INHIBITION OF CHOLESTEROL SYNTHESIS IN RATS

by

James R. Clark

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STATEMENT BY AUTHOR

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SIGNED: 

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

William Frank McCaughey
Professor of Biochemistry and Nutrition
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>EXPERIMENTAL PROCEDURES</td>
<td>5</td>
</tr>
<tr>
<td><strong>I. In Vitro</strong>—A Rat Liver Homogenate for Synthesizing Cholesterol from Acetate</td>
<td>5</td>
</tr>
<tr>
<td><strong>II. In Vivo</strong>—The Effect of Metabolic Analogs on the Incorporation of Intraperitoneally Injected Na-1-C(^{14}) Acetate into Rat Liver Cholesterol at the Time of Maximum Incorporation</td>
<td>7</td>
</tr>
<tr>
<td><strong>III. In Vivo</strong>—Analysis of the Potential Inhibitors' Ability to Change Kinetics of the Rats' Incorporation of Subcutaneously Injected Na-1-C(^{14}) Acetate into Liver</td>
<td>9</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>11</td>
</tr>
<tr>
<td><strong>I. In Vitro</strong>—A Rat Liver Homogenate for Synthesizing Cholesterol from Acetate</td>
<td>11</td>
</tr>
<tr>
<td><strong>II. In Vivo</strong>—The Effect of Metabolic Analogs on the Incorporation of Intraperitoneally Injected Na-1-C(^{14}) Acetate into Rat Liver Cholesterol at the Time of Maximum Incorporation</td>
<td>16</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS--Continued

<table>
<thead>
<tr>
<th>RESULTS AND DISCUSSION (Contd.)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III. In Vivo--Analysis of the Potential Inhibitors' Ability to Change the Kinetics of the</td>
<td>26</td>
</tr>
<tr>
<td>Rats' Incorporation of Subcutaneously Injected Na-1-C(^{14}) Acetate into Liver Cholesterol</td>
<td></td>
</tr>
<tr>
<td>SUMMARY</td>
<td>31</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>32</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>The total cholesterol content of four replications of rat liver homogenate incubated in vitro at 37°C.</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>The effect of various concentrations of acetate on the cholesterol content of a 40% rat liver homogenate</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>The total cholesterol content of incubated homogenates of different concentrations.</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>The biosynthesis of cholesterol by rat liver homogenate prepared with a Potter-Elvejhem homogenizer and incubated in a Dubnoff shaker.</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>The effect of using NADP in place of NAD as a cofactor for the rat liver homogenate.</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>Results of the addition of ATP and the substitution of NADP for NAD on cholesterol biosynthesis by rat liver homogenate.</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>The effect of ATP addition on cholesterol biosynthesis by rat liver homogenate.</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>The incorporation of Na-1-C^{14} acetate into cholesterol by an 8% rat liver homogenate.</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Incorporation of intraperitoneally injected Na-1-C^{14} acetate into the liver cholesterol of a rat.</td>
<td>19</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>10</td>
<td>The effect of 3,3-dimethylglutarimide on the incorporation of Na-1-C\textsuperscript{14} acetate into rat liver cholesterol \textit{in vivo}.</td>
</tr>
<tr>
<td>11</td>
<td>The results of 3,3-dimethylglutaric acid on the incorporation of Na-1-C\textsuperscript{14} acetate into rat liver cholesterol \textit{in vivo}.</td>
</tr>
<tr>
<td>12</td>
<td>Effect of 2,2'-dimethylglutaric acid and 2,2-dimethylglutaric acid on Na-1-C\textsuperscript{14} acetate incorporation into rat liver cholesterol \textit{in vivo}.</td>
</tr>
<tr>
<td>13</td>
<td>Incorporation of Na-1-C\textsuperscript{14} acetate into rat liver cholesterol after the intraperitoneal injection of 2,2'dimethylglutaric anhydride.</td>
</tr>
<tr>
<td>14</td>
<td>The effect of 3,3-dimethylglutaric anhydride on the incorporation of Na-1-C\textsuperscript{14} acetate into rat liver cholesterol \textit{in vivo}.</td>
</tr>
<tr>
<td>15</td>
<td>The effect of 3-ethyl-3-methylglutarimide on the incorporation of Na-1-C\textsuperscript{14} acetate into rat cholesterol \textit{in vivo}.</td>
</tr>
<tr>
<td>16</td>
<td>Results of the subcutaneous injection of 2-phenylglutaric anydride on the incorporation of Na-1-C\textsuperscript{14} acetate into cholesterol \textit{in vivo}.</td>
</tr>
<tr>
<td>17</td>
<td>3-Ethyl-3-methylglutaric anhydride's effect on the incorporation of Na-1-C\textsuperscript{14} acetate into rat liver cholesterol \textit{in vivo}.</td>
</tr>
<tr>
<td>Figures</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>The effect of 3,3-dimethylglutarimide on the kinetics of Na-1-C(^{14}) acetate incorporation into rat liver cholesterol \textit{in vivo}</td>
<td>29</td>
</tr>
<tr>
<td>19</td>
<td>30</td>
</tr>
<tr>
<td>The incorporation of subcutaneously injected Na-1-C(^{14}) acetate into the liver cholesterol of a rat \textit{in vivo}.</td>
<td>30</td>
</tr>
</tbody>
</table>
The purpose of this research was to find a derivative of 3-hydroxy-3-methylglutaric acid which would prevent the reduction of 3-hydroxy-3-methylglutaric Coenzyme A to mevalonic acid by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaric Coenzyme A reductase. Reduction of this reaction would inhibit the synthesis of cholesterol at a strategic site since this is the first irreversible step in the biosynthesis of cholesterol. This is the only site which will not interfere with the synthesis of fatty acids or ketone bodies and will not cause a build up of precursors which are potentially pathological. Accumulation of precursors has occurred when inhibitors blocked reactions past the formation of mevalonic acid. Two examples of this type of inhibitor are Mer-29 and AY9944.

