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INHIBITION OF IN VITRO CHOLESTEROL SYNTHESIS BY FATTY ACIDS

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Summary

Inhibitory effect of 44 species of fatty acids on cholesterol synthesis has been examined with a rat liver enzyme system. In the case of saturated fatty acids, the inhibitory activity increased with chain length to a maximum at 11 to 14 carbons, after which activity decreased rapidly. The inhibition increased with the degree of unsaturation of fatty acids. Introduction of a hydroxy group at the α-position of fatty acids abolished the inhibition, while the inhibition was enhanced by the presence of a hydroxy group located in an intermediate position of the chain. Branched chain fatty acids having a methyl group at the terminal showed much higher activity than the corresponding saturated, straight chain fatty acids with the same number of carbons.

With respect to the mechanism for inhibition, tridecanoate was found to inhibit acetoacetyl-CoA thiolase specifically without affecting the other reaction steps in the cholesterol synthetic pathway. The highly unsaturated fatty acids, arachidonate and linoleate, were specific inhibitors of 3-hydroxy-3-methylglutaryl-CoA synthase. On the other hand, ricinoleate (hydroxy acid) and phytanate (branched-chain acid) diminished the conversion of mevalonate to sterols by inhibiting a step or steps between squalene and lanosterol.

Introduction

The biosynthesis of cholesterol starts with acetyl-CoA and is accomplished via 3-hydroxy-3-methylglutaryl-CoA, mevalonate, and squalene in a reaction sequence comprising more than 20 individual steps, in which 3-hydroxy-3-methylglutaryl-CoA reductase that catalyzes the reduction of 3-hydroxy-3-methylglutaryl-CoA by NADPH to form mevalonate represents the major control point of cholesterogenesis. In normal individuals, the cholesterol synthesis in liver is regulated by a variety of factors and physiological conditions, includ-

ing ingestion of cholesterol and bile acids, starvation, and hormones [1]. For example, the ingestion of cholesterol causes a strong depression of cholesterol synthesis in liver.

On the other hand, in vitro sterol synthesis has been shown to be inhibited by several bile acids and fatty acids [2—6]. Wood and Migikovsky observed that erucate and linoleate were potent inhibitors of in vitro cholesterol synthesis by rat liver homogenates [6]. The mcheanisms for inhibition, however, have not been established.

In the present studies, we have determined more fully the inhibitory effect of 44 species of naturally and unnaturally occurring fatty acids on the in vitro sterol synthesis by a rat liver enzyme system. In an attempt to elucidate the inhibitory mechanism by fatty acids, we have also determined the site(s) of action of several fatty acids in the cholesterol synthetic pathway.

Materials and Methods

Materials

All fatty acids used in this study were products of Applied Science Laboratories, Inc. The purity of saturated, unsaturated, and hydroxy fatty acids was over 99%, except for arachidonate, the purity of which was 90% or more. Among the branched chain fatty acids, phytanate (purity, 96%) was obtained as free acid, and the remainder as methyl ester (purity, 96%), which were saponified before use.

[1-^{14}C]Acetate (54.9 Ci/mol) was obtained from Daiichi Pure Chemicals Co., [1-^{14}C]acetyl-CoA (51.5 Ci/mol) and DL-[3-^{14}C]3-hydroxy-3-methylglutaryl-CoA (18.8 Ci/mol) from New England Nuclear Co., and DL-[2-^{14}C]-mevalonate (lactone) (4.7 Ci/mol) from Radiochemical Centre (Amersham). Acetyl-CoA and CoA were purchased from Boehringer Corp., and acetyacetyl-CoA, ATP, NAD, NADP, NADPH, glutathione (reduced), gluocse 1-phosphate, digitonin, lanosterol, cholesterol, and DL-mevalonate (lactone) were from Sigma Chemical Co. Squalene was obtained from Tokyo Kasei Co., dithiothreitol from British Drug Houses Chemicals, and 3-hydroxy-3-methylglutaryl-CoA from P-L Biochemicals.

