THE ISOLATION AND IDENTIFICATION OF THE STEROL FOUND IN CORK

By

Gordon F. Dittmar

Thesis submitted to the faculty of the Graduate School of the University of Maryland in partial fulfillment of the degree of Doctor of Philosophy
1942

Acknowledgment

The author wishes to express his appreciation to Dr. Nathan L. Drake, who proposed the problem, and offered countless suggestions during the carrying out of the work.

Table of Contents

Introduction

A. The Cork Problem....................1

B. General Remarks on Steroids..........2

Historical Development of Sitosterol.3

The Chemistry of Sitosterol..........8

II. Experimental

A. Isolation............................15

B. Identification.......................24

III. Discussion

A. On the Isolation.....................29

B. On the Identification................31

IV. Conclusions...............................34

V. Bibliography..............................35

INTRODUCTION

A. The Cork Problem

For the past decade compounds which have been extracted from cork have been under investigation in the organic chemistry laboratory at the University of Maryland. (c.f. Drake and Co-workers, 1935 to present). Chief among these compounds are cerin and friedelin, two triterpenoids, which have been subjected to prolonged study; and are today still under investigation.

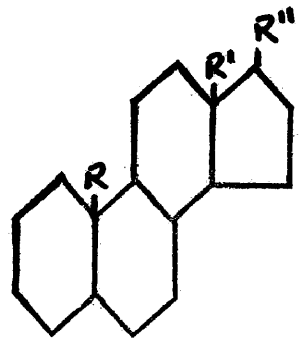
Recently phellonic acid was isolated from cork and it's structure proved (32). Still more recently a series of high molecular weight aliphatic hydrocarbons were extracted and their structures demonstrated (38).

The presence of a sterol in cork was first shown by Drake and Jacobsen (34) in 1935. A portion of crude ethyl acetate extract was saponified with alcoholic potassium hydroxide, and the residue obtained on cooling was a gel-like substance. This was recrystallized many times from absolute alcohol and then sublimed in a high vacuum. The product was still amorphous and melted 65-70°C to a turbid liquid which cleared at 145°C. On the basis of the carbon and hydrogen analysis the formula $C_{29}H_{50}O_2$ was assigned to the compound.

However, no attempts were made to ascertain the purity of the compound, nor was its structure investigated. Therefore, this research was undertaken in order not only to isolate the pure compound, but to investigate its structure as well. The source of the material was to be the residues from the ethyl acetate extraction of cerin and friedelin from cork.

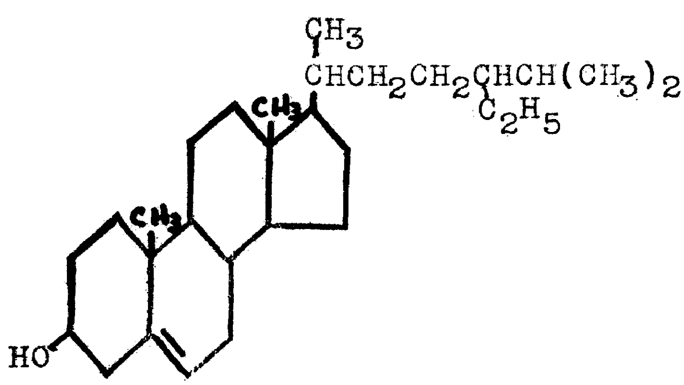
B. General Remarks on Steroids

The hydrocarbon, cyclopentanoperhydrophenanthrene, serves as the nucleus for a family of naturally occurring compounds. These are known as the steroid group, and the recognized members are: the sterols, the bile acids, the aglucons of the cardiac glycosides, the genins of the toad poisons, the sapogenins of the digitalis saponins, the sex hormones, and certain adrenal substances. The structure of the hydrocarbon is:



All the naturally occurring members are oxygenated, alkyl substituted derivatives of this parent hydrocarbon and vary according to the structure of the various R groups.

The compound isolated from cork, sitosterol, belongs to the sterol classification and has the formula:



C. Historical Development of Sitosterol

Sitosterol was first discovered by R. Burian (1) in 1897. He isolated from rye and wheat germ oil an unsaponifiable material, which when dissolved in alcohol, could be separated into two fractions. The lesser soluble of the two; m.p. 137.5, $(\alpha)_D$ -26.8; he called sitosterol, and considered it to be a single chemical entity isomeric with cholesterol, and possessing the formula $C_{27}H_{44}O$. The portion more soluble in alcohol was represented by a sterol called para-sitosterol m.p. 132.5, $(\alpha)_D$ -20.8.

In 1902 Ritter, (2) working on the unsaponifiable fraction of wheat germ oil, obtained a product m.p. 136.5, $(\alpha)_D$ -33.9 which was similar to the sitosterol of Burian. He also obtained a second sterol, the more soluble in alcohol, which he also called para-sitosterol.

In 1906 Windaus and Hauth (3) isolated from calabar beans a sitosterol melting at 133. From this extraction, however, he was able to separate a doubly unsaturated sterol by preparing its sparingly soluble tetrabromo-acetate. This proved to be stigmasterol and was the first discovery of this particular sterol. The sitosterol obtained by removing the stigmasterol has a melting point of 136-7 and was decided to be identical with that of Burian and Ritter. Windaus, however, gave sitosterol the formula $C_{27}H_{46}O$.

In 1908 Pickard (4) isolated sitosterol from wheat hulls of m.p. 138. He decided his compound to be identical with that of Windaus and also gave it the formula $C_{27}H_{46}O$.

With these works and several others of lesser importance research on sitosterol was at a stand-still. The only conclusions being that from the unsaponifiable fraction of vegetable matter there was a laevo rotatory sterol whose melting point was in the vicinity of 135°C.

Interest in Sitosterol was reawakened by Anderson and Moore in 1923 (5). They worked on the sitosterol from corn oil, from cottonseed oil, and from linseed oil and found that by numerous recrystallizationsof the crude sterol instead of obtaining one pure compound as did Windaus and Pickard, they obtained two compounds. From cottonseed oil the two compounds were (a) m.p. 138-9° C, $(\alpha)_D$ -34.19; acetate m.p. 124° C; (b) m.p. 134-5° C, $(\alpha)_D$ -33.61; acetate m.p. 119° C. Linseed oil gave (a) m.p. 138°C, $(\alpha)_D$ -34.22; acetate m.p. 129-30° C; (b) m.p. 134° C, $(\alpha)_D$ -31.16; acetate m.p. 124° C. The authors, however, were inclined to believe that neither of the two compounds was homogeneous, but this work served to rekindle interest in a compound which was considered up to this time to be a single chemical entity.

In 1924, continuing his work with Nabenhauer, Anderson (6) inaugurated an analytical application of the Liebermann-Burchard reaction. This reaction employs acetic anhydride and concentrated sulfuric acid on a chloroform solution of the sterol. If the unsaturated sterol is present, a green coloration appears in the chloroform layer. The saturated sterol does not react with the above reagents and thus can be separated unchanged. Accordingly, by this method, dihydrositosterol was isolated from the original sitosterol mixture and is, therefore, present as an impurity. Dihydrositosterol was given the formula $C_{27}H_{48}O$.

In 1926 Anderson and various collaborators worked on sitosterol obtained from rye (7) corn oil (8), and wheat germs and wheat germ oil (9). They first eliminated the stigmasterol and dihydrositosterol; then by numerous recrystallizations of the acetates were able to separate three distinct fractions of different melting points and different $(\alpha)_D$ values.

This work showed for the first time the complexity of the sitosterol problem which appeared at this moment to be composed of three isomers.

α	m.p. 133-5° C	$(\alpha)_D$ -23 to -30
β	m.p. 137-9	$(\alpha)_D$ -36 to -37
γ	m.p. 143-6	$(\alpha)_D$ -42 to -45

In 1927 it was shown that on irradiation sitosterol obtained somewhat of an antirachitic property (10) (11). It this contains a provitamin D as an impurity. Much later, in 1938, Lobert (12) showed the presence of very small amounts of erogosterol which he detected by spectrographic absorption measurements.