3,3-Dimethylglutarimide, 3,3-dimethylglutaric acid, 2,2'-dimethylglutaric acid, 2,2'-dimethylglutaric anhydride, 3,3-dimethylglutaric anhydride, 3-ethyl-3-methylglutarimide, 2-phenylglutaric anhydride and 3-ethyl-3-methylglutaric anhydride were tested. Since all attempts to effect cholesterol synthesis in a rat homogenate failed, the derivatives were examined in vivo. None of these compounds effectively lowered the incorporation of Na-1-C$^{14}$ acetate into cholesterol in the in vivo studies.
INTRODUCTION

Cardiovascular disease is now, and has been since 1920, the leading cause of death in the United States. According to H. J. Sanders (1965), cardiovascular disease caused 983,504 deaths or 54.1 per cent of all deaths in the United States during 1963. Without question this disease is the biggest single medical and public health problem facing Americans today. Although the term cardiovascular disease is a very ambiguous one covering almost every form of heart disease, the major killer in this category is a more specific disease called atherosclerosis. Atherosclerosis, Sanders says, accounted for 56 per cent of the cardiovascular deaths in 1963.

In atherosclerosis there is a fatty degeneration and an infiltration of lipids into the walls of arteries. This lipid infiltration is especially dangerous when it is allowed to continue over many years. In these acute cases the lipid deposits cause arterial obstruction which is generally fatal.

Although advances in the diagnostic and therapeutic management of atherosclerosis have been made, no important reduction of mortality has occurred (Kannel et al. 1961, p. 33). The fact that mortality rate is high in spite of modern therapeutic efforts clearly demonstrates the urgent need for additional research.

The pathogenesis of atherosclerosis is as yet unidentified, but many factors have been shown to be closely associated. Of these
factors three have been shown by Kannel et al. (1961) to be associated with the proneness to develop atherosclerosis. They are elevated serum cholesterol, hypertension and left ventricular hypertrophy. To the biochemist, the depression of elevated serum cholesterol represents the most challenging approach to the control of atherosclerosis. In a six year follow-up study by Kannel's group the association between hypercholesterolemia and coronary heart disease was shown quite clearly. His study, comprising some seven thousand people, showed that those individuals with serum cholesterol levels over 244 g per 100 ml had more than three times the incidence of coronary heart disease as did those with levels less than 210 g per 100 ml. It should be mentioned that while falling levels of serum cholesterol may be considered successful therapy, high serum cholesterol is not invariably associated with clinical coronary disease. At the same time, it should be kept in mind that normal cholesterol levels are necessary for the production of bile acids, certain hormones and membrane structures. With these facts in mind, the control of abnormally high plasma cholesterol levels is not intended to be a miraculous cure for atherosclerosis, but it would be a counterthrust against this mass killer.

Several approaches to the control of abnormally high cholesterol levels have become exceptionally popular in recent years. These are (1) to block cholesterol absorption, (2) to prevent its reabsorption, (3) to sequester it, and (4) to inhibit its endogenous production. Of these four approaches, it is this researcher's belief that controlling the endogenous production is the most favorable approach. This method
takes into consideration the body's own mechanism for synthesis control and uses it to bring about a more favorable balance. Abnormally high concentrations of cholesterol in the body inhibit the body's ability to produce more. This inhibition has been found by Siperstein and Guest (1960) to occur at the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid. This is the only site which will not interfere with the synthesis of fatty acids or ketone bodies and will not cause a build up of precursors which are potentially pathological. Such a build up has occurred following the administration of Mer-29 (Avigan et al. 1960), AY9944 (Givner and Dvornik 1965) and Tolbutamide (Dalidowicz and McDonald 1965).