Rat liver enzyme preparations

Male Wistar-Imamichi rats weighing about 150 g were housed in environmentally controlled rooms where the lights were automatically regulated to go off at 7 p.m. and on at 8 a.m. daily. For elevation of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase activity, the rats were fed 2% cholestyramine diet with 8% added corn oil for 5 days [7] and then killed by decapitation at 11—12 p.m., the time when the peak activity occurs in the diurnal cycle of the enzyme. The livers were rapidly removed to ice cold beakers, and then homogenized in 2 vols. of the cold buffer containing 100 mM potassium phosphate, pH 7.4, 15 mM nicotinamide, and 2 mM $MgCl_2$ in a Potter-Elvehjem type homogenizer with a loose fitting Teflon pestle. After homogenization with only four strokes, the mixture was centrifuged at 700 × *g* for 5 min, and the supernatant solution was then centrifuged at 12 000 × *g* for 30 min. The supernatant from this centrifugation was further fractionated by centrifugation at 105 000 × *g*

for 60 min. The resultant supernatant (cytosolic fraction) and pellet (microsomes) were pooled separately. The cytosolic fraction was then subjected to $(NH_4)_2SO_4$ precipitation, and the precipitate obtained at 40—80% saturation was collected. This fraction (hereafter referred to as the $(NH_4)_2SO_4$ fraction) was dissolved in 100 mM potassium phosphate buffer, pH 7.4 and dialyzed against the same buffer at 4°C for 6 h. The microsomal fraction was washed once with the aforementioned buffer. These two preparations were stable at −80°C and retained their catalytic activity for at least 4 months, unless freezing and thawing were repeated which caused a loss in activity. All the experiments reported were performed with the same preparations of microsomes and $(NH_4)_2SO_4$ fraction, except for the experiments shown in Fig. 2, in which different preparations were used.

Incorporation experiments

Each incubation tube contained in a final volume of 0.2 ml: 1 mM ATP; 10 mM glucose 1-phosphate; 6 mM glutathione; 6 mM $MgCl_2$; 40 μM CoA; 0.25 mM NAD; 0.25 mM NADP; 100 mM potassium phosphate buffer, pH 7.4; 1 mM [1-^{14}C]acetate (3.0 Ci/mol); microsomes (0.15—0.20 mg protein); and $(NH_4)_2SO_4$ fraction (1.5—2.0 mg protein). In some experiments, where indicated, [1-^{14}C]acetate was replaced by 0.44 mM DL-[2-^{14}C]mevalonate (0.59 Ci/mol) or 30 μM [1-^{14}C]acetyl-CoA (14.3 Ci/mol). The tubes were incubated at 37°C for 60 min without stopper, unless otherwise mentioned. At the end of the incubation period, 1 ml of 15% alcoholic KOH was added to the incubation tubes.

The synthesized nonsaponifiable products, sterols, and fatty acids were measured as described by Knauss et al. [8]. The reaction mixtures were saponified at 75°C for 60 min and extracted with light petroleum. Aliquots of the extracts (nonsaponifiable fraction) were evaporated to dryness at 75°C for 10 min and counted. Sterols were isolated from another aliquot of these extracts as digitonide, dissolved in 0.5 ml of hydroxide of Hyamine 10-X (Packard) and counted. To determine fatty acids, the residues remaining after the light petroleum extractions were acidified, and the fatty acids were extracted with light petroleum and counted.

Enzyme assays

To assay 3-hydroxy-3-methylglutaryl-CoA reductase, the reaction mixture contained in a total volume of 50 μl: 0.11 mM DL-[3-^{14}C]3-hydroxy-3-methylglutaryl-CoA (1.6 Ci/mol), 100 mM potassium phosphate buffer, pH 7.4, 10 mM EDTA, 10 mM dithiothreitol, 5 mM NADPH, and 0.03 to 0.04 mg of microsomal protein. The reaction was started by addition of the enzyme (microsomes), and the mixtures were incubated at 37°C for 30 min. After termination of the reaction by addition of 10 μl of 2 M HCl, the mixture was incubated at 37°C for 15 min and then applied to a Silica Gel F-254 plate (Merck). The chromatograms were developed in benzene/acetone (1 : 1) by the procedure of Shapiro et al. [9]. Labeled mevalonolactone was used as reference standard. Sections of the thin layer plates where the mevalonolactone was located were scraped and assayed for radioactivity.

