the diet enhanced the ability of liver homogenates to incorporate mevalonate-2-C14 into cholesterol. Unless otherwise specified, all animals were maintained on a Purina fox chow diet supplemented with 20% corn oil.

Preparation of Homogenates—The liver homogenates were prepared by the method of Bucher (4) in phosphate buffer (0.08 M, pH 7.2) containing MgCl2 (0.004 M), and nicotinamide (0.03 M). The turbid supernatant solution obtained after centrifuging the homogenate at 500 × g for 2 minutes was used as the source of enzymes.

Incubation—The standard reaction mixtures contained either mevalonate-2-C14, or mevalonate-1-C14, in the amount specified, 1.75 μmoles of ATP, 2.0 μmoles of TPN, 10.6 μmoles of MgCl2, 276.0 μmoles of phosphate to give pH 7.2, and 0.5 ml of rat liver homogenate, in a final volume of 2.5 ml.

Studies of the incorporation of mevalonate-2-C14 into nonsaponifiable lipid were carried out aerobically in the Dubnoff metabolic shaker at 37°. The reaction was stopped by the addition of 3 ml of 15% KOH in 50% ethanol to each reaction mixture followed by heating to 65-70° for 10 minutes.

Studies of the decarboxylation of mevalonate-1-C14 were carried out aerobically in 15-ml Warburg vessels. The enzymatic reaction was stopped by the addition of 0.2 ml of 3.5 M H2SO4 from the side arm of the flask. Shaking was continued for 1 hour after the addition of the H2SO4 to ensure complete distillation of the C14O2 from the reaction mixture into 10% KOH in the center well.

Isolation and Separation of Nonsaponifiable Lipids—The partially saponified reaction mixture was transferred to a 30-ml screw-capped culture tube, the beaker was rinsed with 3 ml of the alcoholic KOH solution which was then added to the contents of the culture tube. Saponification was completed by heating the tightly capped tube in a water bath (75-80°) for 60 minutes.

The saponified reaction mixtures were extracted twice with 10 ml portions of light petroleum ether. The combined extracts were washed once with 10 ml of water, dried over anhydrous Na2SO4, and finally made up to a volume of 25 ml with petroleum ether. This solution contains the total nonsaponifiable lipid.

A suitable aliquot of the petroleum ether extract was evaporated to dryness in a 12-ml, heavy duty centrifuge tube. The residue was dissolved in 3 ml of acetone-absolute ethanol (1:1) volume for volume, and 2.5 mg of carrier cholesterol in 0.5 ml of ethanol was added to this solution. The 3β-hydroxy sterols were precipitated by the addition of 7 ml of 0.5% digitonin in 50% ethanol. After standing at room temperature for 16 hours, the digitonide precipitate was recovered by centrifugation, washed once with 99% ethanol, once with acetone, once with acetone-ether (1:2, volume for volume), and finally with petroleum ether.

Measurement of Radioactivity—C14O2 was precipitated as the barium salt. The C14 activity, measured with a Nuclear D-47 thin window gas flow counter, was corrected to infinite thinness. The 3β-hydroxy steroid digitonide was transferred quantitatively to a counting vial with 10 ml of phosphor solution (0.4% 2,5-diphenyloxazole in toluene). The digitonide suspension was stabilized by shaking with 3.0% Thixcin for 1 hour. For determination of the C14 activity of the total nonsaponifiable fraction, suitable aliquots (1 to 2 ml) of the petroleum ether extract were added directly to 9.0 ml of the phosphor solution. The radioactivity in the Thixcin gel and the liquid samples was determined with a Packard Tri-Carb liquid scintillation counter. A suffi-
cient number of counts was taken to reduce the statistical error of counting to less than 5%. Cholesterol-4-C14 was used as an internal standard to correct the counts for quenching.

Materials—Mevalonate-2-C14 was obtained from the Volk Radiochemical Company and from Tracerlab. Mevalonate-1-C14 was kindly supplied by Dr. S. Gurin.

A solution of the inhibitor was prepared freshly each day. A suitable dilution of this material in water was neutralized to a slight turbidity (pH about 5.5) immediately before its addition to the reaction mixtures.

RESULTS

Effect of Drug on Conversion of Mevalonate to Nonsaponifiable Lipids—The results of early experiments showed that the drug inhibited the conversion, by rat liver homogenate, of mevalonate-2-C14 to cholesterol during an incubation period of 75 minutes. Furthermore, the distribution of radioactivity between the digitonide-precipitable sterols and the other components of the nonsaponifiable fraction indicated that the inhibitor blocked the metabolism of mevalonate at an early stage.

