CHEMICAL INVESTIGATIONS ON THE STEROLS OF FIVE TROPICAL OILS

A THESIS SUBMITTED TO THE FACULTY OF THE
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INTRODUCTION

The sterols[#] constitute a large group of complex organic compounds found in the unsaponifiable portion of the oils and fats of almost all natural cell material. Although they are distributed widely they seldom occur abundantly in any material. In early classifications the sterols included all higher alcohols occurring in the unsaponifiable portion whereas the modern view-point restricts the term to compounds having a particular molecular structure. They are classified for convenience into two large groups, "zoo-" and phyto-" sterols, in accordance with their respective origin in animal and vegetable tissue. Those of vegetable origin occur in small amounts in all parts of the plant but are found most concentrated in the oil-bearing seeds and pollen grains. Researches extending over more than a century have elucidated the biological and chemical significance of the sterols. The outstanding features of these investigations, with particular emphasis upon their chemical aspects, have been set forth in following sections of this thesis.

In undertaking the present research it was recognized that the sterols occupied a very important place in biology and chemistry and that knowledge of these compounds was by no means complete. A search of the literature and a tabulation of known sterols according to their sources and

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[#] A glossary is included for convenience in the Appendix.

properties showed that the tropical oils, as a whole, were unstudied as sterol sources.

Five tropical oils, namely avocado (Persea gratissima), kukui (Aleurites moluccana), china-wood (Aleurites fordii), chaulmoogra (Taraktogenos kurzii), and cocoanut (Cocos nucifera) were investigated as sources of phytosterols. The object was to establish methods for isolating and purifying the sterols, to establish methods for separating closely related sterols as found in component mixtures, to determine their physical characteristics and constants, to determine their chemical properties and formulae, and to determine relationships, if any existed, between these sterols and the vitamins.

The importance of taking into consideration the "component nature" of the sterol portion from any source has received particular emphasis during the past fifteen years, while the general study of the sterols has covered more than a century. Although sterols are individual substances, definitely crystalline and homogeneous, they are obtained from most sources as mixtures of substances very closely related to each other. Because of similarities in properties such as solubility, unusually complicated procedures are involved in the separation of sterol mixtures into pure, homogeneous substances. In the present investigation particular emphasis has been laid on a study of methods for separating phytosterols which are closely related physically and chemically.

HISTORICAL REVIEW

The important contributions made by the biological sciences in developing and extending a knowledge of the sterols have been recognized in reviewing the history of these compounds. However, inasmuch as the present investigation has been fundamentally a study in chemistry, the development of the chemical constitution of these compounds has received major emphasis.

The chemical history of the sterols began with the isolation of cholesterol (1) from gall stones by Pouelletier about the year 1769. Beginning with this pioneer step, the history of the investigation of plant and animal sterols has fallen into three periods. During the first period, extending to the close of the nineteenth century, methods of isolation and analysis of these compounds became well established. The second period was initiated at about the turn of the century by Windaus and others who devoted themselves during the following thirty years to determining the chemical constitution of cholesterol and closely related substances. The work of Windaus (2) was supplemented by the valuable work of Wieland (3) who by 1912 had begun separate investigations on the bile acid series. The third period, comprising the relatively few years from 1932 to the present time, has seen a wide extension of interest in the chemistry of sterols because of their connection with certain closely related, naturally occurring substances recognized as poss-

essing great physiological significance. Impetus was given to this recent work by the revision and correlation of the elaborate researches of Windaus and Wieland in the light of the contributions of Rosenheim and King (4).

During the first period attention was directed toward the isolation and characterization of various sterols from many sources including animal products, plants, and lower forms of life. The name cholesterine, synonomous with cholesterol, was given by Chevreul (1) in 1816 to the characteristic substance occurring in most of the divisions of the animal kingdom. Coprosterol, occurring in human faecal matter, was recognized as distinct from cholesterol by Flint (1) in 1862. Sterols closely resembling cholesterol were isolated from a number of plant sources; crude phytosterol was prepared by Hesse (5) in 1878 from Calabar beans and later was shown to be a mixture of sterols; the most common vegetable sterol, sitosterol, was discovered by Burian (6) in 1897 in the germ of cereal grains. The sterols occurring in the lower plants such as the yeasts, fungi, and algae, were studied extensively with the result that Tanret (7), working over a period of years from 1879 to 1889, discovered and purified ergosterol from the oil of ergot. During the years 1892 to 1898 Gerard (8) proposed a taxonomic relationship in the occurrence of sterols, suggesting that just as cholesterol was the typical sterol of animals so might phytosterol be considered characteristic of the higher plants and

ergosterol of the lower plants. The analysis and characterization of many of these substances followed immediately upon their isolation. Remarkably accurate analyses of cholesterol were made by Chevreul (9) as early as 1823. The presence of the characteristic hydroxyl group in cholesterol was established by Berthelot (9) in 1859 through the preparation of acyl derivatives. The formula calculated by Berthelot in 1859 from Chevreul's results differed from the accepted formula established by Reinitzer (10) in 1888 by the small margin of one $-CH_2$ group. Even today, with the vastly improved technical and analytical procedures available, selection of the correct formula from a number of homologues is open to debate. Toward the close of this pioneer period the foundations of modern sterol chemistry were laid (11) by the researches of Mauthner on cholesterol.

The second period covering the first thirty years of the twentieth century was devoted chiefly to the characterization of the sterols as substances possessing definite chemical constitution and properties. The goal of all these investigations conducted by Windaus, Wieland, and others, was to establish the structures of the large, complex molecules involved.

The secondary alcoholic nature of the characteristic hydroxyl group was proven by Diels and Abderhalden (12) in 1903 when they succeeded in dehydrogenating cholesterol to form a ketone. The presence of a double bond in the chole-

sterol molecule was demonstrated by Wislicenus and Moldenhauer (13) in 1868 through the formation of the dibromide. Both Mauthner (14) and Diels (15) obtained the saturated hydrocarbon cholestane from 1907 to 1909. The empirical formula $C_{27}H_{48}O$ for cholestane, compared with $C_{27}H_{56}$ of an open chain paraffin hydrocarbon, established the presence of four alicyclic rings in the cholesterol molecule.