3-Hydroxy-3-methylglutaryl-CoA synthase was assayed as described by Clin-

kenbeard et al. [10]. The reaction mixture contained in a total volume of 0.1 ml: 50 mM Tris · HCl, pH 7.4, 50 μM EDTA, 0.3 mM [1-^{14}C]acetyl-CoA (2.0 Ci/mol), 0.1 mM acetoacetyl-CoA, and 0.02 mg of $(NH_4)_2SO_4$ fraction. The reaction was initiated by addition of [^{14}C]acetyl-CoA to the otherwise complete reaction mixture and terminated by addition of 0.2 ml of 6 M HCl after incubation at 37°C for 30 min. The acidified mixture was transferred into a counting vial and taken to dryness at 95°C in a forced draught oven. The non-volatile radioactivity (as [^{14}C]3-hydroxy-3-methylglutaryl-CoA) was determined with a liquid scintillation spectrometer.

Acetoacetyl-CoA thiolase activity (thiolytic cleavage activity) was determined by the method of Clinkenbeard et al. [11]. The assay mixture contained in a total volume of 2.0 ml: 50 mM Tris · HCl,pH 8.2, 0.03 mM EDTA, 0.2 mM acetoacetyl-CoA, 0.25 mM CoA, and 0.9 mg of $(NH_4)_2SO_4$ fraction protein. After a 3 min preliminary incubation of thiolase in the above reaction mixture without CoA, the reaction was initiated by addition of 0.25 mM CoA. The rate of thiolysis was followed at 30°C by the disappearance of acetoacetyl-CoA absorbance at 300 nm.

The sequential reaction of acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase was followed as described by Sugiyama et al. [12]. The reaction mixture contained 20 mM Tris · HCl, pH 8.4, 0.03 mM EDTA, 0.15 mM [1-^{14}C]acetyl-CoA (2.0 Ci/mol), and 0.02 mg of $(NH_4)_2SO_4$ fraction protein in a total volume of 0.1 ml. Following a 10 min incubation at 37°C, the radioactivity incorporated into 3-hydroxy-3-methylglutaryl-CoA was determined as described above.

Other determinations

Protein was determined by a modification of the method of Lowry et al. [13], with bovine serum albumin as a standard. Radioactivity was measured with a Packard Tri-carb Model 3390 or Beckman LS-250 spectrometer in a toluene-based counting fluid. Samples insoluble in toluene were counted in a toluene-dioxane (1 : 4) fluid.

Results

Properties of enzyme system

As mentioned by Knauss et al. [8], the incorporation of [^{14}C]acetate into nonsaponifiable lipids was dependent upon the presence of both microsomes and $(NH_4)_2SO_4$ fraction, and the maximum incorporation was achieved with a ratio of 0.15—0.2 mg of microsomal protein to 1.5—2.0 mg of $(NH_4)_2SO_4$ fraction protein. The synthesis of nonsaponifiable lipids was linearly proportional to the enzyme protein incubated when the ratio between these two fractions was kept constant. Further, the incorporation of the labeled substrates was proportional to time (up to 180 min). The system was highly reproducible, as the results of duplicate incubations differed by less than 5%. Repeated studies of the same preparations of microsomes and $(NH_4)_2SO_4$ fraction gave identical results.