In 1928 Bonstedt (13) extracted crude phytosterol from soybeans and used the bromination technique for purification. Like Anderson, he isolated dihydrositosterol and sitosterol of melting point 142° C and $(\alpha)_D$ -44.8. This is very similar to Anderson's γ sitosterol.

Sanqvist (14), in 1930, and his collaborators analyzed the unsaponifiable fraction of pine hulls. They were of the opinion that the material extracted consisted of a mixture of more than two substances. From the mixture they identified a sterol which compared to Anderson's an sitosterol, m.p. 139° C $(\alpha)_D$ -29.5 and to which he assigned the formula of $C_{29}H_{50}O$. This is the first use of the C_{29} formula. Previously all the other investigators had used the $C_{27}H_{46}O$ formula as proposed by Windaus (3).

Windaus (15) himself, in 1932, confirmed the $C_{29}H_{50}O$ formula by analyzing numerous derivatives. (acetate; 3,5-dinitrobenzoate; 2-chloro-3,5-dinitrobenzoate; 4-bromo; 3-nitrobenzoate; and the bromoacetate)

Wallis and Fernholz (16), in 1936, isolated from the sitosterol mixture gotten from wheat germ oil two more fractions hitherto unreported. They were gotten from the portion of the original mixture which was the most soluble in alcohol through the recrystallization of the m-dinitrobenzoates. They were named α_1 which was isomeric with stigmasterol ($C_{29}H_{48}O$), and α_2 which was a homolog of stigmasterol ($C_{30}H_{50}O$). α_1 m.p. 164-6° C; $(\alpha)_D$ -1.7; α_2 m.p. 156° C; $(\alpha)_D$ + 3.5.

Another sterol, belonging to the $C_{29}H_{50}O$ group was isolated by Simpson and Williams (17) from Sarsaparilla roots. This compound, called ε-sitosterol m.p. 143-4° C; $(\alpha)_D$ -38.7, was gotten by recrystallizing the 3,5-dinitrobenzoates of the crude sitosterol from cyclohexane. This work was done in 1937.

Also in 1937, Karrer and Salomon (18), carried out a very thorough investigation on wheat germ oil. After removing the crude phytosterols from the saponified mixture, the mother liquor was concentrated to a dark colored oil. This was taken up in petroleum ether, washed, and then dried over anhydrous sodium sulfate. This solution was adsorbed on activated alumina and developed with 60-90° C petroleum ether. Part of the column was then eluted with an 80-20 mixture of methyl alcohol-ether. The eluate was evaporated to dryness, and the red-brown oil resulting was readsorbed on alumina. It was again eluted, the eluate evaporated and then taken up in 96% alcohol. To this was added a 1% digitonin solution in 90% alcohol, and a voluminous precipitate resulted. The digitonides were then cleaved, and after elaborate precipitation and crystallization procedures, two new sterols were gotten. They were isomeric and were given the names α-and β-tritisterol. (α-tritisterol m.p. 114-15° C, $(\alpha)_D$ + 54.3; β-tritisterol m.p. 97° C, $(\alpha)_D$ + 49.2). Analysis showed the formula to be $C_{30}H_{50}O$. The compounds are isomeric with amyrin, and, unlike the other known pytosterols, give neither the Liebermann-Burchard nor the Salkowski reactions although they contain at least one double bond.

The announcement of the isolation of another sterol, (α_3 sitosterol), from the sitosterol mixture was made simultaneously and independently by Gloyer and Schuette (19), and by Wallis and Bernstein (20), in 1939. Gloyer and Schuette separated their compound from rye germ oil by fractional recrystallization of the m-dinitrobenzoates of the more soluble portion of the sitosterol mixture. Wallis and Bernstein found that the α_1 sitosterol fraction when converted to the acetates could be recrystallized into two fractions. The new one being the α_3 sitosterol, m.p. 142-3° C, $(\alpha)_D$ + 5.2. Gloyer and Schuette give their constants as m.p. 142° C, $(\alpha)_D$ + 1.65.

While the preponderance of the work that has been done shows that sitosterol is a mixture, there have been some investigators who claim it is not. In 1938 Castille and Ruppol (21) made numerous derivatives of crude sitosterol which they put through elaborate recrystallization schemes. They concluded that only one compound existed which has m.p. 146.5-147.5° C; $(\alpha)_D$ -45.26. The source of their material was barley rootlets.

In the same year, Lobert (12) carried out a very extensive research on sitosterol. He employed a combination of recrystallization and chromatographic adsorption methods. By these procedures he also isolated but one sitosterol: m.p. 140.8-41° C; $(\alpha)_D$-38.8. This material, as does that of Castille and Ruppol, compares best with Anderson's γ-sitosterol.

These are the major investigations undertaken on sitosterol up to this writing, exclusive of the actual proof of structure. This will be considered in a separate section.

The sitosterol problem is still a very open one, and more work must be done to clarify some of the conflicting date. Nevertheless, important advances have been made, and no doubt some day the problem will reach a satisfactory conclusion.

D. The Chemistry of Sitosterol

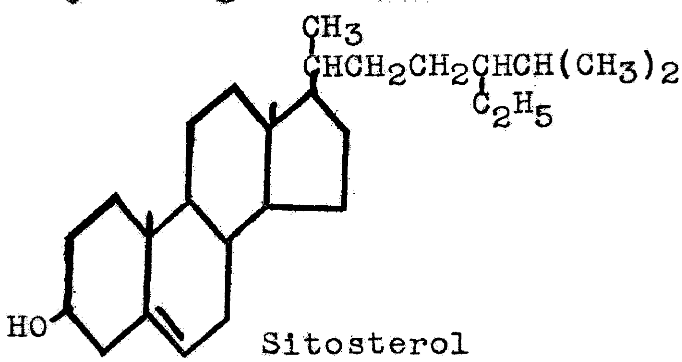
The fact that sitosterol, as it is first obtained from nature, can be separated into so many different compounds, might seem to complicate its chemistry and proof of structure. However, exclusive of the α_1-, α_2-, and α_3-sitosterols and the α- and β-tritisterols, all the rest are apparently isomeric because they show the same reactions. Consequently a proof for any one is a proof for all.

With the establishment of the correct structure for cholesterol in 1932, work on the structure proof of various other sterols was greatly facilitated. In fact it was not until 1935 that any work at all was done on the structure proof of sitosterol. The scheme used involved the conversion of the already proved stigmasterol into sitosterol or the conversion of both into the same compound.

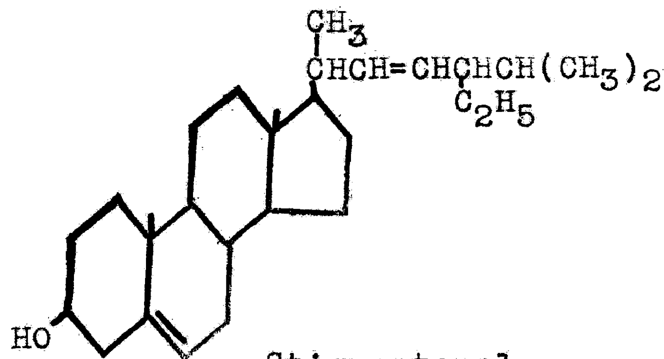
Sitosterol had long been known to be a sterol of formule

$C_{29}H_{50}O$ containing a secondary hydroxyl group and one double bond. Stigmasterol was similar in all respects except it had two double bonds. Its formula is $C_{29}H_{48}O$.

Bengtsson (22), working on β-sitosterol, showed that both it and stigmasterol could be completely hydrogenated to give the same compound ($C_{29}H_{52}O$). He believed that sitosterol was 22-dihydrostigmasterol.