An effective and safe inhibition must stop the reduction of 3-hydroxy-3-methylglutaryl-CoA. Due to such work as Durr and Rudney (1960), Brodie and Porter (1960) and Brodie, Wasson and Porter (1963), considerably more is now known about this strategic reaction.

While many inhibitors have received the attention of countless researchers over the last decade, no drug is on the market today which will effectively lower cholesterol levels without producing unwanted side effects. Included in the long list of compounds which have been studied recently for their ability to inhibit the synthesis of cholesterol are antimetabolites of mevalonic acid (Stewart and Woolley 1964), 2-p-chlorophenoxyisobutyryl ethyl ester (Avoy, Swyryd and Gould 1965), thyroalkanols (Wechter, Phillips and Kagan 1965), basic carbinols and related compounds (Short et al. 1965), 4-(2-chlorophenyl)-2-(p-methoxyphenoxymethyl)-1-piperazine ethanol (Rodney, Black and Bird 1965) and W-398 (Douglas 1964).
The research reported in this thesis is an effort to apply the antimetabolite principles that Pletscher, Grey, and Wursch (1961) used on later intermediates of cholesterol synthesis to the reduction of 3-hydroxy-3-methylglutaryl-CoA. This work uses various metabolites of 3-hydroxy-3-methylglutaryl-CoA to competitively inhibit the enzyme which causes 3-hydroxy-3-methylglutaryl-CoA reduction to mevalonic acid and thereby inhibit cholesterol synthesis.
EXPERIMENTAL PROCEDURES

One in vitro and two in vivo procedures were developed to test the effect of a variety of analogs of 3-hydroxy-3-methylglutaric acid on cholesterol biosynthesis. The in vitro procedure was based on the ability of a rat liver homogenate to synthesize cholesterol to the extent that there was a net gain in its concentration large enough to be determined colorimetrically. Both in vivo procedures were concerned with testing the ability of the analogs to lower the rat liver incorporation of Na-1-C\(_{14}\) acetate into cholesterol.

I. In Vitro--A Rat Liver Homogenate for Synthesizing Cholesterol from Acetate--Essentially, the in vitro procedure was that reported by Frantz and Bucher (1954). It consisted of sacrificing a Sprague-Dawley rat, excising, chilling, weighing, and homogenizing the liver for two minutes with two and one half volumes of 0.08 M potassium phosphate buffer pH 7.4 in an Omni Mixer at 0°C. In the fourth and all succeeding experiments the Omni Mixer was replaced by a Potter-Elvejhem homogenizer. The liver homogenate was centrifuged at 500 X g for ten minutes in a Sorvall RC-2 automatic refrigerated centrifuge in a SS-34 head and the supernatant fractions were pooled. Subsequently, the cofactors were added and 2.5 ml aliquots were transferred to 50 ml incubation flasks. Acetate, and in some cases the metabolic analog, were added to the flasks and the mixture was incubated in a Dubnoff shaker.
at 37°C under 100% oxygen for varying lengths of time (four to twelve hours). Each incubation flask contained the following solutions:

2.5 ml of 8% rat liver homogenate
0.5 ml of MgCl₂, 0.0048 M
0.5 ml of nicotinamide, 0.03 M
0.5 ml of NAD, 0.0008 M
0.5 ml of inhibitor solution or water
5.0