The microsomes prepared as described above contained approximately 34.5 μg of palmitate, 26.4 μg of stearate, 62.1 μg of arachidonate and 39.4 μg of

linoleate/mg of protein, respectively, as determined by gas-liquid chromatography [14] after saponification of lipids extracted with chloroform/methanol (2 : 1). The $(NH_4)_2SO_4$ fraction had less than 1 μg of total fatty acid/mg of protein. Both preparations seemed to be free of contamination with acetyl-CoA and acetate, since these preparations were washed with or dialyzed against buffer as described in Materials and Methods. Further, no detectable amounts of free fatty acids were present in the reaction mixture incubated for 60 min under standard incubation conditions. When labeled squalene formed from [1-^{14}C]acetate under standard conditions in the presence of arachidonate (50 μg/ml) was isolated and determined [15], its specific radioactivity was 81—92% of the theoretically expected if no endogenous dilution occurred. In addition, over 90% of arachidonate and oleate added to the reaction mixture were recovered after 60 min of incubation. On the basis of these data, the amount of labeled substrates incorporated into nonsaponifiable lipids were calculated from the specific activities of the substrates and on the assumption that the labeled substrates were not diluted in the assay mixtures with unlabeled material, and that 33% of the radioactivity in [1-^{14}C]acetate and [1-^{14}C]acetyl-CoA were converted into CO_2 during their incorporation into nonsaponifiable lipids. Under standard conditions, the incorporation of [1-^{14}C]acetate into nonsaponifiable lipids was 7.9 nmol/assay (0.080 nmol/min per mg protein), and the incorporation into digitonin-precipitable sterols was 5.9 nmol/assay (0.060 nmol/min per mg protein).

Both microsomal and $(NH_4)_2SO_4$ fractions had no detectable activity of β-oxidation of fatty acid, as determined by the method of Lee and Fritz [16].

Incorporation of [^{14}C]acetate into nonsaponifiable lipids

The inhibitory effect of saturated fatty acids on the [^{14}C]acetate incorporation into nonsaponifiable lipids is shown in Table I. The data indicate that the inhibitory activity increased with chain length, reaching a maximum between 11 to 14 carbons, after which it decreased sharply. Both even- and odd-numbered acids showed inhibition to the same degree. Acids with 6 (caproate) and 7 (heptanoate) carbon atoms had no detectable effect on cholesterol synthesis at a concentration as high as 1 mg/ml.

The inhibitory activity of fatty acids with double bonds is shown in Table II. Introduction of double bonds in the chain elevated the inhibitory potency of acids except for myristoleate, the inhibition by which was lower than that by the corresponding saturated acid (myristate) (Table I). Palmitelaidate was a more potent inhibitor than palmitoleate, and linolelaidate than linoleate, thus indicating that the *trans* forms exerted a stronger inhibition than did the corresponding *cis* forms. The data in Table II also show that the inhibitory potency of acids increased with the number of double bonds, with the exception seen in the relation between linoleate and linolenate. It should be noted that the three essential fatty acids, linoleate, linolenate, and arachidonate, were potent inhibitors, diminishing cholesterol synthesis by 50% at concentrations from 20 to 38 μg/ml.

All fatty acids with a hydroxy group at the α-position of carbon chain were weakly inhibitory. Thus, α-hydroxymyristate showed less inhibitory activity than did myristate, suggesting that the presence of a hydroxy group at the α-

TABLE I

INHIBITION OF [^{14}C]ACETATE INCORPORATION INTO NONSAPONIFIABLE FRACTION BY SATURATED FATTY ACIDS

Experiments were carried out as described under Materials and Methods. In parentheses: the number of C atoms : number of double bonds, in the order described.

Fatty acid		Concentration required for 50% inhibition (μg/ml)
Caproate	(6:0)	>1000
Heptanoate	(7:0)	>1000
Caprylate	(8:0)	500
n-Nonanoate	(9:0)	300
Caprate	(10:0)	75
n-Undecanoate	(11:0)	53
Laurate	(12:0)	47
Tridecanoate	(13:0)	49
Myristate	(14:0)	48
Pentadecanoate	(15:0)	300
Palmitate *	(16:0)	>500
Heptadecanoate *	(17:0)	>500
Stearate *	(18:0)	>500
Nonadecanoate *	(19:0)	>500
Arachidate *	(20:0)	>500

* Reaction mixtures containing these fatty acids (500 μg/ml) were partly cloudy due to their low solubility.