Sitosterol



Stigmasterol

Bengtsson also showed that a favorable comparison could be made between the two ketones, hydrocarbons, and various esters.

Marker and Wittle (23) extended this work even further with a more complete series of interconversions (Figure I). Both stigmasterol (I) and sitosterol (X) yield the same fully saturated compound, and both can be converted into their corresponding unsaturated ketones (II and IX). The two ketones can be converted into the identical compound by reduction with Pt and H_2 with subsequent epimerization of the C_3-OH group to the epi form. The compound gotten from both is 24-ethyl-epi-coprostanol (V). Oxidation of this compound to the corresponding ketone (VII), followed by bromination gave 4-bromo-24-ethyl-coprostanol (VIII). This compound could be reconverted into sitostenone (IX) by heating with pyridine.

More work on the comparison of sitosterol and stigmasterol was done by Marker and Oakwood (24) and by Marker and Lawson (25), all showing that sitosterol is 22-dihydrostigmasterol.

The proof of structure of stigmasterol has been the product of the researches of quite a few workers. Following the determination of the empirical formula ($C_{29}H_{48}O$) (15), Guiteras (26) established the structure of part of the side chain and the location of one of the double bonds. This was accomplished through ozonolysis and hydrolysis. As a product of this reaction, he obtained ethyl-isopropyl-acetaldehyde, $(CH_3)_2$ $CHCH(C_2H_5)CHO$.

Fernholz (27) completely established the carbon framework of the sterol by an oxidative deradation study on stigmasteryl acetate (Figure II). Stigmasteryl acetate (I) is first brominated using one mole of bromine. The 5-6 double bond is more active than the 22-23 one and can be brominated while the other one remains untouched. The dibromide (II) is then oxidized with ozone and debrominated with zinc to give the unsaturated acid (III). This is then reduced to the saturated compound (IV), oxidized to the ketone, and reduced by the Clemmensen method to bisnorallocholanic acid (V). This compound was identical with bisnorallocholanic acid prepared from allocholanic acid.

Thus, with the carbon skeleton and the side chain definitely established, there remained only the proof of the position of the hydroxyl group. Although there were indications that the hydroxyl was at C_3, it was definitely proved by Fernholz and Chakrovorty (28). (Figure III)

Chromic anhydride oxidation of stigmasteryl acetate (I) yielded acetyl β-3-hydroxynorallocholanic acid (II). This same compound can be prepared in a similar amanner from cholesterol. In this latter case, however, the first acid isolated contains one carbon atom too many and must be degraded by that amount before it

FIGURE I

R = $-\underset{CH_3}{CH}CH_2CH_2\underset{C_2H_5}{CH}CH(CH_3)_2$ R' = $-\underset{CH_3}{CH}CH{=}CH\underset{C_2H_5}{CH}CH(CH_3)_2$

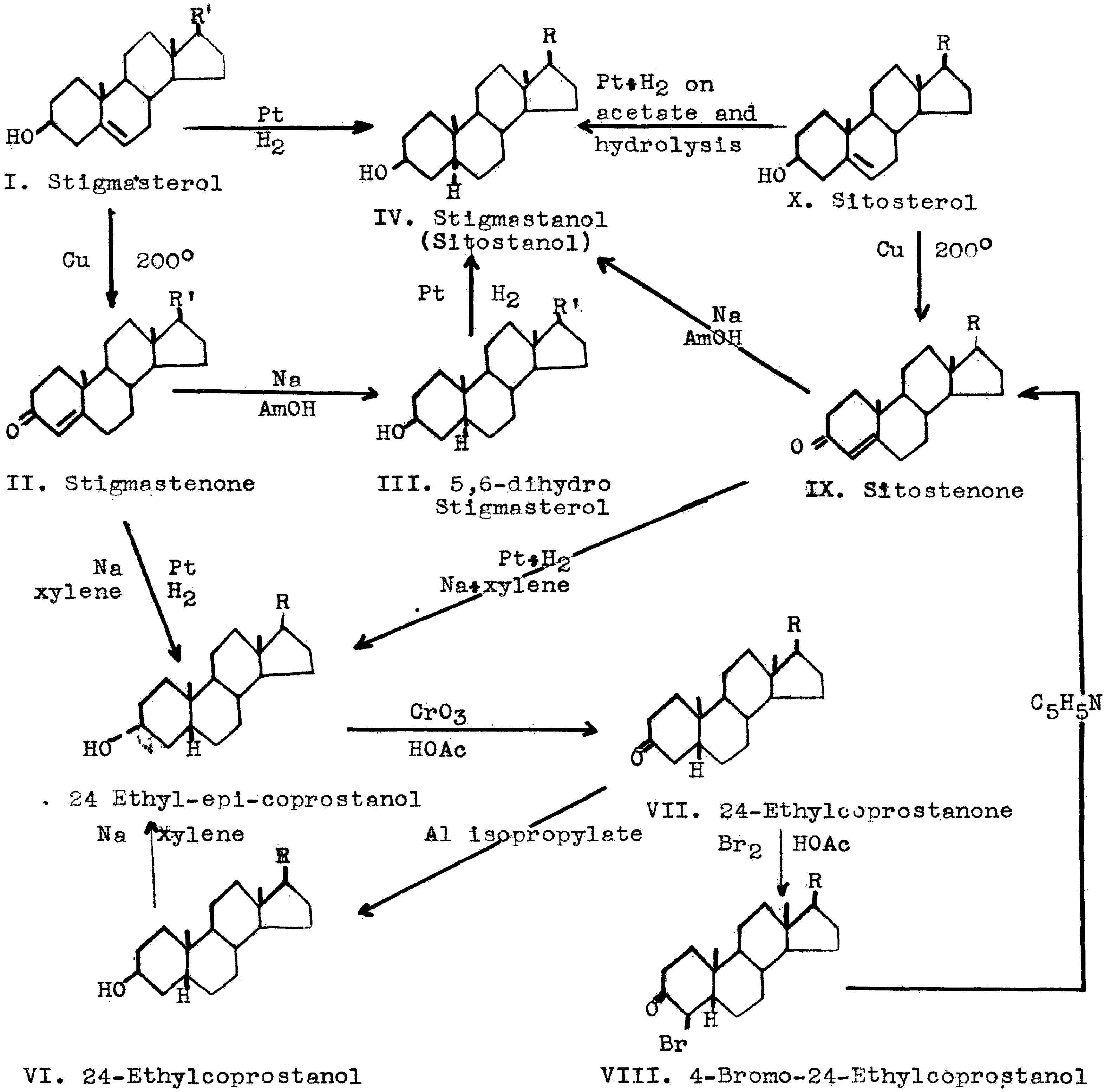
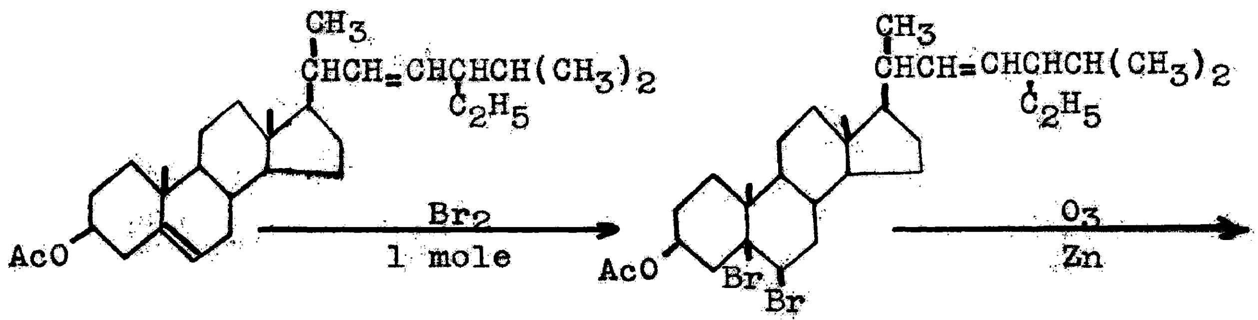
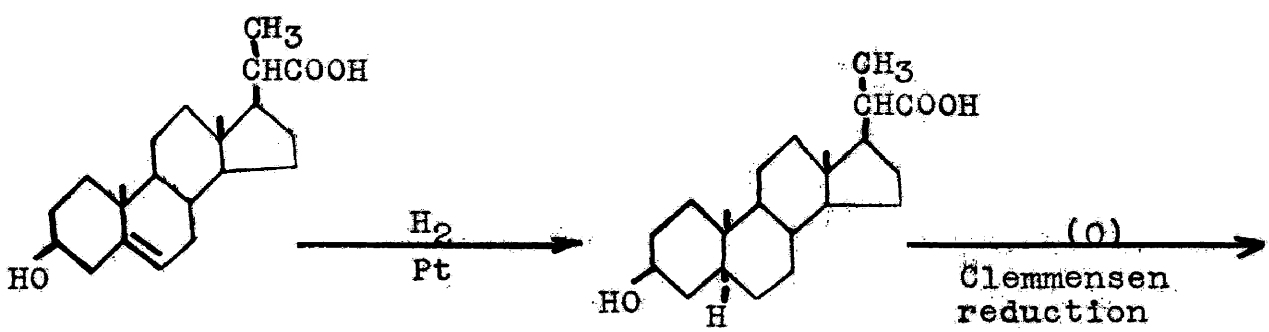