At incubation intervals of one hour, the contents of four to eight replicate incubation flasks were quickly washed into 30 ml screw top hydrolysis tubes with three washings of 11.5% potassium hydroxide. The samples were hydrolyzed in an incubator at 37°C overnight. Cholesterol was extracted with petroleum ether and analyzed after Abell et al. (1952). This method involved the treatment of tissue with alcoholic potassium hydroxide to liberate the cholesterol from the lipoprotein complexes and to saponify the cholesterol esters. Dilution of the alcoholic solution with water was followed by extraction of the cholesterol with a measured volume of petroleum ether. An aliquot of this fraction was analyzed for cholesterol by measuring the optical density at 620 nm exactly thirty minutes after the initiation of the color reaction of cholesterol with the Libermann-Burchard reagent. This reagent was a combination of twenty volumes of cold acetic anhydride, one volume of concentrated sulfuric acid and ten volumes of glacial acetic acid.
Due to the variation of the moisture content of various livers, the homogenates of any two experiments did not contain the same quantity of liver tissue. This made a comparison between experiments unreliable. Consequently, from the fourth experiment on, the protein content of the homogenates was determined and the results reported as mg of cholesterol per g of dry protein. To determine the protein content, eight 2.5 ml aliquots of homogenate were transferred to fifteen ml round bottom centrifuge tubes with an equal volume of 10% trichloroacetic acid. The samples were centrifuged at top speed for ten minutes in an International clinical centrifuge and the supernatant discarded. The precipitate was resuspended in 4% trichloroacetic acid, recentrifuged and the supernatant discarded. This was repeated four times with cold and once with hot (90°C) 4% trichloroacetic acid. The precipitate was washed once with warm 95% ethanol, twice with warm 2:2:1 alcohol-ether-chloroform solution and once with warm ether. After air drying, the protein was resuspended in acetone, transferred to a tared dish, dried and brought to constant weight.

II. In Vivo--The Effect of Metabolic Analogs on the Incorporation of Intraperitoneally Injected Na-1-C¹⁴ Acetate into Rat Liver Cholesterol at the Time of Maximum Incorporation--A.) To determine the time at which the maximum amount of Na-1-C¹⁴ acetate was incorporated into cholesterol, six rats were injected intraperitoneally with 4.9 µc of Na-1-C¹⁴ acetate and were sacrificed with ether at five, ten, fifteen, thirty, forty-five and sixty minute intervals after injection. The
livers were excised, two 0.5 g samples were taken from each liver and the samples were quickly frozen in acetone-Dry Ice bath. The sampling procedure took less than three minutes and was consistent in all experiments. The samples were later homogenized in 5 ml of 11% potassium hydroxide, transferred to screw top vials and hydrolyzed overnight at 37°C. After cooling to room temperature, the cholesterol was extracted from each sample with petroleum ether (BP=30-60°C) and transferred to centrifuge tubes. The petroleum ether was driven off in a stream of air and the residue was resuspended in 5 ml of 1:1 acetone-ethanol. Carrier cholesterol (0.4mg) was added and the digitonin-precipitable sterols were precipitated with an excess of 0.5% digitonin in 50% ethanol. The precipitate was centrifuges at top speed for one hour in an International clinical centrifuge and for one more hour at 12,000 RPM in a Servall SS-4 centrifuge with a SS-34 centrifuge head. The supernatant was discarded and the precipitate resuspended in anhydrous methanol and quantitatively transferred into planchets. All samples were counted in a Nuclear-Chicago 181A thin window Geiger-Muller tube counter and corrected for background. The results were recorded as counts per minute per 0.5 g of liver or mg of dry protein.

B.) To determine the effect of the metabolic analogs, twelve rats were given seven daily intraperitoneal injections of an inhibitor dissolved in either distilled water or 2% dimethylsulfoxide. Three sets of three animals received 30, 60 or 90 mg/kg, respectively. Also, three animals received the same volume of inhibitor solvent. On the seventh
day, six hours after inhibitor injection, each animal received 4.9 μc of Na-1-C¹⁴ acetate intraperitoneally. At exactly thirty minutes after injection, all rats were killed with ether and their livers excised. Two 0.5 g samples were taken from each liver and quickly frozen in an acetone-Dry Ice bath. The samples were later analyzed as described in part A of this procedure.

III. In Vivo--Analysis of the Potential Inhibitors' Ability to Change the Kinetics of the Rats' Incorporation of Subcutaneously Injected Na-1-C¹⁴ Acetate into Liver Cholesterol--A.) The intraperitoneal injection was originally preferred because of the ease with which it was performed and the fast rate of absorption following its use. However, later it was found that better agreement between samples could be achieved using subcutaneous injections. Consequently, another incorporation study was performed to establish the kinetics of the incorporation of Na-1-C¹⁴ acetate into cholesterol by this method of administration.

Six rats were injected subcutaneously with 9.8 μc of Na-1-C¹⁴ acetate. At thirty, sixty, ninety, and one hundred twenty, one hundred fifty and one hundred eighty minutes after the label injection, the animals were sacrificed. Sampling and analysis were performed as described for the determination of incorporation from the intraperitoneal injection.