position diminished the inhibitory activity. α-Hydroxy acids (saturated) having 16, 18, 20, 22 and 24 carbons diminished sterol synthesis by less than 10% at a concentration of 200 μg/ml. However, fatty acids having a hydroxy group located in an intermediate position of the chain showed a strong inhibition. For example, ricinoleate, which has one hydroxy group at the 12th carbon of

TABLE II

INHIBITION OF [^{14}C]ACETATE INCORPORATION INTO NONSAPONIFIABLE FRACTION BY UNSATURATED FATTY ACIDS

Experiments were carried out as described under Materials and Methods. In parentheses: number of C atoms : number of double bonds, in the order described.

Fatty acid		Concentration required for 50% inhibition (μg/ml)
Myristoleate (*cis*-9) *	(14:1)	80
Palmitoleate (*cis*-9)	(16:1)	100
Palmitelaidate (*trans*-9)	(16:1)	40
Oleate (*cis*-9)	(18:1)	100
Elaidate (*trans*-9)	(18:1)	35
Vaccenate (*cis*-11)	(18:1)	60
Linoleate (*cis,cis*-9-12)	(18:2)	30
Linolelaidate (*trans,trans*-9-12)	(18:2)	21
Linolenate (*cis,cis,cis*-9-12-15)	(18:3)	38
cis-5-Eicosenoate	(20:1)	100
cis-11-Eicosenoate	(20:1)	50
Arachidonate (*cis,cis,cis,cis*-5,8,11,14)	(20:4)	20
Erucate (*cis*-13)	(22:1)	150
Nervonate (*cis*-15)	(24:1)	200

* Configuration and position of double bonds in the carbon chains.

oleate, was a much more potent inhibitor than the latter (Table II). Its concentration giving 50% inhibition was 8 μg/ml. 12-Hydroxystearate and 9,10-dihydroxystearate inhibited the synthetic reaction 50% at concentrations of 50 and 40 μg/ml, respectively.

Branched chain fatty acids were found to be potent inhibitors of cholesterol synthesis, as shown in Table III. Of the acids having one methyl group, 12-methyltetradecanoate was the most inhibitory. Phytanate (3, 7, 11, 15-tetramethyl-hexadecanoate), a multi-branched fatty acid, showed the strongest inhibition among all the fatty acids tested. The concentration required for 50% inhibition by this acid was 4.2 μg/ml.

Incorporation of [^{14}C]mevalonate and [^{14}C]acetyl-CoA into nonsaponifiable products

To elucidate the site(s) of inhibition by fatty acids in cholesterogenesis, five fatty acids, tridecanoate, linoleate, arachidonate, ricinoleate, and phytanate, were tested for the effect on the incorporation of [^{14}C]mevalonate and [^{14}C]-acetyl-CoA into the nonsaponifiable fraction. As shown in Fig. 1, the [^{14}C]-mevalonate incorporation was found to be inhibited by ricinoleate and phytanate. The concentration required for 50% inhibition was approximately 30 μg/ml for ricinoleate and 25 for phytanate, respectively. However, no detectable inhibition was observed with tridecanoate and arachidonate at concentrations where these acids reduced the [^{14}C]acetate incorporation by approx. 80%. The [^{14}C]acetyl-CoA incorporation, on the other hand, was inhibited by all fatty acids tested. Experiments with linoleate gave similar results to those with tridecanoate and arachidonate (data not shown). As can be seen in Fig. 1, inhibition of [^{14}C]acetyl-CoA incorporation was the same as that of [^{14}C]-acetate incorporation at various concentrations of individual fatty acids, indicating that acetyl-CoA synthetase, the enzyme which activates acetate to acetyl-CoA, was not an enzyme responsible for the inhibition by these fatty acids. The results suggested that the site of inhibition by tridecanoate, linoleate and arachidonate in cholesterol synthetic pathway was between acetyl-CoA and mevalonate where three enzymes, acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, and 3-hydroxy-3-methylglutaryl-CoA reductase, are known to be involved.

