THE CHEMICAL ACTIVATION OF STEROLS

III. THE CHEMICAL ACTIVATION OF CHOLESTEROL*

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The chemical antirachitic activation of sterols originated with the discovery of Bills (1) that cholesterol could be activated to a slight extent by heating a carbon tetrachloride solution of cholesterol with floridin. Later Yoder (2) found that cholesterol could also be converted into an active product by treatment with sulfur trioxide or with a mixture of sulfuric acid and acetic anhydride. In a study of the activation with sulfuric acid-acetic anhydride (3), it has been shown that a specific relationship probably exists between chemical configuration and chemically activatable cholesterol or cholesterol derivatives. The activation of cholesterol with sulfuric acid-acetic anhydride was investigated in an attempt to determine the mechanism of the reaction.

It has been found (3) that the provitamin D of cholesterol is not the precursor of the active substance produced by the action of sulfuric acid-acetic anhydride on cholesterol. The possibility of the provitamin D of heated purified cholesterol being the precursor of the active substance was investigated and the effect of ultraviolet irradiation on the potency of the reaction product was determined.

EXPERIMENTAL

The effect of various proportions of acetic anhydride and sulfuric acid on the activation of cholesterol was studied. In a series of 50 cc. Erlenmeyer flasks were placed 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid (99.5 per cent), and various

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quantities of acetic anhydride (98 per cent) and concentrated sulfuric acid (95 per cent). The reaction mixtures were heated to 90°, stoppered with a cork, and then the heating was continued in an oven at 85–90° for 3 hours. The reaction products were concentrated and aliquots of the ether-alcohol solutions of the residues containing the equivalent of 6 mg. of the original cholesterol were tested biologically with rats for antirachitic activity. It was found that the maximum antirachitic potency produced by this procedure was obtained when the molar proportions were 0.0025 mole of acetic anhydride and 0.002 mole of sulfuric acid per 0.001 mole of cholesterol.

The effect of time during which 0.001 mole of cholesterol was heated in 4 cc. of glacial acetic acid solution at 85–90° with 0.0025 mole of acetic anhydride and 0.002 mole of sulfuric acid was investigated. It was found that the maximum potency was obtained in about 3 hours and that the potency was about the same when the reaction mixture was heated any length of time between 3 hours and 40 hours.

The effect of temperature in the production of antirachitically active material from cholesterol also was studied. It was found that the product obtained by a 3 hour treatment of cholesterol with sulfuric acid-acetic anhydride at room temperature was inactive at the 24 mg. level, slightly active if treated at 55°, and most potent when produced at about 85–90°. Although the product obtained when the temperature was maintained at 24° for 3 or 24 hours failed to induce noticeable calcification when fed to rachitic rats at the 24 mg. level, it was found that when the reaction mixture which had stood at 24° for 3 or 24 hours was then heated at 85–90° for 3 hours the product was found to possess about the same antirachitic activity as if the heating had been carried out at once.

Furthermore, it was observed that sulfur dioxide was slowly evolved when the reaction mixture was allowed to stand at room temperature for a few hours and the evolution of a trace of carbon dioxide was detected when the reaction mixture had stood for 40 hours. Both sulfur dioxide and carbon dioxide were evolved when the reaction mixture was heated at 85–90° for 3 hours.

The evolution of sulfur dioxide was detected by passing nitrogen through the reaction mixture and into a barium hydroxide solu-
tion. The white precipitate which separated from the barium hydroxide solution was removed by filtration and dissolved in dilute hydrochloric acid; the odor of sulfur dioxide was unmistakable. Bromine water was added to the hydrochloric acid solution and a white precipitate was formed, demonstrating that sulfur dioxide was formed in the reaction mixture. The quantity of evolved sulfur dioxide was determined and the evolution of carbon dioxide was detected by passing nitrogen through the reaction mixture and collecting the sulfur dioxide in dilute hydrogen peroxide and the carbon dioxide in barium hydroxide solution. The nitrogen was bubbled through a barium hydroxide solution and passed through a calcium chloride tube before it was passed into the reaction mixture. The stream of gas from the reaction mixture was bubbled through 50 cc. of a 2 per cent hydrogen peroxide solution to collect the sulfur dioxide and then through 50 cc. of a saturated barium hydroxide solution to collect the carbon dioxide. The system was flushed out with nitrogen and then a slow stream of nitrogen was passed through while the reaction mixture in a 50 cc. flask was heated at 85–90° in a water bath. The hydrogen peroxide solution acidified with 1 cc. of 6 N hydrochloric acid was heated to boiling, and a solution of barium chloride was added dropwise with stirring. After cooling, the barium sulfate was filtered, ignited, and weighed. The white precipitate in the barium hydroxide solution was soluble in acetic acid and no precipitate was obtained by the addition of bromine water to a hydrochloric acid solution of the precipitate, demonstrating that the white precipitate was barium carbonate.