B.) To determine the effect of the analogs on the kinetics of incorporation, the following procedure was used. Every hour, for three
hours proceeding the injection of labeled acetate, 30 mg/kg of inhibitor were injected subcutaneously into each of sixteen rats. Another sixteen rats received a similar volume of saline solution. Three hours after the injection, 12.2 μc of Na-1-C\textsuperscript{14} acetate were injected subcutaneously into each rat. Eight rats, four of which had received the inhibitor and four of which had received saline, were immediately sacrificed. Liver samples from these animals provided the level of Na-1-C\textsuperscript{14} acetate incorporation at 0 time. Similarly, animals were sacrificed one, two, and three hours after labeling. In all cases the animals were killed with ether, the livers excised, two 0.5 g samples taken and the samples quickly frozen. The analysis of the samples was performed as described in IIA.
RESULTS AND DISCUSSION

I. In Vitro--A Rat Liver Homogenate for Synthesizing Cholesterol from Acetate--The effect sought in this study was the stimulation of cholesterol synthesis in the rat liver homogenate system of Frantz and Bucher (1954) to such an extent that the cholesterol synthesis could be determined by the Abell-Kendall technique. According to Abell et al. (1952), this procedure is capable of detecting 0.2 mg of cholesterol with less than 10% error. This represents approximately two thirds of the cholesterol that is normally found in 2.5 ml of 8% rat liver homogenate (200 mg of liver tissue). It was believed that the addition of cofactors and substrate concentrations above physiological concentrations would increase the level of cholesterol beyond that initially found in the homogenate.

Eight independent experiments were performed in which concentrations of homogenate and acetate, incubation conditions and cofactors were varied. The first experiment using an 8% rat liver homogenate gave some indication that the homogenate could effectively synthesize cholesterol. In this study, an homogenate was prepared from a single rat liver and four replicate runs were made. The results (Figure 1) showed that one of the four replicates increased in total cholesterol content. It appeared from this that the system was capable of synthesizing the required concentration of cholesterol. Other
Figure 1:--The total cholesterol content of four replications of rat liver homogenate incubated in *vitro* at 37°C. All flasks are identical replications and contain homogenized tissue from the same liver. The incubation solution contained the following: 2.5 ml of 8% rat liver homogenate, 2.4 μ moles MgCl₂, 15 μ moles nicotinamide, 6 μ moles sodium acetate, 0.4 μ moles NAD and water to make 5.0 ml.
experiments were designed to determine if this effect could be repeated. In a second experiment, a 40% homogenate was used to produce larger differences. In addition, three of the incubation flasks contained two, four or eight times the standard acetate concentration. As shown in Figure 2, none of the incubation flasks showed an increase in cholesterol.

In order to determine if the concentration of homogenate was critical, a third experiment was designed to compare the effect of using 5, 10, 20 and 40% liver homogenate as the incubation medium. Figure 3 shows that none of these concentrations produced favorable results. Furthermore, the cholesterol concentration appeared to decrease upon incubation in all four flasks.

The use of a liver homogenate prepared under more gentle conditions did not result in an increase in cholesterol synthesis. In the fourth experiment the homogenate was prepared using a Potter-Elvejhem homogenizer instead of the Omni-Mixer which was used in the three previous experiments. The results showed no increase in total cholesterol. These results (Figure 4) were reported as mg of cholesterol per g of dry protein. The protein content of the incubation flasks was determined following its precipitation with trichloro-acetic acid as described in part I of the procedures.

A substitution of NADP for NAD did not yield an apparent increase in cholesterol content (Figure 5). This substitution was made as NADP (not NAD) is the cofactor used in the reduction reactions in the synthesis of cholesterol. Moreover, the addition of ATP to the
Figure 2:--The effect of various concentrations of acetate on the cholesterol content of a 40% rat liver homogenate. Except for the acetate and liver homogenate concentrations, the conditions were identical to those listed in Figure 1.

Figure 3:--The total cholesterol content of incubated homogenate of different concentrations. The incubation flasks contained twice the volume of the incubation solutions described in Figure 1 and, except for the homogenate, the concentration of the contents were those listed in Figure 1. Three 2 ml aliquots were pipetted out of the incubation flasks at the second, fourth and sixth hour of incubation, and the cholesterol contents averaged.
Figure 4:--The biosynthesis of cholesterol by rat liver homogenate prepared with a Potter-Elvejhem homogenizer and incubated in a Dubnoff shaker. The contents of the incubation flasks were those described in Figure 1. Every hour six incubation flasks were removed from the Dubnoff shaker and analyzed for cholesterol content according to Abell et al. (1952). Each point represents an average of the six samples.

Figure 5:--The effect of using NADP in place of NAD as a cofactor for the rat liver homogenate. Every hour four incubation flasks were removed and analyzed as described in part I of the procedures. The points represent an average of the four determinations. The incubation solution was that described in Figure 1. NADP was used in the same concentration as was NAD.
system containing either NAD (Figure 6) or NADP (Figure 7) did not effect an increase in cholesterol concentration.