FIGURE II



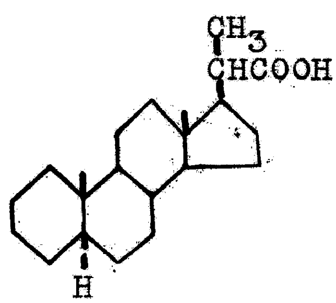
I. Stigmasteryl acetate

II. 5,6-dibromostigmasteryl acetate



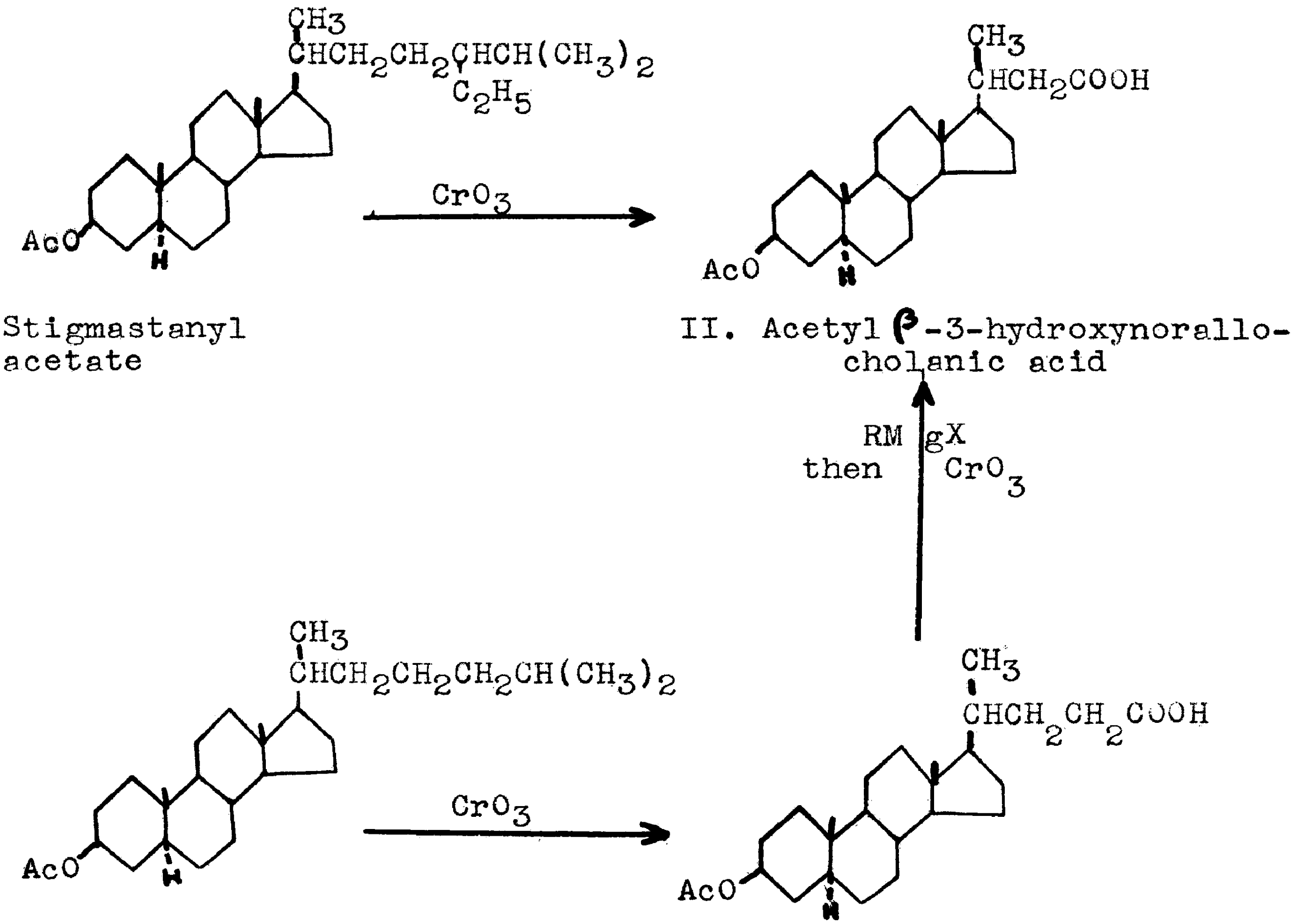
III. 3-hydroxy-5-bisnor-cholenic acid

IV. 3-hydroxybisnor-allocholanic acid



V. Bisnorallocholanic acid

FIGURE III



I. Stigmastanyl acetate

II. Acetyl β-3-hydroxynorallocholanic acid

III. Acetyl β-cholestanol

IV. Acetyl β-3-hydroxyallocholanic acid

is identical with the acid from the stigmasteryl acetate oxidation.

Thus it is proved that the hydroxyl group in stigmasterol is located at C_3, as it is in cholesterol, and that it also has the same configuration as in cholesterol.

With this evidence, the structure of stigmasterol is proved in full.

EXPERIMENTAL

A. Isolation

(1) Attempts on Crystallization

The ethyl acetate residue from the cerin and friedelin extraction of cork was the starting product. The ethyl acetate was first removed in vacuo and the dark brown, gummy mass remaining was saponified with 10% alcoholic KOH. After removal of the alcohol, portions of the residue were taken up in various solvents in an attempt to get crystalline material. The solvents used were:

petroleum ether	chloroform
benzene	carbon tetrachloride
acetone	amyl alcohol
dioxane	ether
propylaalcohol	pyridine
ethyl alcohol	ethyl alcohol-water
butyl alcohol	ethyl-acetate
i-propyllalcohol	methyl alcohol

acetone-water

It was impossible to get crystalline material from any of the above. When acetone, ethyl alcohol, methyl alcohol, or ethyl acetate was used, a dark brown, amorphous solid would precipitate from solution on cooling. However, no amount of recrystallization would give crystalline material.

(2) Attempts at Separation via the Succinic Anhydride Method.

This method was first used by Marker (29) in separation of the total sterol fraction from human non-pregnancy urine.

Four hundred grams of crude cork tar, which had been previously saponified with 10% alcoholic KOH, was diluted with water and extracted with ether. The ether was evaporated and the brown residue taken up in methyl alcohol. On cooling a brown amorphous solid precipitated. A portion of this was subjected to the Liebermann-Burchard test and was found to contain no sterol. Consequently it was filtered out and discarded.