The observation that heat is essential in the production of maximum activity led to a study of the sulfuric acid-acetic anhydride reagent. Below 0° sulfuric acid reacts with acetic anhydride to form acetylsulfuric acid which, if warmed, is converted to sulfoacetic acid (4). Sulfoacetic acid can be prepared by heating a mixture of sulfuric acid and an excess of acetic anhydride to 80° to the disappearance of free sulfuric acid (5, 6) and by treating acetic acid with fuming sulfuric acid (7).

Cyclohexene and camphor can be converted into sulfonic acids by means of a mixture of sulfuric acid and acetic anhydride (acetylsulfuric acid). Friese (8) found that cyclohexene in acetic acid solution could be converted into o-cyclohexanolsulfonic acid
by the action of a molar equivalent of sulfuric acid and an excess of acetic anhydride at $-20^\circ$ and then warmed up to room temperature. When $d$-camphor (1 mole) is treated with a well cooled mixture of sulfuric acid (1 mole) and acetic anhydride (2 moles), it is converted into $d$-camphor-10-sulfonic acid (9, 10), whereas it yields $dl$-camphor-8(or 9)-sulfonic acid by the action of either fuming sulfuric acid or chlorosulfonic acid (11). A sulfonic acid group enters a (different) methyl group in these camphorsulfonic acids.

Carbon dioxide was evolved when a solution of sulfoacetic acid prepared from 0.002 mole of sulfuric acid and 0.005 mole of acetic anhydride, dissolved in 4 cc. of glacial acetic acid, was heated at 85–90$^\circ$ for 3 hours. Melsens (7) found that carbon dioxide was evolved when heat was applied during the preparation of sulfoacetic acid by the action of fuming sulfuric acid on acetic acid. In addition to carbon dioxide a trace of sulfur dioxide was evolved when a solution of 0.002 mole of sulfuric acid or a mixture of 0.002 mole of sulfuric acid and 0.0025 mole of acetic anhydride dissolved in 4 cc. of glacial acetic acid was heated at 85–90$^\circ$ for 3 hours.

**Various Reagents**

The effect of sulfuric acid, sulfoacetic acid, fuming sulfuric acid, and chlorosulfonic acid in imparting antirachitic activity to cholesterol was investigated. Data obtained to determine the antirachitic activity of the products prepared by the action of various reagents on cholesterol are presented in Table I.

The action of the sulfuric acid on cholesterol in the ratio of 2 moles per mole was studied. In a 50 cc. Erlenmeyer flask were placed 0.001 mole (0.386 gm.) of dry cholesterol, 4 cc. of glacial acetic acid, and 0.002 mole (0.112 cc.) of concentrated sulfuric acid. The reaction mixture was heated at 85–90$^\circ$ for 3 hours, during which time a color change through violet to green to a brownish purple color was observed, a solid separated, and sulfur dioxide and carbon dioxide were evolved. After the reaction product was concentrated, the residue was found to be less active than the product which has been produced consistently by the action of the sulfuric acid-acetic anhydride reagent.

Cholesterol was treated with sulfuric acid and the reaction...
product was heated with acetic anhydride. A mixture of 0.001 mole by dry cholesterol, 4 cc. of glacial acetic acid, and 0.002 mole of concentrated sulfuric acid was heated at 85–90° for 3 hours. Then 0.0025 mole (0.24 cc.) of acetic anhydride was added and the reaction mixture was heated an additional 3 hours, during which time no change in appearance of the reaction mixture was observed. This reaction product was about as potent as that produced by the action of sulfuric acid.

**Table I**

*Antirachitic Activation of Cholesterol by Heat Treatment with Various Reagents*

The line test gave average plus values of 2.5 for each reagent. A 2 plus value here denotes approximately 5 vitamin D units, United States Pharmacopoeia XI (12).

<table>
<thead>
<tr>
<th>Reagents used</th>
<th>Quantity of product fed per rat</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂SO₄</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>&quot; then Ac₂O</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Ac₂O, then H₂SO₄</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>HO₃SCH₂COOH</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Ac₂O, then HO₃SCH₂COOH</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>HO₃SO₄OSO₂OH</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>ClSO₄H</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>H₂SO₄·Ac₂O</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

* Based on the weight of the original cholesterol.