The question as to what compounds should be classified under the heading of sterols is still a subject for debate. The exact structure required by a cyclic compound for inclusion as a sterol seems unsettled. For a long time the sterols were classified as terpenoid compounds. For example, the empirical formula assigned to stigmasterol (16) was $C_{30}H_{48}O$, an even multiple of the fundamental isoprene unit, and thereby analagous to those sapogenins which were recognized as being terpenoid. Also cholesterol and cholic acid, as well as camphor and turpentine oil gave small amounts of rhizocholic acid (17) on oxidation. A recent authority (110) has used a similar argument in declaring that successive steps in the oxidation of the sterols finally produce terpenic acids, and thus relates the sterols to the terpenes. Other authorities, however, consider the true steroid structure not to be terpenoid. One criterion adopted (18) depends upon the final products of selenium dehydrogenation. Triterpenoid compounds are presumed to contain a reduced picene ring system on the basis of this criterion. Thus there is no indication of either a structural or biogenetic relationship to the sterols and the other aetiocholane derivatives which came to be classified by the presence of a reduced cyclopentenophenanthrene ring system (9,9a).

Substances originally regarded as sterols, such as lanosterol and onocerin, were reclassified on the basis of this distinction.

The study of the sterols has gone hand in hand with investigations of certain closely related groups of substances notably the bile acids. The study of the bile acid series was begun in 1912 by Wieland as a separate field but by 1919 was correlated with the sterol studies of Windaus through the interconversions involving coprosterol (19). The results of this correlation of two separate fields have been presented in more detail in a following section of this thesis entitled, "The Chemical Constitution of the Sterols."

The third period, in which occurred extensive developments in the chemistry of sterols from 1932 to the present time, was characterized by a revision of the Windaus and Wieland formulation. This revision followed a suggestion made by Rosenheim and King (4) based upon results obtained by Diels (20) in 1927 from dehydrogenation experiments. The presence of the fundamental phenanthrene grouping became the criterion in extensive studies on the inter-relationships of the sterols with the bile acids, carcinogenic hydrocarbons, sex hormones, cardiac poisons, saponins, antirachitic vitamins and other naturally occurring compounds of great physiological importance. This very young and significant field has been described briefly in the last part of the following section in which the development of the chemical constitution of the

sterols has been reviewed under three natural headings, the work of Windaus and Wieland, the revision of their ideas in the light of a suggestion of Rosenheim and King, and recent developments.

THE CHEMICAL CONSTITUTION OF THE STEROLS

A. The Important Contributions of Windaus and Wieland

By far the most difficult phase of determining the chemical constitution[#] of the sterols was the investigation of the large, unwieldy nuclei which frequently reacted contrary to expectations. The various investigators adopted certain working hypotheses which were applied to the degradations and transformations of the compounds involved. These hypotheses have been set forth herewith.

The fundamental procedure in examining the nature of the complicated ring structure was to force open a ring at a vulnerable point, usually at a hydroxyl group. In order to prevent other reactive groupings from taking part in the oxidation, hydroxyl groups were protected by forming the acetyl derivative, while double bonds were saturated either with hydrogen or bromine. These protected compounds then could be converted into the corresponding ketones by mild oxida-

[#] In the discussion that follows, the evidence has been given in terms of the modern accepted formulae rather than the older, partial, and incorrect formulae in use at the time of each investigation. Examples of these incomplete and misleading formulations have been given merely to illustrate the process of revision.

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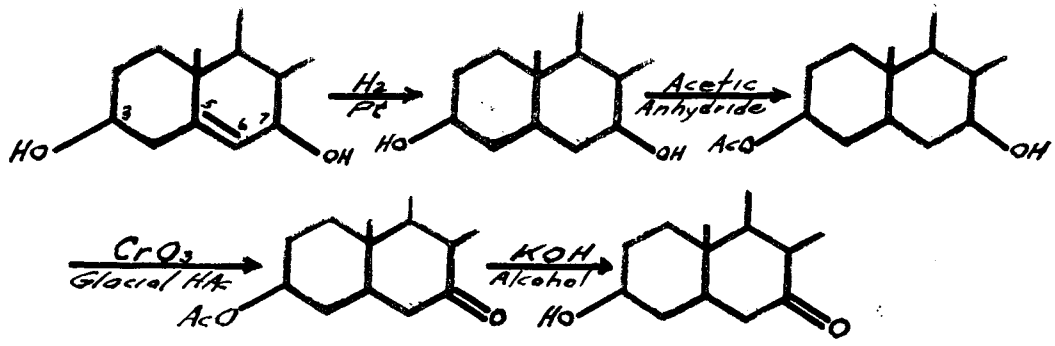
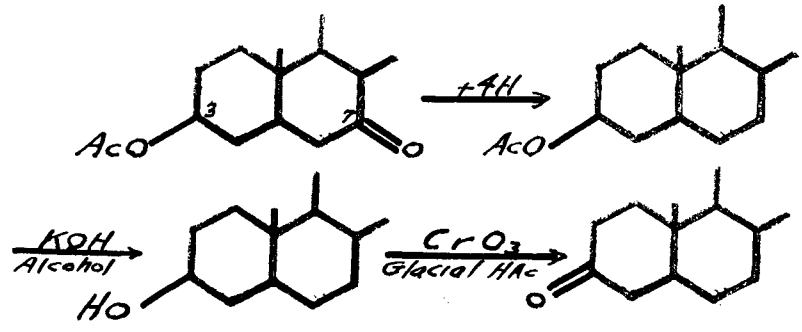
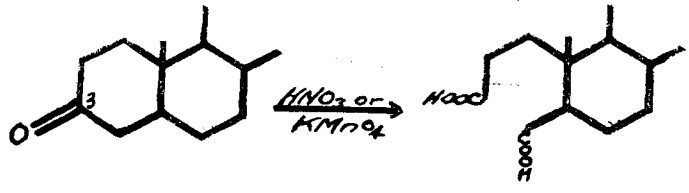