The alcohol solution was then evaporated to dryness and taken up in benzene. The solution was then refluxed into a water trap until dry.

Twenty-five grams of succinic anhydride was added along with 50 ml. of pyridine. The solution was then refluxed for two hours on the steam bath. It was washed with 5% hydrochloric acid to remove the pyridine, then with water, and finally with a 10% solution of sodium carbonate to remove the succinic esters.

The aqueous layer was then acidified and the precipitated succinic esters extracted with ether. The ether solution was evaporated to dryness and 300 ml. of 10% alcoholic KOH was added to saponify the esters. This was refluxed for two hours, then evaporated to dryness and extracted with ether. This is the sterol rich fraction.

Again crystallization methods were tried, but the mixture refused to yield any crystalline material.

A portion of the sterol rich material was evaporated until it was concentrated to the point of precipitation. To this was added a 1% solution of digitosin in 90% ethyl alcohol. However, no precipitation occurred even though the sterol rich solution gave a very strong Liebermann-Burchard test.

(3) Attempted Dibromide Separation

Haenni (30) reports the analytical determination of cholesterol by precipitating it as the dibromide. This procedure was adapted to the cork sterol.

A portion of the sterol rich solution was evaporated to dryness and taken up in the minimum amount of anhydrous ether possible. To this was added 0.2 ml. of a carbon tetrachloride solution containing 0.25 grams of bromine per ml. of solution. After ten minutes, 15 ml. of an 80% acetic acid solution was added rapidly. The mixture was stirred well and allowed to stand in an ice bath. However, there was no precipitated bromide even though the solution was cooled in a carbon dioxide-acetone bath.

(4) Preliminary Purification Method

This method was attempted in order to concentrate the sterol, and to remove some of the undesired material.

The crude (189 g.) was saponified with 300 ml. of 5% methanoic potassium hydroxide and evaporated to dryness. After washing with water, the dark brown solid weighed 138 g. It was taken up in three liters of 85% ethyl alcohol. This solution was placed in a five liter flask that was fitted with a stirrer, a cold finger condenser, and a thermometer. The solution was then extracted four times with 500 ml. portions of 50-70° petroleum ether. The temperature was maintained at 45° C. When these petroleum ether extracts were evaporated, 70 g. of solid resulted. Again the material was dark and amorphous, and attempts to cause it to crystallize or to extract crystalline material from it were unsuccessful.

However, it was possible to concentrate the sterol by using methyl alcohol. A portion of the material from the petroleum ether extract was dissolved in methyl alcohol and then cooled. The material deposited, although not crystalline, was only slightly colored, and was free from sterol as shown by the Liebermann-Burchard test. Therefore, it was discarded. Upon further concentration of the mother liquors, more solid deposited. The second fraction had only a small amount of sterol, and this could be removed by leeching with a small amount of methyl alcohol. However, as the solution was concentrated still further, hot methyl alcohol was required to render the precipitated solids sterol free. Since this treatment also dissolved most of the non-sterol material, only a slight enrichment could be gotten. No amount of recrystallization by various solvents could give crystalline material.

Fifteen grams of the enriched material from the methyl alcohol treatment was dissolved in acetic anhydride. After heating for two hours on the steam bath, the solution was cooled. The first crop to deposit weighed 4 g. It was amorphous and sterol free, and consequently was discarded. The solution was then evaporated to one-half volume under reduced pressure and allowed to stand in the refrigerator over night. Close examination of the precipitated material disclosed the presence of crystals in the predominantly amorphous mass. These crystals were filtered out on suction. The Liebermann-Burchard test was strongly positive. Recrystallization from ethyl alcohol several times gave a constant melting point of 121-122° C. Yield 52 mg.

However, this procedure could not be repeated although attempted many times.

(5) Isolation of Sterol from Methyl Alcohol

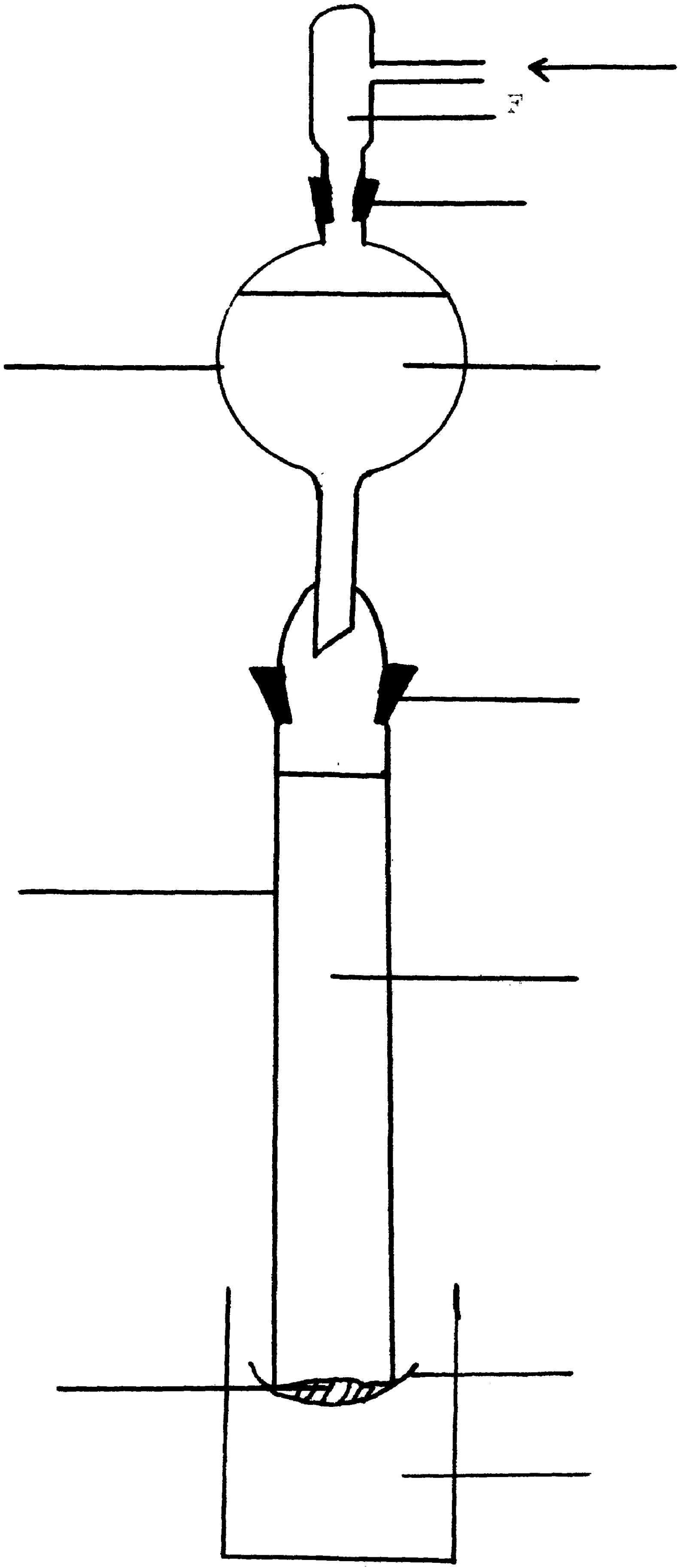
Some of the solid that had been purified by the petroleum ether extraction, and enriched by the methyl alcohol treatment was further concentrated. The concentration was carried to a volume of about 200 ml. The original volume was 1500 ml. This solution was put aside for further consideration; but after standing over night, a light yellow oil settled out of solution. This oil gave a very strong sterol test, and attempts were made to crystallize it from various solvents. These were unsuccessful. The oil was then redissolved in the minimum amount of methanol possible and allowed to stand in the refrigerator. After standing for a week, crystallization was again attempted. This time crystalls were gotten from methanol. These proved to be the sterol being sought for as evidenced by the strong sterol test. Recrystallization from methanol gave a constant melting point of 138-40° C. Yield 47 mg.