Further studies suggested that the lack of apparent increase in cholesterol content upon incubation with the additives was due to the absence of detectable biosynthesis rather than the presence of degradative enzymes. In an experiment where 0, 0.25, 0.50 and 1.0 mg of standard cholesterol were added separately to four complete homogenate solutions and the solutions incubated for four hours, there was no significant loss of cholesterol in any of the four levels. This suggested degrading enzymes of cholesterol were absent. Studies using acetate-1-C\textsuperscript{14} showed no incorporation of the label in the rat liver homogenate (Figure 8). This absence of biosynthesis \textit{in vitro} suggested that formidable problems would require resolution before testing of the new inhibitors could be initiated. Consequently, this approach was abandoned in favor of an \textit{in vivo} approach.

\textbf{II. In Vivo--The Effect of Metabolic Analogs on the Incorporation of Intraperitoneally Injected Na-1-C\textsuperscript{14} Acetate into Rat Liver Cholesterol at the Time of Maximum Incorporation--A.)} The \textit{in vivo} procedures were successful in showing the incorporation of labeled acetate into cholesterol. Following the intraperitoneal injection of 4.9 µc of Na-1-C\textsuperscript{14} acetate, maximum incorporation was obtained within thirty minutes (Figure 9). This period of incorporation was then adopted to determine the effect of the inhibitors on the incorporation of acetate into cholesterol.
Figure 6:--Results of the addition of ATP and the substitution of NADP for NAD on cholesterol biosynthesis by rat liver homogenate. The conditions described in Figure 1 were used except the incubation solution contained 0.8 μ moles of ATP and NADP was substituted for NAD. Four flasks were removed per hour and the average of the analysis of these four flasks was plotted. Four aliquots of liver homogenate identical to those used in the incubation flasks were analyzed for protein content according to part I of the procedures.

Figure 7:--The effect of ATP addition on cholesterol biosynthesis by rat liver homogenate. The incubation conditions were identical to those in Figure 1 except for the addition of 0.8 μ moles of ATP. Four flasks were removed every hour, analyzed for cholesterol content and averaged.
Figure 8:--The incorporation of Na-1-C\textsuperscript{14} acetate into cholesterol by an 8% rat liver homogenate. The incubation solution used in this experiment was that described in Figure 1 except for the substitution of 147 µc of Na-1-C\textsuperscript{14} acetate for the unlabeled acetate. Two incubation flasks were removed every hour for three hours and analyzed for cholesterol content as described in part IIA of the procedures.
Figure 9: Incorporation of intraperitoneally injected Na-1-C\textsuperscript{14} acetate into the liver cholesterol of a rat. Twelve rats were injected intraperitoneally with 4.9 \( \mu \)c of Na-1-C\textsuperscript{14} acetate. At exactly five, ten, fifteen, thirty, forty-five and sixty minutes after the label injection, two rats were sacrificed and two 0.5 g samples taken from each liver. The samples were quickly frozen and later analyzed as described in part IIA of the procedures.
B.) The following criteria were used to determine the effectiveness of an inhibitor of cholesterol biosynthesis: (1) the inhibitor must reduce the incorporation of Na-1-C\textsuperscript{14} acetate into cholesterol at the point of maximum acetate incorporation, (2) it must be effective within seven treatments over a seven day time span and (3) one of three dosage levels, all less than 100 mg/kg, must reduce the incorporation of acetate into cholesterol at a point six hours after the last injection. The reasons for these conditions were two-fold. First, it was thought best to test many inhibitors to a limited extent and then test the most promising inhibitor more thoroughly. Second, this research was intended to be a search for a pharmaceutical compound which would have the characteristics required by this procedure.

The first of the analogs tested was 3,3-dimethylglutarimide which varied from the natural precursor 3-hydroxy-3-methylglutaric acid by the addition of an imide and a methyl group.

\[
\begin{align*}
\text{CH}_3 \\
\text{HOOC-CH}_2-\text{C-CH}_2-\text{COOH} \\
\text{OH} \\
3\text{-Hydroxy-3-methylglutaric Acid} \\
\text{CH}_3 \\
\text{HOOC-CH}_2-\text{C-CH}_2-\text{CONH}_2 \\
\text{CH}_3 \\
3,3\text{-Dimethylglutarimide}
\end{align*}
\]
The results of the study with this derivative were very encouraging in that definite inhibition occurred (Figure 10). Since the analog differed from the substrate by two substitutions, it was of interest to determine which of the substitutions was responsible for the inhibitor's effectiveness. The compound 3,3-dimethylglutaric acid which contained only the methyl substitution of the preceding compound produced an increase rather than a decrease in the incorporation of labeled acetate into cholesterol (Figure 11). Possibly, rats are able to convert this derivative to the natural precursor resulting in additional synthesis.