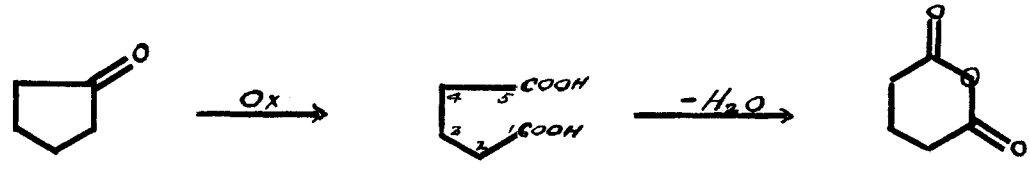
If hydroxyl groups in other rings were involved, carefully controlled reactions could be carried out that would make use of the order of reactivity: C#3 > C#7 > C#12. Corresponding keto groupings resulting from the oxidation could be removed by a form of reduction known as the Wolff-Kishner method (9), involving the treatment of the semicarbazone with sodium ethylate:



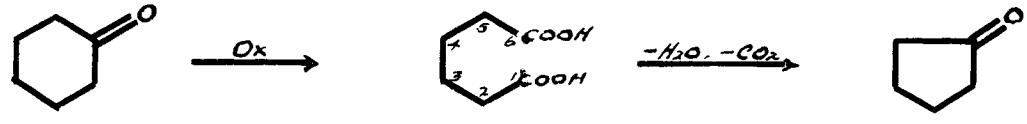
The chosen keto compound could be oxidized more vigorously whereupon the ring was opened at the carbonyl group with the production of two new carboxyl groups:



Cyclization then could be brought about by heating the dicarboxylic acid at higher temperatures and distilling under reduced pressure. The nature of the product often gave good indications as to the size of the original ring through the application of what is known as the Blanc Rule (21). This rule states that if two carboxyl groups occupy the 1,3-, 1,4-, or 1,5- positions in a chain the acid, on treatment with acetic anhydride or by heat distillation, is converted into an anhydride; while 1,6- and higher acids yield cyclic ketones. Blanc's Rule found its application in determining the size of the rings; i.e. whether they contained five or six carbon atoms.



5-C Ring 1,5- Dicarboxylic Acid Acid Anhydride

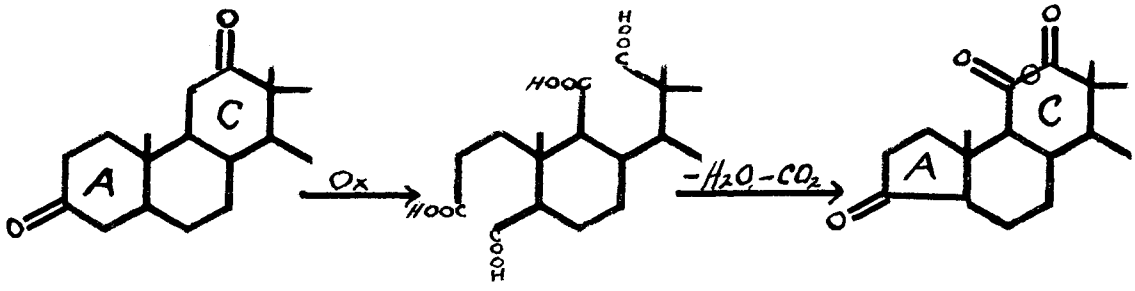


6-C Ring 1,6- Dicarboxylic Acid Ketone

A five-membered ring containing a hydroxyl group could be converted successively into a ketone by mild oxidation, a dicarboxylic acid by vigorous oxidation, and an anhydride by pyrolysis with no evolution of carbon dioxide. A six-membered ring containing a hydroxyl group could be converted successively into a ketone by mild oxidation, a dicarboxylic acid by vigorous oxidation, a ketone by pyrolysis with the

evolution of both carbon dioxide and water, a dicarboxylic acid by vigorous oxidation, and finally an anhydride by pyrolysis with the evolution of only water. The formation of the intermediate five-membered ring furnished strong evidence that the original ring must have been six-membered.

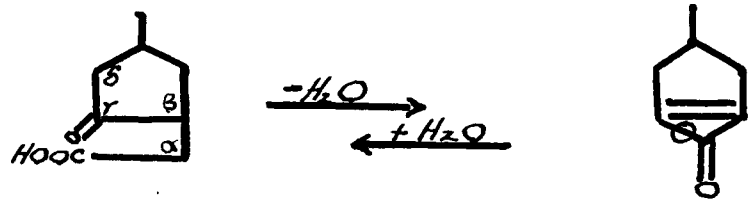
It was recognized later (22) that the Blanc Rule was not always applicable if the two carboxyl groups involved were located between two rings:



Diketone Tetracarboxylic Acid Pyrolysis Product

Rings A and C were originally six-membered, yet the pyrolysis product contained a five-membered ketone ring in place of Ring A and a six-membered anhydride ring in place of Ring C.

Another rule applied in the determination of the size and structure of a ring was Bredt's Rule (23) stating that alpha- and beta- keto acids cannot form the enol-lactone grouping while gamma- and delta- keto acids may do so:



Keto Acid Hydrate Enol-lactone

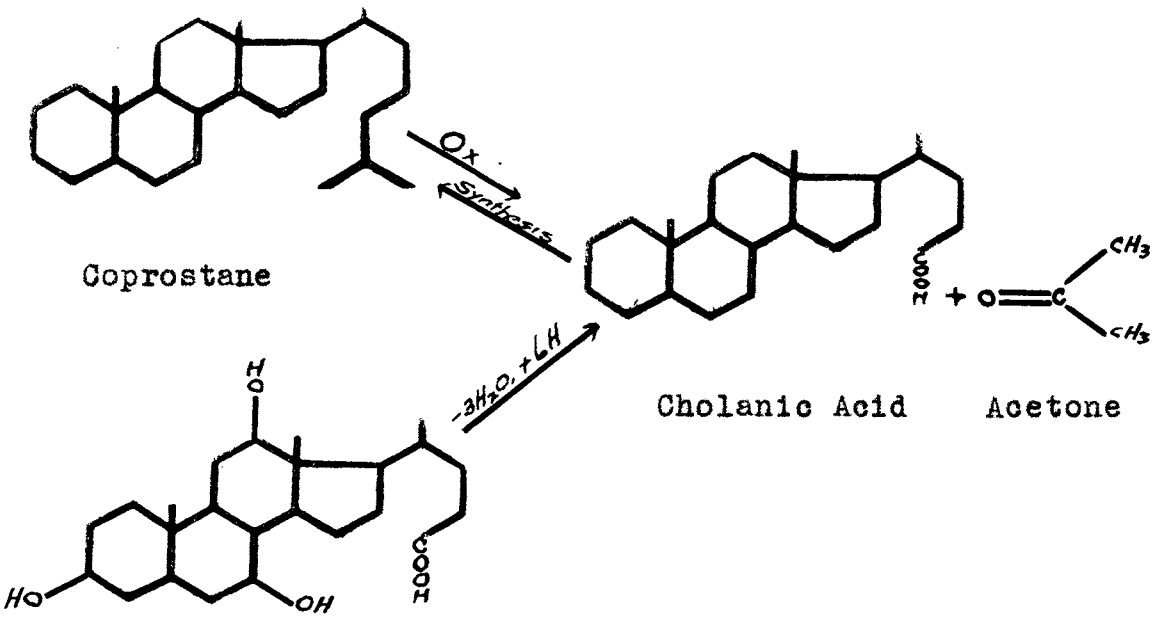
At one time the application of this rule seemed to favor the presence of a seven-membered ring in the cholane nucleus. Later developments eliminated this possibility.

A third criterion of structural relationships was the precipitability of a substance with digitonin. This glycosidic saponin, $C_{56}H_{92}O_{29}$, forms a characteristic, stable, and sparingly soluble addition product with all natural sterols. This reaction formed the basis for quantitative gravimetric determinations (24) of sterol content and at the same time served as a guide to stereochemical relations. No exceptions were discovered to the rule (25) that the beta-compounds were precipitated with digitonin, while the alpha-compounds were not. This relation was maintained among the degradation products in which the sterol side chain was completely oxidized away.

A method which would have given precise information concerning the carbon skeleton and which was tried probably many times with negative results, was the dehydrogenation of the saturated ring systems to produce aromatic substances which then could be characterized by comparison with known samples. The successful development of this method depended upon the work of Diels which has been discussed at the end of this section in connection with the final clarification of the entire problem.

The investigations of the sterols and of the bile acids had been undertaken originally as separate problems. The two series due to their natural occurrence together, their

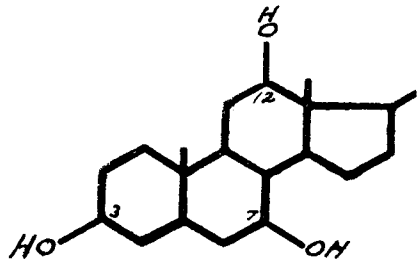
resemblance in composition and molecular complexity, and their similarity in certain characteristic color reactions had been suspected for some time of being related to each other. Windaus succeeded in establishing a connection (19) between the two series in 1919 by oxidizing the saturated hydrocarbon coprostane. He secured an acid that was identical in every respect with cholanic acid derived from a bile acid. The evidence that the carbon skeleton of the bile acids was identical with that of a large part of the sterol molecule was confirmed (26) by the reverse process of reforming coprostane from cholanic acid:



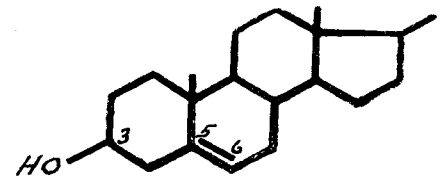
Cholic Acid

The two separate problems were correlated in this way and conclusions drawn in one series could be applied directly to the other series. The bile acids yielded information about

the cholane ring system more readily than could cholesterol with its single hydroxyl group. The distribution of the hydroxyl groups in different rings made it possible for each ring containing such a group to be opened at the vulnerable point by oxidation and its particular character investigated:



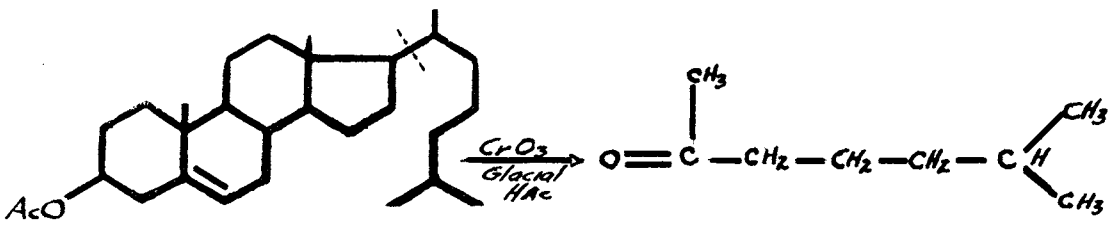
Cholane Ring System



Cholesterol Ring System

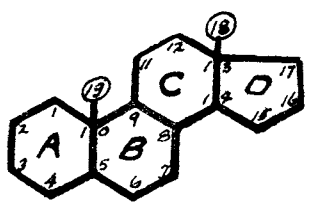
The entire problem thus resolved itself into two parts; the characterization of the side chain and the establishment of the structural relationships of the nuclear ring system. Oxidative degradation was the most effective means of accomplishing these ends.

The characterization of the side chain was the first part of the constitutional problem to be solved definitely. Both the sterols and the bile acids yielded a variety of oxidation products depending upon the conditions under which the oxidations were carried out. Several workers had noticed a pleasant odor during the oxidation of cholesterol and its derivatives. In 1913 Windaus (27) isolated and identified this substance as methylisohexyl ketone:

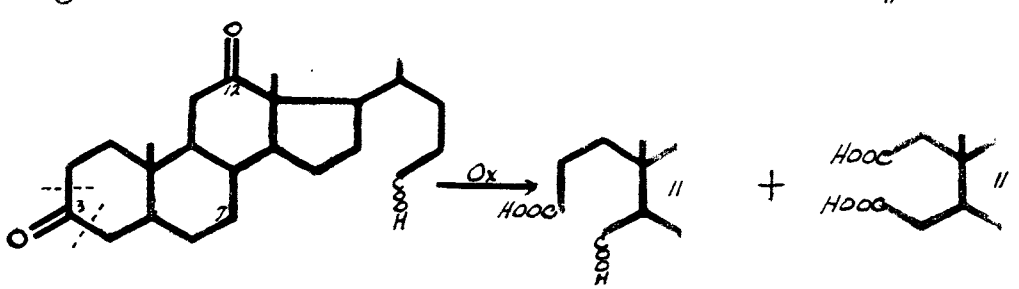


Cholesteryl Acetate Methylisohexyl Ketone

In the difficult task of characterizing the four carbocyclic rings the secondary alcoholic groups were indispensable for the manipulation of the complex structures. A ring containing such a group could be opened by oxidation and its structure studied. The modern method (9) of naming and numbering the rings has been established as follows:

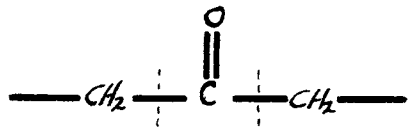


In the oxidation of the diketo acid (28) derived from desoxycholic acid the reactive group at #3 formed the first point of attack. It occupied a beta position in the ring and therefore was not subject to steric interference by adjacent rings or substituents as would be the case at #7 and #12.

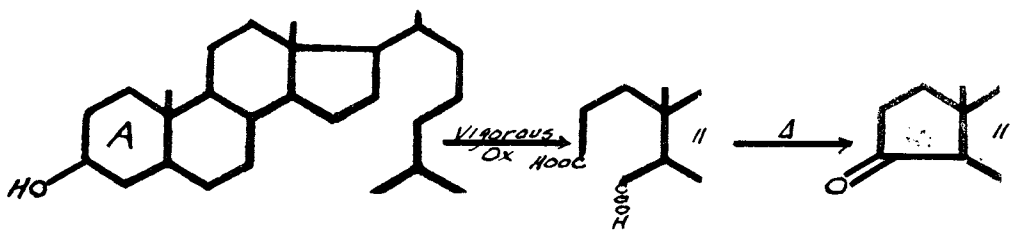


Dehydrodesoxycholic Acid Desoxybilianic Acid Isodesoxybilianic Acid

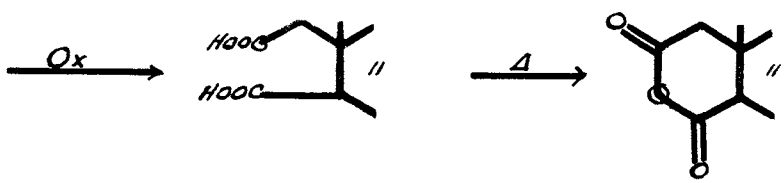
The formation of isomeric bilianic acids revealed the presence of a methylene group flanking each side of the reactive carbonyl group:



The size of Ring A was deduced by applying Blanc's Rule to the pyrolysis products of the two isomeric acids. Both acids lost carbon dioxide and water and formed ketones. This proved that Ring A of the original bile acid was a six-membered ring. The same conclusion (29) was reached regarding Ring A in the sterol series which contained the sole alcoholic group. The acid resulting from the oxidation of dihydrocholesterol yielded a ketone on pyrolysis. This in turn yielded an acid on oxidation that could be converted into an anhydride by further pyrolysis. Both observations clearly indicated an original six-membered ring in cholesterol.

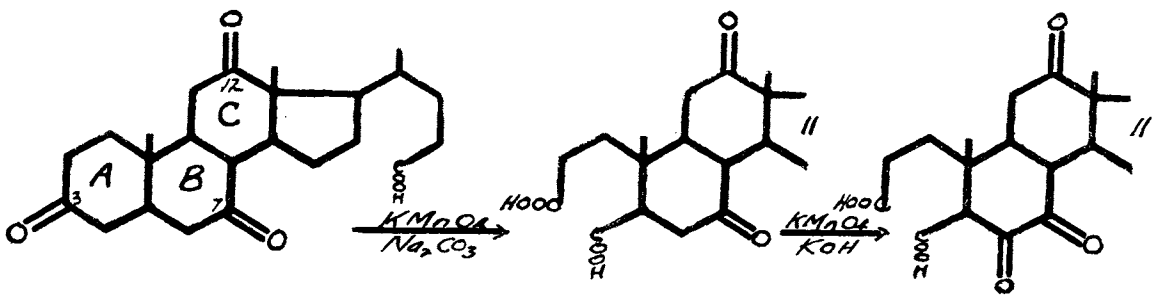


Dihydrocholesterol Acid Ketone

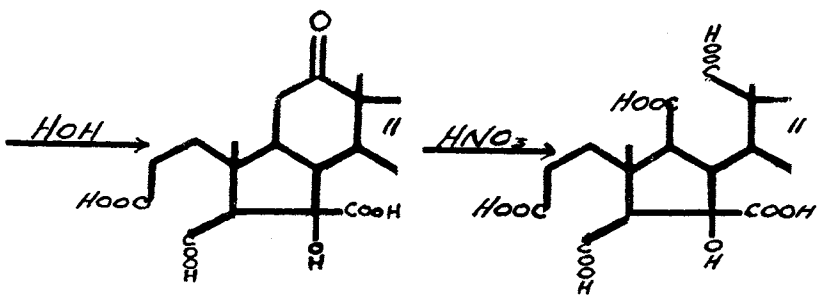


Acid Anhydride

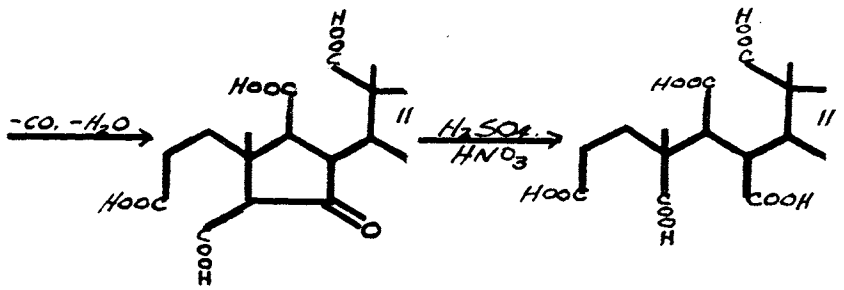
Before methods suitable for the selective opening of Ring B had been developed which would allow the application of Blanc's Rule, the systematic degradation of desoxycholic acid (9) gave evidence that this ring was six-membered. Further analysis (30) of the degradation series definitely placed the first hydroxyl group at #3 and the second hydroxyl group in Ring C. These conclusions left Ring B without oxygen in it. The nature of Ring B was confirmed by the series (31):