TABLE III

INHIBITION OF [^{14}C]ACETATE INCORPORATION INTO NONSAPONIFIABLE FRACTION BY BRANCHED CHAIN FATTY ACIDS

Experiments were carried out as described under Materials and Methods. In parentheses: number of C atoms : number of double bonds, in the order described.

Fatty acid		Concentration required for 50% inhibition (μg/ml)
12-Methyltridecanoate	(14:0)	23
12-Methyltetradecanoate	(15:0)	7
14-Methylpentadecanoate	(16:0)	19
14-Methylhexadecanoate	(17:0)	27
16-Methylheptadecanoate	(18:0)	40
Phytanate (3,7,11,15-tetramethylhexadecanoate)	(20:0)	4.2

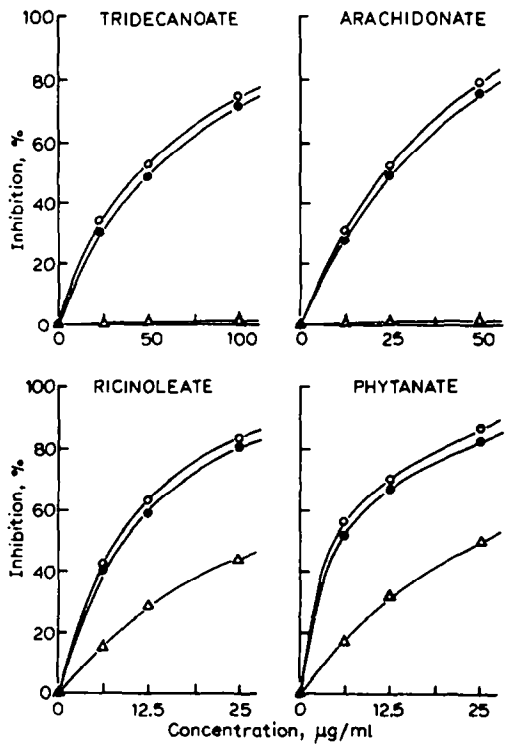

Fig. 1. Inhibitory effects of fatty acids on the incorporation of [^{14}C]acetate, [^{14}C]acetyl-CoA, and [^{14}C]-mevalonate into nonsaponifiable lipids. Experiments were carried out as described in Materials and Methods. The values for control experiments were: 0.076 nmol of [^{14}C]acetate incorporated/min per mg protein; 4.6 pmol of [^{14}C]acetyl-CoA incorporated/min per mg protein; 0.24 nmol of [^{14}C]mevalonate incorporated/min per mg protein. ○———○, [^{14}C]acetate; ●———●, [^{14}C]acetyl-CoA; △———△, [^{14}C]-mevalonate.

Site(s) of action of ricinoleate and phytanate

The data in Fig. 1 indicated that two fatty acids, ricinoleate and phytanate, inhibited post-mevalonic site(s) in cholesterol synthetic pathway. Fig. 2 shows the inhibitory effects of varying concentrations of phytanate and ricinoleate on the [^{14}C]acetate incorporation into digitonin-precipitable sterols. The incorporation was strongly inhibited by these two acids at concentrations of 10—25 μg/ml. The concentration required for 50% inhibition was approximately 3.0 μg/ml for phytanate and 6.0 for ricinoleate, respectively. The [^{14}C]acetate incorporation into squalene fraction was, however, much less sensitive to these two acids (Fig. 2); the inhibition was less than 20% for both acids at concentrations where the incorporation into digitonin-precipitable sterols was diminished by over 80%. Thus, these findings indicated that both ricinoleate and phytanate inhibited specifically the site(s) between squalene and lanosterol, that is, one of the two enzymes, squalene monooxygenase and squalene oxide cyclase, or both.