The action of acetic anhydride followed by sulfuric acid was studied. A mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, and 0.0025 mole of acetic anhydride was heated at 85–90° for 3 hours. Then 0.002 mole of concentrated sulfuric acid was added and the reaction mixture was heated an additional 3 hours, during which time the colorless solution changed through a green to a brown color, sulfur dioxide and carbon dioxide were evolved, and a resin separated on the sides of the flask. This product was as active as that produced by the action of the sulfuric acid-acetic anhydride reagent.
The action of sulfoacetic acid on cholesterol was investigated. A sample of sulfoacetic acid was prepared in a cut-off sample tube by heating a mixture of 0.002 mole of sulfuric acid and 0.005 mole of acetic anhydride at 85° for 3 minutes. The mixture was stirred before and after heating with a short stirring rod. The tube containing the sulfoacetic acid was dropped into a flask containing 0.001 mole of dry cholesterol and 4 cc. of glacial acetic acid and the sulfoacetic acid on the stirring rod was washed into the flask by means of a few drops of glacial acetic acid. The reaction mixture was heated at 85–90° for 3 hours. The reaction mixture changed through a green to a brown color, a solid separated, and sulfur dioxide and carbon dioxide were evolved. The reaction product was found to be about as active as that obtained by the action of sulfuric acid on cholesterol.

The action of acetic anhydride followed by sulfoacetic acid was studied. A mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, and 0.0025 mole of acetic anhydride was heated at 85–90° for 3 hours. Then a sample of sulfoacetic acid prepared from 0.002 mole of sulfuric acid and 0.005 mole of acetic anhydride was added and the reaction mixture was heated at 85–90° for 3 hours, during which time the colorless solution changed through a green to a brownish purple color, sulfur dioxide and carbon dioxide were evolved, and a resin separated on the sides of the flask. The product was found to be slightly more active than that produced from cholesterol by the action of the sulfuric acid-acetic anhydride reagent.

The action of fuming sulfuric acid and of chlorosulfonic acid was investigated. In a 50 cc. flask a mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, and 0.186 gm. (equivalent to 0.002 mole of 100 per cent sulfuric acid) of 25 per cent fuming sulfuric acid was heated at 85–90° for 3 hours and the product was found to be active. Likewise, a mixture of 0.001 mole of dry cholesterol, 6.25 cc. of glacial acetic acid, and 0.002 mole (0.13 cc.) of chlorosulfonic acid (about 10 per cent excess over that amount which should be hydrolyzed by the water present in the acetic acid) was heated at 85–90° for 3 hours and the product was found to be active. Both of these reaction products possessed about the same activity as that produced by the sulfuric acid-acetic anhydride reagent.
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Irradiation of Product

To determine whether a provitamin D as well as an antirachitic substance is produced by the action of sulfuric acid-acetic anhydride on cholesterol, the potency of the reaction product was compared with the potency following irradiation. In a 50 cc. flask a mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, 0.0025 mole of acetic anhydride, and 0.002 mole of sulfuric acid was heated at 85–90° for 3 hours and the reaction product was concentrated. The residue was dissolved in 75 cc. of anhydrous ether to which 5 cc. of absolute alcohol had been added and one-half of the solution was irradiated in a 200 cc. quartz Erlenmeyer flask provided with a reflux condenser for 30 minutes at the distance of 1 foot from a Cooper Hewitt mercury arc lamp. The solution was slowly heated by means of a hot-plate to effect a more uniform irradiation. Aliquot portions of the greenish amber solution of the non-irradiated and the amber solution of the irradiated products equivalent to 6 mg. of the original cholesterol were tested biologically. Both products possessed about the same activity, which indicated that no provitamin D activatable by ultraviolet irradiation was produced by the action of sulfuric acid-acetic anhydride on cholesterol.

Heated Purified Cholesterol

The comparative efficacies of ultraviolet irradiation and sulfuric acid-acetic anhydride in imparting antirachitic activity to purified cholesterol (3) and to heated purified cholesterol were studied. The heated purified cholesterol was prepared by heating 1 gm. of purified cholesterol from room temperature to 210° during a period of 8 minutes and then heating at 210° for 2 hours in a 1 X 8 inch Pyrex tube stoppered with cotton. The oxygen of the air was not removed from the tube since Hathaway and Koch (13) found the presence of oxygen to be essential. The sublimate on the sides of the tube and the unsublimed residue were combined as it was found by Koch, Koch, and Ragins (14) that both the sublimate and the residue acquire the same degree of antirachitic potency following irradiation.

The purified cholesterol and the heated purified cholesterol were irradiated in a manner similar to the irradiation procedure de-
A solution of 0.4 gm. of each cholesterol dissolved in 50 cc. of anhydrous ether was irradiated for 30 minutes. An aliquot of each solution was tested biologically for antirachitic activity and about 5 times as much irradiated purified cholesterol were required to produce the same degree of healing as irradiated, heated purified cholesterol. This showed that the provitamin D of heated purified cholesterol had been produced, although an accurate assay was not made, since the provitamin D was not of primary importance in this work.