Dehydrocholic Acid — Bilianic Acid — Triketo Acid (Unstable)

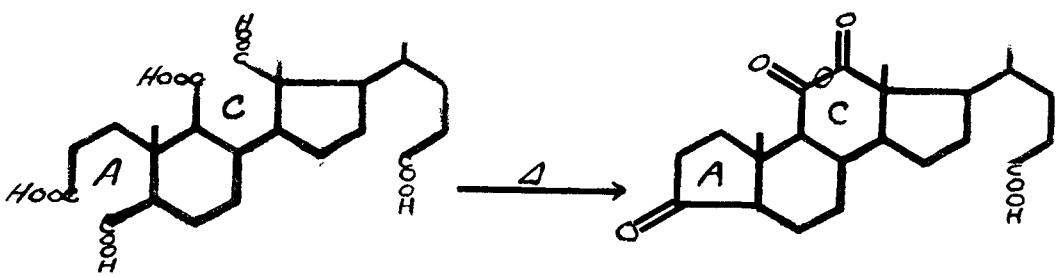


Cilianic Acid — Ciloidanic Acid



Beta-Keto Acid — Biloidanic Acid

Ring C was established at first (32) erroneously as being five-membered. The pyrolysis series was carried out on cholo-idanic acid which yielded pyrocholoidanic acid anhydride. This compound contained a ketone grouping in Ring A and an anhydride grouping in Ring C. This meant that Ring A was six-membered and Ring C five-membered according to Blanc's Rule. The Blanc Rule did not hold for Ring C because of the complex steric relationships present there. Both of the carboxyl groups were attached to rings thus producing steric hindrance.

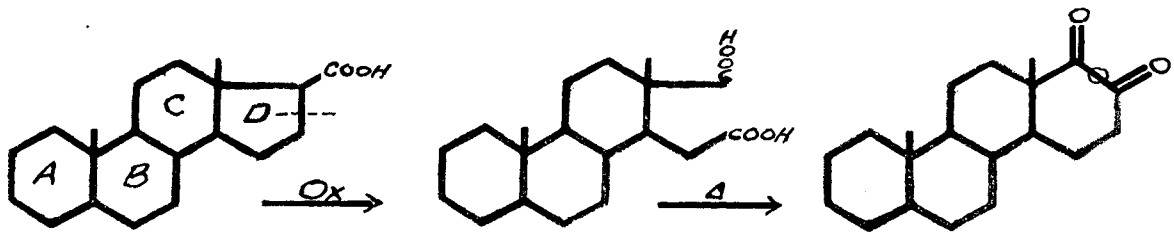


Choloidanic Acid Pyrocholoidanic Acid Anhydride

Another contributing error (33) was that the supposedly five-membered Ring C which carried the second hydroxyl group of desoxycholic acid was assumed to be attached to Ring A and to share two carbon atoms with this ring. Both of these errors contributed in large part to the misleading formulae that were used for many years to describe the sterols and bile acids.

The fourth and last ring, Ring D, presented special difficulties because there was no oxidizable group in this part of the molecule. The first plan (34) was to oxidize away

Rings A, B and C, leaving Ring D attached to the side chain. The very small yield and the difficulties encountered made it necessary to work from the side chain toward the ring. The side chain was degraded by one carbon atom at a time (35) until the base of the chain was reached in the form of aetiocholanic acid. This acid split upon oxidation to yield a dicarboxylic acid named aetiobilianic acid. This dibasic acid could have arisen only by the opening of Ring D which contained the carboxyl group. This final acid was subjected to the test of the Blanc Rule from which it was concluded correctly and unexpectedly that Ring D was five-membered.

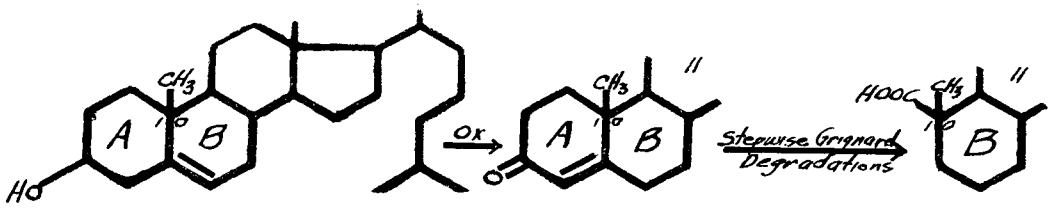


Aetiocholanic Acid Aetiobilianic Acid Anhydride

With the nature of the ring structures elucidated there remained the problem of locating the substituent groups. The positions earlier assigned (36,37) to the methyl groups were chosen more or less arbitrarily in an effort to correlate the two "homeless" carbon atoms with the rest of the now obsolete formula. However, with the acceptance of the revised formula only a limited number of positions were available for the two methyl groups. These positions were #1 and those carbon atoms shared by two rings. These positions were assigned definitely

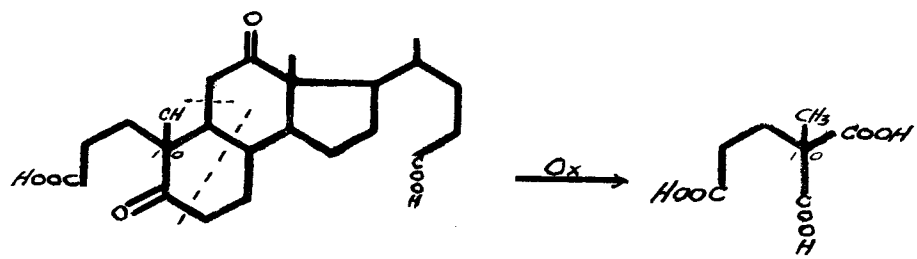
according to the following evidence.