More of the oil gotten from methanol was prepared and treated in the same manner, but in every case crystallization did not occur. However, a total of 0.55 g. of sterol was extracted by the above method. This represents about 2500 g. of crude material.

(6) Isolation of the Sterol by Adsorption on Alumina

(a) Description of the Apparatus (Figure IV)

Tube (B) (2 cm. in diameter; 35 cm. long) contains the alumina (J) which is packed to a height of about 28-29 cms. At the exit end, a plug of cotton (C) is inserted and held in place by a copper wire gauze (D). The tube is connected to the reservoir (A) by a standard taper joint (H) ($ 24/40). The reservoir holds the solution (L) to be run through the alumina.



Attachment (F) is a plug containing a side arm which admits the compressed air used to force the solution through the adsorbent. The container (E) collects the exit solution.

(b) Preparation of the Column.

The exit end of the adsorption tube is plugged with cotton which is held in place by copper wire gauze, and clamped in an upright position. A small amount of alumina (activated alumina made by Brockman's method) * is placed in small beaker, and alcohol free and dry 50-70° C. petroleum ether is added so as to make a slurry. This is poured directly into the column and then is washed down with more petroleum ether. The alumina must not be allowed to become dry once it has been wet or it will be inactive. When the height of the alumina has reached about 2 cms., dry material may be added directly. There must be an excess of petroleum ether of several centimeters over the alumina already in the tube, and the amount of alumina added must not exceed the height of the liquid. As the dry material is being added, the tube is gently tapped to insure even settling. After each batch has been completely added, more petroleum ether is introduced, and the process is repeated until the alumina is packed to a height of about 28-29 cms. A small layer of petroleum ether is left on top of the alumina, and the reservoir bulb is quickly attached so the sterol rich solution may be run in before any of the alumina becomes dry.

(c) Preparation of the Solution Containing the Sterol.

Five hundred and eighty-seven grams of the crude

---

* prepared in this country by Merck & Co.,Inc. Rahway, N.J.

cork tar was subjected to the petroleum-ether purification method. Evaporation of the petroleum ether solution yielded 324 gms. of solid. This material was dissolved in two liters of methanol and the sterol concentrated as previously described. The final volume of the methanol solution was 275 ml. When evaporated to dryness, this solution yielded 94 gms. of sterol rich material. A solution of this was made using 50-70° C. petroleum ether. This was carefully washed with water by gently swirling in a separatory funnel. Vigorous shaking results in unbreakable emulsions and was therefore avoided. The solution was then dried by allowing it to stand over anhydrous sodium sulfate for two hours. It was light-brown in color and occupied a volume of 420 ml.

(d) The Adsorption Proper

When the adsorption column has been prepared and the sterol rich solution is ready, the adsorption is started by pouring the solution into the reservoir. In order to speed up the rate of flow, excess pressure of about 18 cms. of mercury is applied to the column. Under these conditions the solution runs through at a rate of two to three drops per second at the start and then gradually slows down. Nevertheless, the time to treat 120 ml. of the solution is from fifteen to twenty minutes.

The pressure on the column is maintained until the liquid column is about one-half centimeter above the alumina, and is then released. The remaining liquid is then allowed to run through under atmospheric pressure. When the height of the two columns is the same, the apparatus is quickly dismounted and the alumina pushed out with a wooden rod. This procedure was repeated four times, until all the sterol rich solution had been treated.

(e) Division of the Column

The adsorption of the various substances in the sterol rich solution gives rise to four colored bands in the column. Starting from the top they are: (1) a yellow band usually about five centimeters long containing no sterol; (2) a dull grey band about one centimeter long containing only a trace of sterol; (3) a light pink band one-half centimeter long, very rich in sterol; and (4) a white band occupying the rest of the column also rich in sterol. The bottom two layers are separated, placed in a Soxhlet apparatus, and extracted exhaustively with methanol. The extraction is usually complete in one hour.

(f) Crystallization of Sterol.

The methanol extract gotten by the above extraction is evaporated to one-half its volume and filtered to remove any suspended matter that may have been carried over such as alumina. The solution is then cooled in an ice bath. The first solid to seperate is usually amorphous, but on occasional runs crystalline material appeared at this point. The amorphous solid is filtered out and redissolved in methanol. On cooling nice white crystals of sterol separate. (m.p. 132-6° C.) One recrystallization from methanol yields crystals m.p. 136-8° C, and on the third recrystallization the melting point is 138-9° C. This does not change on further recrystallization. The usual yield for each adsorption is from 0.15 gms. to 0.30 gms. of sterol. Total amount from 587 gms. of crude was 0.85 gms. sterol (yield 0.14%)

(g) Reactivation of Alumina

The used alumina, both that which had contained sterol and that which had not, was placed in a pyrex glass tube about five feet long and twenty-five millimeters in diameter. The solid was evenly spread out along the entire length of the tube so that it occupied about one-half the diameter of the tube. Oxygen was blown through and the tube was heated progressively with a bunsen burner while the tube was rotated. The adsorbed organic material first carbonized and then burned completely. Pure white alumina resulted, and this could be used in subsequent adsorptions.

B. Identification

(1) Physical Constants.

(a) Melting Point.

The melting point was observed in a capillary tube on material which was purified by conversion to the acetate, recrystallized to a constant melting point, saponified back to the free sterol and again recrystallized to a constant melting point. Observed m.p. 139-39.5° C.

(b) Optical Rotation

$(\alpha)_D^{21}$ -31.2; $CHCl_3$ as solvent (l=2; c=2.08)

(c) Carbon and Hydrogen Analysis

Calculated for $C_{29}H_{50}O$: C,83.98; H,12.16

Found: C,83.64, 83.70; H, 12.36, 12.22.

(d) Molecular Weight

The acetate was saponified with sodium propoxide and titrated with standard acid.

Calculated for $C_{31}H_{52}O_2$: 456

Found: 458, 460

(e) Number of Double Bonds

The number of double bonds in the molecule were determined by titration with monoperphthalic acid. The per-acid was made according to the method of Böhme (40).

A known amount of the sterol acetate was weighed into a 25 ml. volumetric flask which was then filled to the mark with an ether solution of the per-acid. Another flask containing no other material was filled at the same time. The latter is for the blank titration. Both flasks are then placed in the refrigerator and allowed to remain for at least an hour. Each is then titrated with standard sodium thiosulfate solution, one after the other. The titration is accomplished by pipetting a 1 ml. aliquot of the ether solution into a 125 ml. erlenmyer flask and adding 10 ml. of a 20% potassium iodide solution and 10 ml. of a 10% sulfuric acid solution. After ten minutes standing, 25 ml. of water is added and the titration begun. Starch is added as the indicator. The titrations are repeated until there is no more uptake of the per-acid.

Time in hrs.	Titration in ml. Blank	Titration in ml. Sterol	Difference	No. of double bonds
	2.42	2.30	0.12	0.39
	2.42	2.20	0.22	0.72
	2.41	2.16	0.25	0.80
	2.39	2.14	0.25	0.80
o	2.39	2.14	0.25	0.80
13	2.35	2.10	0.25	0.80
24	2.30	2.06	0.24	0.78

$Na_2S_2O_3$ = 0.0561 N. Weight of sterol acetate = 0.0982 gm.

From this data the presence of only one double bond can be deduced.