The purified cholesterol and the heated purified cholesterol were treated under the same conditions with sulfuric acid-acetic anhydride. In a 50 cc. flask a mixture of 0.001 mole of the cholesterol, 4 cc. of glacial acetic acid, 0.0025 mole of acetic anhydride, and 0.002 mole of concentrated sulfuric acid was heated at 85-90° for 3 hours. After the reaction products were concentrated, an aliquot of each residue was tested biologically for activity. It was found that both reaction products gave the same degree of calcification, i.e. an average 2.5 plus value at the 6 mg. level. These results indicate that the provitamin D of heated purified cholesterol is not the precursor of the antirachitic substance produced by treating cholesterol with sulfuric acid-acetic anhydride.

**DISCUSSION**

The activation of cholesterol with the sulfuric acid-acetic anhydride reagent was investigated. It was found that to obtain the maximum potency the proportions of 0.0025 mole of acetic anhydride (98 per cent) and 0.002 mole of concentrated sulfuric acid (95 per cent) per 0.001 mole of dry cholesterol dissolved in 4 cc. of glacial acetic acid (99.5 per cent) were required. The length of time required to obtain the maximum potency, when the reaction mixture was heated at 85-90°, was found to be about 3 hours; continued heating of the reaction mixture did not influence the potency of the reaction product.

It was observed that heat was essential in the production of the antirachitic substance. When sulfuric acid and acetic anhydride are mixed below 0°, they form acetyl sulfuric acid (4) which is a sulfonating reagent (8, 9). However, if acetyl sulfuric acid is warmed, it is converted into sulfoacetic acid (HO3SCH2COOH), which has been used as a condensing and an
acetylating reagent (5, 6, 15). It was found that sulfoacetic acid was effective in converting cholesterol into an active product.

Although concentrated sulfuric acid converts aromatic compounds into sulfonic acids, it yields sulfate esters from alcohols and olefins. Fuming sulfuric acid and chlorosulfonic acid are sulfonating reagents (11). It was found that sulfuric acid, fuming sulfuric acid, and chlorosulfonic acid were effective in producing active products from cholesterol. Since fuming sulfuric acid reacts with acetic acid to produce sulfoacetic acid, the quantity of fuming sulfuric acid sufficient to react with about one-half of the water in the acetic acid was used in a comparison with concentrated sulfuric acid. Furthermore, it was found that, if the solution of cholesterol in acetic acid was first heated with acetic anhydride, the potency of the product obtained by the action of sulfuric acid or sulfoacetic acid was increased. The study of the various reagents indicated that the best activity was obtained when the reaction mixture was anhydrous.

Sulfur dioxide was found to be evolved when cholesterol was treated with sulfuric acid-acetic anhydride in acetic acid solution at room temperature and sulfur dioxide and carbon dioxide were evolved when cholesterol was heated in acetic acid solution with sulfuric acid, sulfoacetic acid, or sulfuric acid-acetic anhydride. The evolution of sulfur dioxide indicates that an oxidation occurs in the reaction mixture although it does not necessarily indicate that an oxidation is involved in the conversion of cholesterol into the antirachitic substance. No correlation was obtained between the potency of the various products and the quantity of sulfur dioxide evolved. This indicates that sulfur dioxide is evolved in a side reaction and not during the conversion of cholesterol into the antirachitic substance. The carbon dioxide could arise as a result of an oxidation or from a decomposition of sulfoacetic acid to form methanesulfonic acid, since carbon dioxide is evolved when the reagents are heated in acetic acid solution.

Ultraviolet irradiation of the reaction product obtained by the action of sulfuric acid-acetic anhydride on cholesterol did not affect the potency, indicating that a provitamin D was not produced. It was found that the reaction products obtained by the action of sulfuric acid-acetic anhydride on purified cholesterol and heated purified cholesterol, possessed about the same potency, demon-
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strating that the provitamin D of heated purified cholesterol is not the precursor of the active substance.

SUMMARY

Cholesterol can be treated to acquire antirachitic properties by heating with sulfuric acid, sulfoacetic acid, fuming sulfuric acid, or chlorosulfonic acid in acetic acid solution. In addition to the active substance produced from cholesterol by sulfuric acid, sulfoacetic acid, or sulfuric acid-acetic anhydride, sulfur dioxide is evolved in a side reaction.

The treatment of cholesterol with sulfuric acid-acetic anhydride produces an antirachitic substance but not a provitamin D which is activatable by ultraviolet irradiation. The provitamin D of heated purified cholesterol is not the precursor of the active substance obtained by the action of sulfuric acid-acetic anhydride on cholesterol.

BIBLIOGRAPHY