The effect of the inhibitor on the conversion of mevalonate to nonsaponifiable lipids by rat liver enzymes, during a 10-minute incubation, is shown in Table I. These data show that the synthesis of nonsaponifiable lipid is inhibited markedly by this compound, even during the brief incubation period used in this study. Also, the magnitude of the inhibition clearly is dependent on the concentration of the inhibitor. The percentage of inhibition of incorporation of mevalonate into the total nonsaponifiable and digitonide-precipitable fractions in the presence of increasing concentrations of inhibitor again suggested an interruption of biosynthesis of cholesterol at an early stage in the reaction sequence. These studies, however, did not provide any information concerning the nature of the inhibition produced by the drug.

Studies of Kinetics of Conversion of Mevalonate to Nonsaponifiable Lipid—The kinetics of the complex multienzyme system which converts mevalonate to nonsaponifiable lipid were studied before investigating the nature of the inhibition caused by the drug. The time course of the conversion of mevalonate to total nonsaponifiable lipid and digitonide-precipitable sterol by two concentrations of rat liver homogenate is shown in Fig. 1 A, B.

TABLE I
Effect of β-diethylaminomethyl diphenylpropylacetate hydrochloride on conversion of mevalonate-2-C14 into nonsaponifiable lipid

The standard incubation mixture described in text was used. Each reaction mixture contained 1.0 µmole of mevalonate-2-C14, 1.0 µmole of cysteine (30 µmoles), glutathione (30 µmoles), DPN (1 µmole), CoA (1 µmole), and additional ATP (1.7 µmoles) were ineffective in overcoming the dilution effect. Mn++ (2 to 4 µmoles) had an effect similar to that of boiled enzyme. The optimal concentration of Mn++ was found to vary from one experiment to another. The effectiveness of Mn++ probably depended on the length of time the homogenate was prepared before use (5).

The effect of varying the substrate concentration (Fig. 2)
data shows that the inhibitor produces approximately the same proportional changes in apparent $K_m$ and $V_{max}$. Although a complex multienzyme system was used to obtain these data, they indicate that the inhibition is of the so-called uncompetitive type (7, 8).

**Attempts to Localize Site of Action**—In an attempt to localize the site of action of the drug, its effect on the decarboxylation of mevalonate $1^4C$ was investigated. The data in Fig. 4 show that this compound had practically no effect on the decarboxylation of mevalonate when tested at concentrations known to markedly inhibit the conversion of this substrate to nonsaponifiable lipid.

The method of Popjak (9) was used to study the distribution of radioactivity among the petroleum ether-soluble $1^4C$-labeled components of the reaction mixture. Rat liver homogenate from which the mitochondria had been removed by centrifugation at 10,000 × $g$ (S$_{10}$ enzyme) (10) was used as the source of enzymes in this study. The total nonsaponifiable fraction was partitioned into petroleum ether from the alkaline incubation mixture after saponification with KOH. The aqueous residue was then acidified to pH 1.0 with $H_2SO_4$. After the solution had stood at room temperature for 1 hour, the pH was readjusted to 10.0 with KOH, and the neutral $1^4C$-labeled substances were extracted with petroleum ether. It is evident (Table II) that the drug markedly inhibited the incorporation of mevalonate $2^4C$ into both the total nonsaponifiable lipid and digitonide-precipitable material. Moreover, the data show a large accumulation of the neutral petroleum ether-soluble fraction in the inhibited reaction mixtures. It is presumed, based on the findings of others (9, 11, 12), that this fraction is comprised of presqualene branched chain alcohol intermediates, i.e. the polyprenols.

Chromatography of an alcohol-ether extract of reaction mixtures described in Table II on Whatman No. 1 filter paper in the system n-butanol-water-formic acid (77:10:13) (13) revealed the accumulation in the inhibited reaction mixture of a compound with an RF (0.34) corresponding to that reported for isopentenyl pyrophosphate. An aqueous solution of the alcohol pyrophosphates was prepared from inhibited reaction mixtures by the method of Popjak (9). After hydrolysis of the pyrophosphates with cobra venom at pH 9.0, and addition of small

**Table II**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>Drug inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.p.m.</td>
<td>Recovery</td>
</tr>
<tr>
<td>Total nonsaponifiable</td>
<td>115,000</td>
<td>21.0</td>
</tr>
<tr>
<td>Digitonide-precipitable</td>
<td>38,500</td>
<td>7.1</td>
</tr>
<tr>
<td>Petroleum ether-soluble</td>
<td>9,000</td>
<td>1.6</td>
</tr>
<tr>
<td>Polyprenols</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Recovery expressed as percentage of the initial radioactivity.
amounts of carrier alcohols, the free alcohols were extracted with light petroleum ether. The 3,5-dinitrobenzoates prepared from oil, with 75% acetic acid as the developing solvent. The dinitrobenzoates of farnesol and geraniol were found to have light petroleum ether. The 3,5-dinitrobenzoates prepared from oil, with 75% acetic acid as the developing solvent. The dinitrobenzoates of farnesol and geraniol were found to have light petroleum ether. The 3,5-dinitrobenzoates prepared from oil, with 75% acetic acid as the developing solvent.

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