(2) Preparation of the Acetate

Two-tenths of a gram of the free sterol (m.p. 138-9) was heated on the steam bath with 30 ml. of acetic anhydride for one hour, and then poured into a water-ice mixture. When the excess acetic anhydride had hydrolyzed, the precipitated solid was filtered with suction and washed with a little water. The solid was then dissolved in methanol, and on cooling, glistening white plates appeared of m.p. 120-1° C. Yield 0.21 gms. (94% of theoretical). Further recrystallization gave a constant melting-point of 124-5° $(\alpha)_D^{27}$ - 35.1 (l = I, c = 1.72 in $CHCl_3$).

Analysis; Calculated for $C_{31}H_{52}O_2$: C, 81.56; H, 11.50.

Found: C, 81.38, 81.25; H, 11.60, 11.58.

(3) Preparation of the Dihydro Sterol

Attempts were made to hydrogenate the free sterol in dioxane solution using Raney nickel as a catalyst. At a pressure of 100 pounds per square inch of hydrogen and at room temperature, no hydrogenation occurred. This same was true for 1000 pounds per square inch and a temperature of 50°C. However, when the temperature was raised to 95° C. and the pressure to 3000 pounds per square inch the hydrogenation proceeded smoothly. The reaction was run for four hours under the latter conditions, and after the bomb had cooled, the dioxane solution was removed and evaporated to dryness. The crystalline residue was taken up in methanol and recrystallized to a constant melting point of 139° C. From 0.35 gms. of the free sterol, 0.32 gms. of the dehydro product resulted. This represents 92% of the theoretical. $(\alpha)_D^{26}$ + 29.1 (l = I, c = 1.35 in $CHCl_3$).

Analysis Calculated for $C_{29}H_{52}O$: C, 83.65; H,12.50.

Found: C, 83.35, 83.29; H, 12.61, 12.64..

(4) Preparation of the Dihydro Acetate

Two-tenths of a gram of the dihydro sterol was heated on the steam bath with 30 ml. of acetic anhydride for one hour, and then poured into a water-ice mixture. After the hydrolysis of the excess acetic anhydride, the crystals were filtered off and washed with a little water and then recrystallized from methanol. Yield 0.18 gms. melting at 136° $(\alpha)_D^{27}$ + 12.9 (1 = I, c = I.47 in $CHCl_3$)

Analysis; Calculated for $C_{31}H_{54}O_2$: C, 81.22; H, 11.80.

Found: C, 80.85, 80.96; H,11.95, 11.99.

(5) Preparation of the Δ^4 Ketone

This compound was prepared according to the method of Oppenauer (31). In a 100 ml. round-bottom flask was placed 0.50 gms. of the free sterol, 7.5 ml. of acetone, and 10 ml. of benzene. The flask was topped by a condenser with a calcium chloride tube attached. The mixture was first heated to boiling in an oil bath maintained at 75-85° C., and then a solution of 0.80 gms. of aluminum tertiary-butoxide was added in one portion to the boiling solution. Refluxing was continued for eight hours. The mixture was cooled. Then it was treated 2 ml. of water, and 5 ml. of 10% sulfuric acid. After shaking vigorously, it was transferred to a 250 ml. separatory funnel. The benzene extracts were washed with water and then dried by filtration through a layer of anhydrous sodium sulfate. The solvent was then evaporated on the steam bath. An oily-yellow residue resulted, but on placing it in an ice-salt bath, it solidified. This material was then dissolved in a 7-10 mixture of acetone-methanol. The solution was cooled slowly and crystals separated. The first to appear were unchanged sterol and were filtered off. The mother liquor on further cooling gradually deposited the desired ketone. After filtration, it was again taken up in an acetone-methanol solution and allowed to stand in the refrigerator overnight. On filtration and drying the crystals melted 81-82° C. Yield 50 mg.

Analysis; Calculated for $C_{29}H_{49}O$: C, 84.20; H, 11.86.

Found: C, 84.10, 84.15; H, 12.01, 12.05.

(6) Preparation of the Semicarbazone of the Δ^4 Ketone

Aproximately 35 mg. of the above ketone together with 20 mg. of semicarbazide hydrochloride and 35 mg. of sodium acetate were heated to reflux in 20 ml. of ethanol for one hour. The semicarbazone was precipitated from solution by cooling and adding water. After one recrystallization from ethanol, the material melted at 250-2° C. with sintering at 245° C.

Analysis; Calculated for $C_{30}H_{52}ON_3$: C, 76.60; H, 11.06.

Found: C, 76,70, 76.75; H, 11.08, 11.06

(7). Preparation of the Digitonide

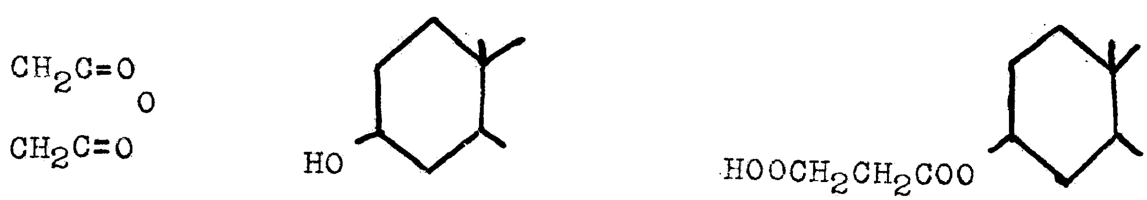
A small amount of the free sterol was dissolved in the minimum amount of hot 90% ethanol. To this was added an equal volume of a 1% solution of digitonin in 90% ethanol. A copius white precipitate appeared after about ten seconds and continued to precipitate even though the solution was maintained hot. The digitonide had no definite melting point, and because of this could be shown to be neither the free sterol nor the unreacted digitonin.

DISCUSSION

A. On the Isolation

For the most part, the methods recorded in the literature for the isolation of sterols, are simple, straight-forward procedures. Of these the simplest is by straight recrystallization. The usual solvents used are alcohol and acetone. However, not only were these solvents unsuccessful in crystallizing the cork sterol, but many others were also (c.f. p. 15). Apparently, either the cork extract is too complex a mixture to allow crystallization to occur, or the sterol is present in such a small amount that an effective concentration of it cannot be obtained without simultaneously concentrating some of the undesired material.

It was decided, therefore, to try some chemical means of separation. Since all the sterols have a free hydroxyl at C_3, this makes a convenient point of attack. Esterification of this free hydroxyl by using succinic anhydride had already been successfully used by Marker (29). This method has the advantage, that after the ester has been formed, there is a carboxyl group in the new molecule.



This compound then can be extracted via the sodium salt in an aqueous solution, precipitated by the addition of mineral acid, and finally saponified to the free sterol. Apparently however, there must be various other hydroxyl containing compounds in the original mixture as the material gotten by this procedure was still an uncrystallizable mixture.

An alternative method for the extraction of the sterol from this sterol rich mixture is by the use of digitonin. With digitonin, the sterols which have the normal C_3-OH group (normal form has the C_3-OH cis to the C_{10}-angular methyl group) form a molecular compound which are sparingly soluble in alcohol. All the naturally occurring phytosterols have such a configuration. Nevertheless, the cork sterol did not form a digitonide. This is perplexing, since a digitonide was later prepared on the pure material. There is a possibility that there was an epimerization of the hydroxyl group during a previous saponification with alkali. There is no doubt about the presence of the sterol as the Liebermann-Burchard was very positive. In subsequent work, all saponifications were carried out in methanoic potassium hydroxide solution in order to lessen the chances of any possible epimerizations.

It was quite apparent by this time that some preliminary purification method or some preferential concentration method must be undertaken before any method for the extraction of the sterol would work.

Consequently, a method was devised whereby the saponified crude was dissolved in 85% ethanol and extracted with 50-70° C. petroleum ether. It was expected that some of the crude material would be more soluble in the alcohol than in the petroleum ether. This was found to be the case, as some of the material would not go into the petroleum ether regardless of the number of extractions. The sterols as a class are much more soluble in petroleum ether than in alcohol. It must be pointed out, however, that by this method all of the sterol could not be removed from the alcohol solution.