Ring A of cholesterol was degraded systematically (38) until only a carboxyl group was left next to a methyl group. This arrangement was shown by comparison with tertiary carboxylic acids which were difficult to esterify and which yielded carbon monoxide when warmed with concentrated sulfuric acid.



Cholesterol Cholestenone Bisnor Acid

This evidence was in accord with a former observation (39) that oxidative degradation of a bile acid product yielded alpha-methylglutaric-alpha-carboxylic acid:

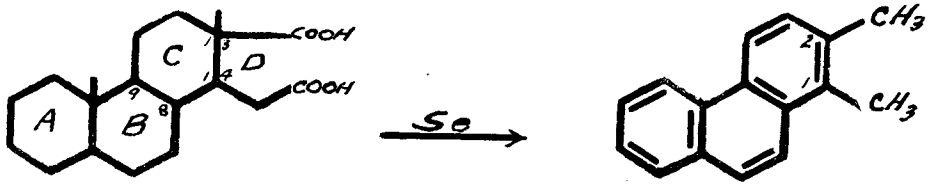


Keto Acid from Pyrodesoxybilianic Acid

Alpha-methylglutaric-alpha-carboxylic Acid

Both of these transformations fixed the methyl group at #10 which was one of the junctions between Ring A and Ring B.

The second methyl group was excluded systematically from positions #8 and #9 by studies (40,41) of Rings B and C. The only positions left were #13 and #14 in Ring D. Position #13 was chosen from stereochemical considerations and final proof (42) was given to this choice by dehydrogenation of aetiobilianic acid:



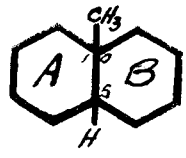
Aetiobilianic Acid 1,2 dimethylphenanthrene

This first clear proof that the cholane structure contained a hydrogenated phenanthrene nucleus also fixed the second tertiary methyl group at #13.

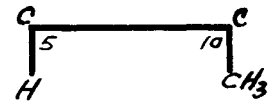
Even when the nature of the four rings and their substituents was determined definitely there remained the problem of determining their arrangement in space. The stereochemistry of the sterols and the bile acids proved to be very complicated due to the large number of asymmetric carbon atoms available for isomerism.

The saturated hydrocarbons coprostane and cholestane, in every other respect identical, were found to differ (43) in the nature of the union between Rings A and B. This type of

isomerism was dependent specifically upon the configuration at the asymmetric center #5. The hydrogen atom at this position bore either the cis or the trans relationship to the methyl group at #10. In coprostane, formerly called pseudocholestane, the configuration of the two rings corresponded to a "cis decalin" or two "bed" cyclohexane rings. This configuration served to define the "normal" series which contained all members of the bile acid series and also coprosterol.

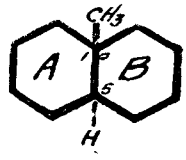


Cis Decalin Arrangement



"Bed" Diagram

In cholestane the two rings corresponded to a "trans decalin" or two "chair" cyclohexane rings. This configuration served to define the "allo" series which contained all the sterol series with the exception of coprosterol.

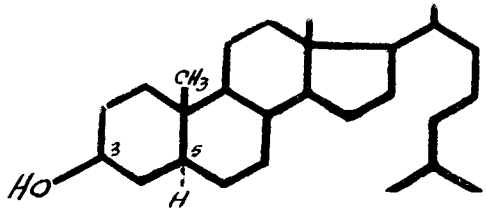


Trans Decalin Arrangement

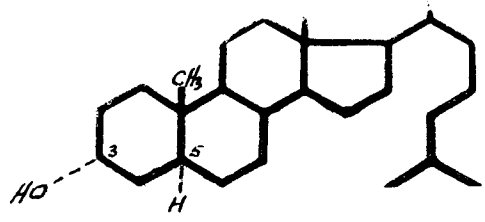
"Chair" Diagram

Another type of stereoisomerism involved the arrangement

of the hydroxyl group at #3 with the hydrogen atom at #5. The convention adopted (44) was based on the Auwers-Sitka Rule which stated that catalytic hydrogenation in an acidic medium usually produced cis forms and in a basic medium, trans forms. The configuration of dihydrocholesterol was assigned the term "Beta". This meant that the hydroxyl group bore the trans relationship to the hydrogen atom. The epimeric form in which the relationship was cis was assigned the prefix "epi-". All of the natural sterols were deduced to be of the "beta" type on the basis of their precipitation by digitonin.

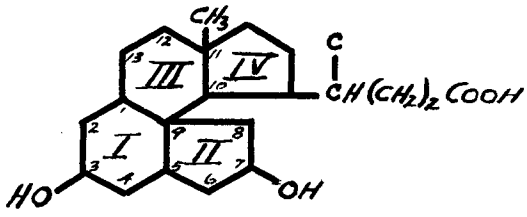


Dihydrocholesterol
(Beta-Cholestanol)

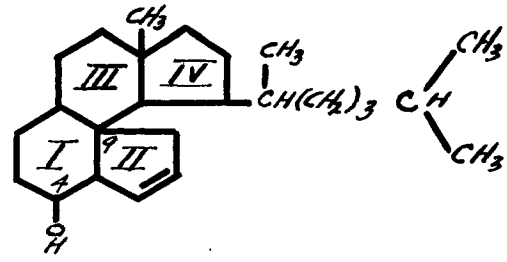


Epidihydrocholesterol

By 1928 the structural problem seemed to have been solved in its most pertinent points and the Nobel Prize was awarded to Windaus for his work on cholesterol begun in 1903, and to Wieland for his investigations in the field of bile acids dating back to 1912. The formulations due to these two men were so firmly entrenched that the ultimate clue to the whole solution was overlooked.