Unfortunately, the derivative with only the imide substitution was not available and its effect could not be determined. Other dimethyl substitution compounds were available and were tested. 2,2'-dimethylglutaric acid (Figure 12) produced what appeared to be an initial decrease in the incorporation at the 10 and 25 mg/kg dosage levels. An analysis of variance, testing the treatment variance against random error with an F test, failed to show any significant change in incorporation with either 2,2'-dimethylglutaric acid or with 2,2-dimethylglutaric acid. The failure of the dimethyl derivatives to inhibit labeled acetate incorporation led to the testing of 3-ethyl-3-methylglutarimide. Figure 15 demonstrates that here again there was no decrease in incorporation.

A search for an entirely new type of derivative resulted in the testing of several anhydride derivatives of 3-hydroxy-3-methylglutaric
Figure 10:--The effect of 3,3-dimethylglutarimide on the incorporation of Na-1-C\textsubscript{14} acetate into rat liver cholesterol in vivo. Two Sprague-Dawley rats were injected intraperitoneally with 5 mg/kg of inhibitor, two with 50 mg/kg and two with an equal volume of saline, once a day for seven days. Six hours after the last injection, all animals were injected intraperitoneally with 4.9 µc of Na-1-C\textsubscript{14} acetate. Exactly thirty minutes later the animals were sacrificed and two 0.5 g liver samples were taken and quickly frozen in an acetone-Dry Ice bath. The samples were analyzed as described in part IIA of the procedures.
Figure 11:--The results of 3,3-dimethylglutaric acid on the incorporation of Na-1-C\textsuperscript{14} acetate into rat liver cholesterol \textit{in vivo}. One rat was injected intraperitoneally with 100 mg/kg, one with 5 mg/kg and two with an equal volume of saline, once a day for seven days. Six hours after the last injection, all rats received 4.9 \mu c of Na-1-C\textsuperscript{14} acetate intraperitoneally. The rats were sacrificed exactly thirty minutes after the labeled acetate injection and two 0.5 g liver samples were taken. The samples were quickly frozen and later analyzed as described in part IIA of the procedures.
Figure 12:--Effect of 2,2'-dimethylglutaric acid and 2,2-dimethylglutaric acid on Na-1-C¹⁴ acetate incorporation into rat liver cholesterol in vivo. Both of these drugs were tested in the same manner. Two animals were injected intraperitoneally with 100 mg/kg of the inhibitor, two with 10 mg/kg and two with saline, once a day for seven days. Six hours after the last injection all the rats were injected with 4.9 µc of Na-1-C¹⁴ acetate. Exactly thirty minutes later all animals were sacrificed, two 0.5 g samples taken and the samples were quickly frozen and analyzed as described in part IIA of the procedures.

Figure 13:--Incorporation of Na-1-C¹⁴ acetate into rat liver cholesterol after the intraperitoneal injection of 2,2'-dimethylglutaric anhydride. The procedure employed in this experiment was identical to that procedure described in Figure 12.
Figure 14:--The effect of 3,3-dimethylglutaric anhydride on the incorporation of Na-1-C^{14} acetate into rat liver cholesterol in vivo. Four sets of three animals were injected with 30, 60, 90 mg/kg of inhibitor and an equal volume of saline, once a day for seven days. Six hours after the last injection, each rat was injected with 4.9 μc of Na-1-C^{14} acetate. Exactly thirty minutes later, all animals were sacrificed and two 0.5 g liver samples taken. The samples were immediately quick frozen and later analyzed as described in part IIA of the procedures.

Figure 15:--The effect of 3-ethyl-3-methylglutarimide on the incorporation of Na-1-C^{14} acetate into rat liver cholesterol in vivo. This experiment was performed identically to that experiment described in Figure 14.
acid. These anhydrides were thought to make effective inhibitors as anhydrides are more reactive with the sulfhydryl groups of the enzyme 3-hydroxy-3-methylglutaryl CoA reductase than is the acetyl coenzyme A group of natural precursor.

Experiments with two anhydrides, 2,2'-dimethylglutaric anhydride and 3,3-dimethylglutaric anhydride, showed no effect on cholesterol synthesis (Figures 13 and 14). A completely different anhydride, 2-phenyl-glutaric anhydride, was also tested. Figure 16 indicated that in doses larger than 30 mg/kg, 2-phenylglutaric anhydride stimulated acetate incorporation. Figures 16 and 17 indicate that 2-phenylglutaric anhydride and 3-ethyl-3-methylglutaric anhydride produced strikingly similar results. Both compounds in doses larger than 30 mg/kg produced a very large increase in incorporation. These two derivatives could have been tested more thoroughly to try to determine where and how this acceleration was accomplished. However, it was not the objective of this research to find an accelerator of cholesterol synthesis.