Inhibitory effects on acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, and 3-hydroxy-3-methylglutaryl-CoA reductase

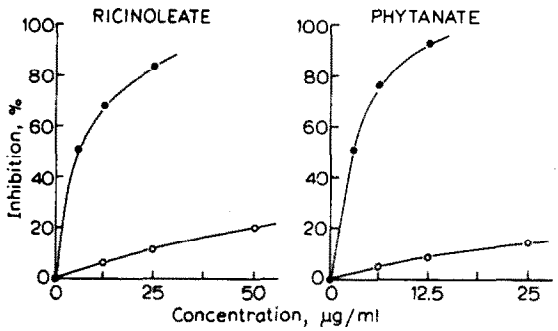

Fig. 2. Inhibition of [^{14}C]acetate incorporation into squalene fraction and sterols by phytanate and ricinoleate. Experiments were carried out as described under Materials and Methods. Microsomes and $(NH_4)_2SO_4$ fraction (0.18 and 1.9 mg protein, respectively) used in these experiments were different preparations from those used in the experiments described above. The reaction mixtures were incubated for 90 min at 37°C. The control incorporation into nonsaponifable fraction was 12.0 nmol/assay. An aliquot of the nonsaponifiable fraction was submitted to thin layer chromatography on a silica gel G plate in benzene/ethyl acetate (4 : 1), and the sections of the plates where squalene located were scraped and assayed for radioactivity. Under these conditions, the control incorporations into sterols and squalene fraction were 5.24 and 5.80 nmol, respectively. o——o, squalene fraction; •——•, sterols.

Of the five fatty acids tested, only tridecanoate was inhibitory to acetoacetyl-CoA thiolase (Table IV). The concentration giving 50% inhibition of the thiolytic cleavage activity was about 150 μg/ml; the value was much larger than that for the inhibition of [^{14}C]acetate incorporation into nonsaponifiable products by this acid (Table I).

The cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase which catalyzes the second step in cholesterogenesis was specifically inhibited by two highly unsaturated fatty acids, linoleate and arachidonate (Table IV). The latter inhibited the reaction by 50% at a concentration of 22 μg/ml.

To determine whether these fatty acids affect the thiolase-catalyzed formation of acetoacetyl-CoA from acetyl-CoA (condensation reaction), the 3-hydroxy-3-methylglutaryl-CoA generating activity was determined by using acetyl-CoA as the sole substrate. As shown in Table IV, tridecanoate was found to inhibit the activity, although much higher concentrations were required than for the inhibition of the thiolytic cleavage reaction. The data were interpreted as indicating that tridecanoate was inhibitory to the condensation reaction of thiolase, since this acid did not affect synthase (Table IV). The inhibitory activity of arachidonate in this generating system was presumably attributed to the inhibition of synthase but not of thiolase, as judged from the fact that arachidonate did not inhibit thiolase (Table IV), but reduced both the synthase reaction and the coupled reaction of thiolase and synthase to the same degree (Table IV).

3-Hydroxy-3-methylglutaryl-CoA reductase, the rate-controlling, key enzyme in cholesterogenesis, was affected by none of the five fatty acids tested at higher concentrations (from 500 to 1000 μg/ml).

Effects on fatty acid synthesis

Tridecanoate, arachidonate and ricinoleate showed no inhibitory activity to-

TABLE IV

INHIBITORY EFFECT OF SEVERAL FATTY ACIDS ON ACETOACETYL-CoA THIOLASE AND 3-HYDROXY-3-METHYLGLUTARYL-CoA SYNTHASE

Experiments were carried out as described under Materials and Methods. The values for control experiments were: 33.2 nmol of acetoacetyl-CoA converted to acetyl-CoA/min per mg protein (acetoacetyl-CoA thiolase); 2.2 nmol of 3-hydroxy-3-methylglutaryl-CoA synthesized/min per mg protein (3-hydroxy-3-methylglutaryl-CoA synthase); 8.4 nmol of acetyl-CoA converted to 3-hydroxy-3-methylglutaryl-CoA/min per mg protein (coupled reaction of acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase).