In fact, its presence there could be demonstrated by the sterol test, when the extraction with petroleum ether was completed.

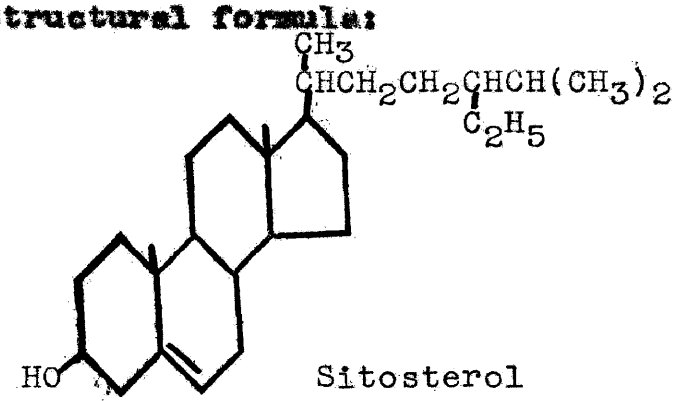
As before, though, the straight crystallization of this purified material was impossible. However, when the sterol was concentrated still further by use of methanol, it was possible to obtain the crystalline product from an oil which settled from solution. This method of extraction was quite laborious and unreliable. It was therefore discontinued, and other more reliable methods sought.

The chromatographic adsorption method was the only method found that could be relied upon to give reproducible results. It is very important though that all materials used be free from any alcohol and dry. Otherwise the adsorption of sterol would not occur.

It was stated earlier in this paper that the two top colored bands were discarded after the adsorption. However, the sterols themselves are colorless and their presence in a colored band, other than perhaps a white one, is purely coincidental, and the color is due to other compounds. By all methods a total of 2.85 gms. of sterol was isolated.

B. On the Identification

The cork sterol has been identified as sitosterol, empirical formula, $C_{29}H_{50}O$, structural formula:



Sitosterol

However, the identity was complicated by the fact that sitosterol, as it is given up by the plants, is a mixture of a number of compounds. Most of these are isomeric sterols of formula $C_{29}H_{50}O$. Some of these compounds, however, contain two double bonds and are isomeric with stigmasterol, $C_{29}H_{48}O$. Because there is a mixture, different plant sources may give sitosterols which do not match exactly in their physical properties. It is quite possible that various plants may produce sitosterols which vary slightly in the amounts of the isomers. There is also a probability that different modes of extraction may cause varying amounts of the components. Consider the following:

Free Sterol m.p.° C.	Acetate m.p.° C.	Source	Investigator
137.5-138	127.1	maize oil	Gill and Tufts (35)
139-40	125.8	cotton seed oil	Seigfeld (36)
139	125	cotton seed oil	Wagner and Clement (37)
137.5	127	corn oil	Anderson (5)
139	124	cotton seed oil	Anderson (5)
138	129-30	linseed oil	Anderson (5)
140-41	134-5	rice bran fat	Anderson (8)
138-9	126-7	wheat germ oil	Anderson (8)
139	125	pine oil	Marker (23)
136	--	cotton seed oil	Windaus (3)
136-7		cotton seed oil	Bomer and Winter (38)
139		cotton seed oil	Matthes and Heintz (39)

Each of the above workers called his compound "sitosterol". Since there is poor agreement, there should be more latitude than usual for the comparison of the physical properties of a compound of this nature.

A more complete comparison of the cork sterol and more or less average values of reported sitosterols and their derivatives follows:

	Cork Sterol		Sitosterol	
	m.p.° C.	$(\alpha)_D$	m.p.° C.	$(\alpha)_D$
Free sterol	139-39.5	-31.2	139	-33
Free sterol acetate	124 -24.5	-35.1	126	-36
Dihydro sterol	138	+29.1	139	+28
Dihydro acetate	135.5	+12.9	136	+13
Ketone Δ^4	81-2		82	
Semicarbazone of ketone	249-51		252-4	

IV. CONCLUSIONS

The sterol contained in cork can be isolated by the chromatographic adsorption method using activated alumina as the adsorbent.

. The sterol is sitosterol.

BIBLIOGRAPHY

(1) Burian, Mon. 18 551, (1897)

(2) Ritter, Z. physiol. chem. 34 461 (1902)

(3) Windaus and Hauth, Ber. 39 4378 (1906)

(4) Pickard, J. Chem. Soc. 93 1928 (1908)

(5) Anderson and Moore, J.A.C.S. 45 1944 (1923)

(6) Anderson and Nabenhauer, J.A.C.S. 46 1717 (1924)

(7) Anderson and Nabenhauer, J.A.C.S. 48 2972 (1926)

(8) Anderson and Shriner, J.A.C.S. 48 2976 (1926)

(9) Anderson, Shriner and Burr, J.A.C.S. 48 2987 (1926)

(10) Hess and Anderson, J. Biol. Chem. 51 746 (1927)

(11) Rosenheim and Webster, Biol. J. 21 389 (1927)

(12) Lobert, Bul. soc. chim. biol. 20 766 (1938)

(13) Bonstedt, Z. physiol. chem. 176 269 (1928)

(14) Sandqvist and Groton, Ber. 63 1935 (1930)

(15) Windaus, Werder and Gschaider, Ber. 65 1006 (1932)

(16) Wallis and Fernholz, J.A.C.S. 58 2446 (1936)

(17) Simpson and Williams, J. Chem. Soc. 151 733 (1937)

(18) Karrer and Salomon, Helv. chim. Acta. 20 424 (1937)

(19) Gloyer and Schuette, J.A.C.S. 61 1901 (1939)

(20) Wallis and Bernstein, J.A.C.S. 61 1903 (1939)

(21) Castille and Ruppol, Bull. soc. chim. biol. 19 1716 (1938)

(22) Bengtsson, Z. physiol. chim. 237 46 (1935)

(23) Marker and Wittle, J.A.C.S. 59 2704 (1937)

(24) Marker and Oakwood, J.A.C.S. 59 2708 (1937)

(25) Marker and Lawson, J.A.C.S. 59 2710 (1937)

(26) Guiteras, Z. physiol. chem. 214 89 (1933)

(27) Fernholz, Ann. 507 128 (1933)

(28) Fernholz and Chakravorty, Ann. 500 270 (1933)

(29) Marker, J.A.C.S. 60 1901 (1938)

(30) Haenni, Ph.D. Dissertation, Univ. of Md. (1940)

(31) Oppenauer, Rec. trav. chim. 56 137 (1937)

(32) Drake, Carhardt, and Mozingo, J.A.C.S. 63 617 (1941)

(33) Love, Ph.D. Dissertation, Univ. of Md. (1941)

(34) Drake and Jacobsen, J.A.C.S. 57 1570 (1935)

(35) Gill and Tufts, J.A.C.S. 25 251 (1903)

(36) Seigfeld, Z. Nahr. Genussm 7 577 (1904)

(37) Wagner and Clement, Z. Nahr. Genussm 17 266 (1909)

(38) Bömer & Winter, Z. Nahr. Genussm 4 865 (1901)

(39) Matthes and Heintz, Arch, Pharm. 247 161 (1909)

(40) Böhme, Ber. 70 379 (1937); Org. Syn. 20 70 (1940)

VITA

Name: Gordon Frederick Dittmar

Permanent address: 2833 Gwynns Falls Parkway
Baltimore, Maryland

Degrees and date: B.S. 1937; M.S. 1938.

Date of birth: November 22, 1915

Place of birth: Baltimore, Maryland

Secondary Education: Forest Park High School, Balto. Md.

Collegiate Institutions attended:	Dates	Degree	Date of Degree
University of Maryland	1933-37	B.S.	1937
University of Maryland	1937-38	M.S.	1938

Employment: Now employed as a post-doctorate fellow in organic chemistry at Northwestern University, Evanston, Illinois.