III. In Vivo--Analysis of the Potential Inhibitors' Ability to Change the Kinetics of the Rats' Incorporation of Subcutaneously Injected Na-1-C\(^{14}\) Acetate into Liver Cholesterol--A.) The first in-vivo procedure (part IIB of the procedures) tested the effect of the analogs on the point of maximum incorporation of Na-1-C\(^{14}\) acetate into cholesterol. The second in vivo procedure (part IIIB of the procedures), a more inclusive examination, tested the effect of the potential inhibitors on the entire kinetics of labeled acetate incorporation.
Figure 16: Results of the subcutaneous injection of 2-phenylglutaric anhydride on the incorporation of Na-1-C\textsuperscript{14} acetate into cholesterol \textit{in vivo}. The procedure used in this experiment was the procedure described in Figure 14.

Figure 17: 3-Ethyl-3-methylglutaric anhydride's effect on the incorporation of Na-1-C\textsuperscript{14} acetate into rat liver cholesterol \textit{in vivo}. The procedure described in Figure 14 was used in this experiment.
It was found from experience with the first in vivo procedure that much biological variation was hampering the results. In an effort to minimize this effect, the intraperitoneal injections were abandoned in favor of subcutaneous injections. This made it necessary to establish the incorporation curve of Na-1-C\textsuperscript{14} acetate from a subcutaneous injection. A comparison of the incorporation curves resulting from the two forms of injection can be obtained by comparing Figures 9 and 19.

3,3-Dimethylglutarimide, which had shown favorable results when tested for its effect on the point of maximum acetate-1-C\textsuperscript{14} incorporation, was tested for its effect on the overall kinetics of incorporation. Statistical analysis\textsuperscript{1} of the control and the treatment group at the one hour time period (Figure 18) showed these groups differed significantly at the 0.01 level. At the two hour time period, the control group was again significantly higher, but at the 0.05 level. Both the treatment and the control group gave statistically similar results at the zero and the four hour time period.

Hence, it could be concluded that 3,3-dimethylglutarimide raised the level of labeled acetate incorporation into cholesterol at these two time periods in the absorption spectrum.

\textsuperscript{1} Analysis of Variance at each time period, testing the treatment variance against random error with an F test, with one and six degrees of freedom.
Figure 18: The effect of 3,3-dimethylglutarimide on the kinetics of Na-1-C\(^{14}\) acetate incorporation into rat liver cholesterol in vivo. Every hour for three hours preceeding the injection of labeled acetate, 30 mg/kg of the inhibitor were injected subcutaneously into sixteen rats. In the same manner, an additional sixteen rats received saline solution. Three hours after the initial injection, 12.2 \(\mu\)c of Na-1-C\(^{14}\) acetate were injected subcutaneously into each rat. Eight rats, four of which had received the inhibitor and four of which had received saline, were immediately sacrificed. Similarly, animals were sacrificed one, two and three hours after labeling. Samples were taken and analyzed as described in part IIA of the procedures. Each point on the graph represents the average of values from four animals.
Figure 19:--The incorporation of subcutaneously injected Na-1-C¹⁴ acetate into the liver cholesterol of a rat in vivo. Six rats were injected subcutaneously with 9.8 µc of Na-1-C¹⁴ acetate. At thirty, sixty, one hundred twenty, one hundred fifty and one hundred eighty minutes after the label injection one animal was sacrificed and two 0.5 g samples taken. The samples were quickly frozen in an acetone-Dry Ice bath and later analyzed as described in part IIA of the procedures.
SUMMARY

Of the compounds 3,3-dimethylglutarimide, 3,3-dimethylglutaric acid, 2,2'-dimethylglutaric acid, 2,2-dimethylglutaric acid, 2,2'-dimethylglutaric anhydride, 3,3-dimethylglutaric anhydride, 3-ethyl-3-methylglutaric amide, 2-phenylglutaric anhydride and 3-ethyl-3-methylglutaric anhydride, only 3,3-dimethylglutarimide lowered the \textit{in vivo} incorporation of Na-1-C$^{14}$ acetate into cholesterol at the time period of maximal incorporation. When this compound was tested for its effect on the entire kinetic spectrum of incorporation, it caused an increase rather than a decrease in incorporation.
LITERATURE CITED