Fatty acid	Concentration (μg/ml)	Inhibition (%)		
		Thiolase	Synthase	Thiolase-synthase
Tridecanoate	50	31.8	0	—
	250	67.3	—	12.9
	500	—	2	45.3
Linoleate	50	3	47.6	—
	250	<10	71.8	—
Arachidonate	50	5	81.9	59.8
	100	—	—	80.6
	250	<10	93.0	—
Ricinoleate	50	6	—	—
	250	<10	4	2
Phytanate	50	5	—	—
	250	<10	3	0

ward the incorporation of [^{14}C]acetate into fatty acids at concentrations where the incorporation into nonsaponifiable lipids was inhibited by more than 50% (Table V). However, phytanate reduced this incorporation by 52% at 10 μg/ml, indicating that this acid was a potent inhibitor of both cholesterol and fatty acid synthesis.

TABLE V

EFFECT OF FATTY ACIDS ON [^{14}C]ACETATE INCORPORATION INTO NONSAPONIFIABLE AND FATTY ACID FRACTIONS

Experiments were carried out as described under Materials and Methods. The control radioactivities incorporated were 8000 dpm (0.081 nmol of [^{14}C]acetate/min per mg protein) for nonsaponifiable fraction and 1125 dpm in fatty acid fraction, respectively.

Fatty acid	Concentration (μg/ml)	Nonsaponifiable lipids (% of control)	Fatty acids (% of control)
Tridecanoate	50	40.9	101.3
	100	5.7	99.6
Arachidonate	10	88.1	80.9
	20	43.8	98.7
Ricinoleate	25	14.8	111.1
	50	5.9	97.8
Phytanate	5	36.6	65.4
	10	16.5	48.0
	20	12.1	31.1

Discussion

The data described in this paper have shown that a specific molecular structure of the fatty acids is necessary for the inhibition of in vitro cholesterol synthesis. First, an appropriate length of the carbon chain was required for the inhibitory activity of saturated fatty acids, giving a maximum activity at 11 to 14 carbon atoms (Table I). This is generally consistent with the results obtained by Wood and Migikovsky [6], except that their data show that fatty acids with an odd number of carbon atoms inhibited cholesterol synthesis to a greater extent than did even-numbered acids. The data in Table I indicate that the inhibitory effect of fatty acids of these two groups, having carbon atoms of 11 to 14, were essentially the same. Secondly, the inhibitory activity increased by the introduction in the carbon chain of a double bond, of a methyl group and of a hydroxy group, except for the case of a hydroxy group located at the α-position of the chain. From these data, it can be concluded that the inhibitory potency of fatty acids increases in parallel with the number of these functional groups.

Of the 44 species of fatty acids tested in this study, phytanate (multi-branched fatty acid) was the most potent inhibitor, which reduced in vitro cholesterol synthesis by 50% at concentrations of 3—5 μg/ml. This acid is known to accumulate in tissues and organs of patients with Refsum's disease as triglyceride and cholesteryl ester [17,18].

The results presented in this paper show that the two fatty acids, phytanate and ricinoleate, inhibited specifically the enzymatic reaction(s) between squalene and lanosterol without affecting the rest of the reactions in the cholesterol synthetic pathway. It is known that two enzymes, squalene monooxygenase and squalene oxide cyclase, catalyze the cyclization of squalene to lanosterol, thus indicating that these two acids are inhibitors of either one of these two enzymes, or of both.

The data presented here also show that the highly unsaturated, essential fatty acids, arachidonate and linoleate, reduced cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA synthase, one of the constituents of the cytosolic 3-hydroxy-3-methylglutaryl-CoA generating system. Thus, arachidonate reduced cholesterol synthesis in vitro by 50% at a concentration of 20 μg/ml ($6.6 \cdot 10^{-5}$ M). According to Muto and Gibson [19], this is 3 to 4 fold the maximum concentration of free arachidonate in whole liver, indicating that it is questionable whether this fatty acid interferes with the cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase reaction in liver. However, the possibility cannot be excluded that under certain physiological conditions, these acids function as a regulator in in vivo cholesterogenesis by inhibiting the 3-hydroxy-3-methylglutaryl-CoA generating system.

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