Desoxycholic Acid

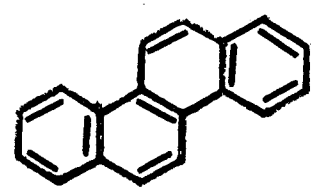


Cholesterol

(Mistaken Formulations Used by Wieland and Windaus in 1928)

B. Revision of the Wieland-Windaus Formulation Based on the Suggestion of Rosenheim and King

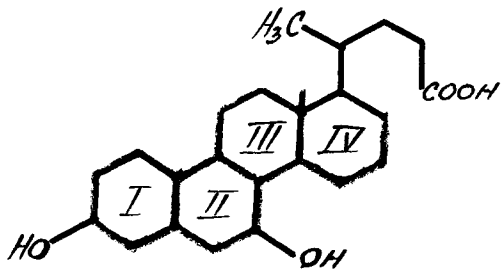
The final clue which served to connect all the foregoing evidence concerning the ring structure was available in an important but overlooked observation made by Diels (20) in 1927. He had discovered that the sterols and bile acids could be dehydrogenated by the action of palladium charcoal at 500° C., or more advantageously by the action of selenium at 360° C. He isolated several hydrocarbons from the resulting mixtures, among them being one which has received his name as well as the familiar chrysene. The value of this observation was ignored at the time on the premise that the formation of chrysene from cholesterol involved the loss of nine carbon atoms together with a probable rearrangement due to the drastic conditions of the reaction.



Chrysene

The formation of chrysene seemed irrelevant until the matter of modifying the current formulation in order to clear up a few discrepancies became urgent. Bernal's X-ray measurements (45) of the crystal structure of the sterols did not

allow the possibility of three rings coming together at a point. The reader is referred to #9 in the formulae of 1928 given on page 25. There were also two carbon atoms which had not been accounted for satisfactorily. Rosenheim and King (4) made the revolutionary step in 1932 by suggesting that chrysene was a normal degradation product and that desoxycholic acid was:

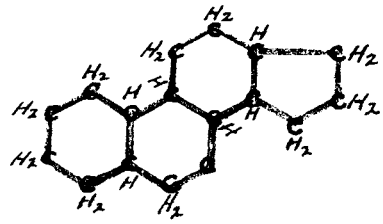


Desoxycholic Acid

(Rosenheim and King -- 1932)

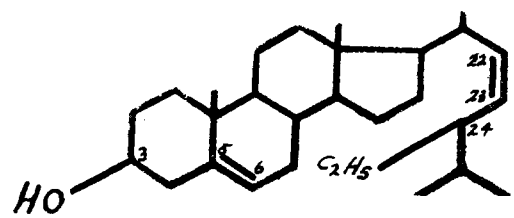
Although the chrysene structure thus represented was an important advance, it was not in accord with certain reliable evidence and required further modification. The result was that the above formula was modified still further by replacing the six-membered Ring IV of chrysene with the recognized five-membered Ring D of the bile acid series. The inclusion of the angular methyl group at #13 during pyrolytic rupture was conceded in order to explain the formation of chrysene. This modified formula recognized the true six-membered nature of Ring II, given erroneously by the formulae on page 25, and placed Ring III on the other side of the corrected Ring II. The formation of the Diels hydrocarbon during selenium dehydrogenation verified this modified formula, and the entire problem was brought to a satisfactory conclusion. The new

formulation consistently and convincingly correlated all previous data and opened the way to the new and larger field of clarifying the chemistry of the biological products related to the sterols which contained the hydrogenated cyclopentenophenanthrene nucleus:



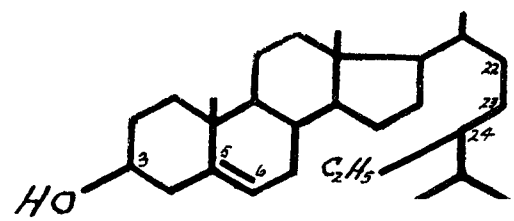
C. Recent Developments

The correct formula for cholesterol was applied to other sterols, among them the phytosterols with which the experimental part of this thesis deals. Stigmasterol was investigated by Fernholz (46,47) and others (48) and proved to be very similar to cholesterol. It differed in having an ethyl group at #24 and a double bond between #22 and #23 in the side chain:



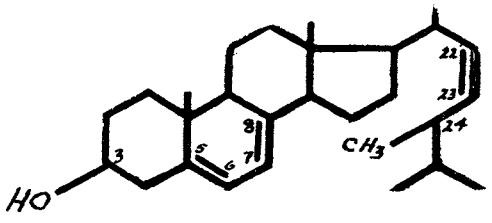
Stigmasterol

Sitosterol, or at least that fraction of "sitosterol" containing a single double bond and designated as Beta-sitosterol, was proven (49) to be 22-dihydrostigmasterol:



Beta-sitosterol

Ergosterol was found to resemble stigmasterol[50], differing in having a methyl group[51] at #24 and an extra nuclear double bond[52] between #7 and #8:



Ergosterol

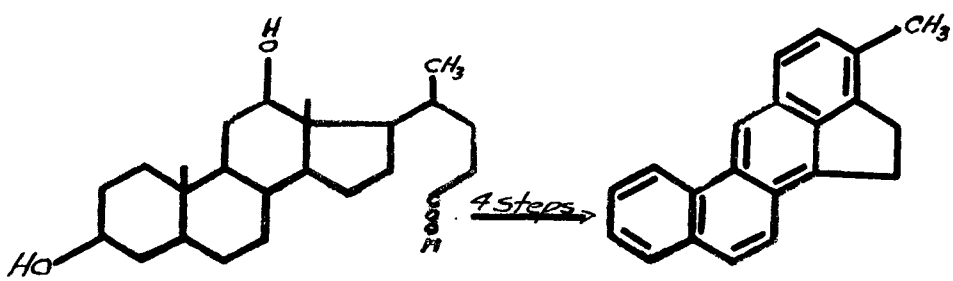
The primary importance of the sterols as starting materials in numerous partial syntheses, degradations and transformations was recognized in the preparation of biologically active substances of closely related composition. Moreover, complex substances were broken down into simpler compounds that could be characterized as steroid in nature. In this way the structures of these complex substances were established with comparative ease. Without attempting to do any more than indicate this pivotal nature of the sterols, the following summary of related compounds has been compiled.

Bile Acids

The first interconversion of the bile acid and sterol series occurred during the correlation of the two series through the oxidation of coprosterol to cholanic acid. The possibility that the bile acids are products of cholesterol metabolism has been postulated (9) through the medium of intermediate substances such as cholestenone and 3-keto-Δ^4-cholenic acid.

Carcinogenic Hydrocarbons

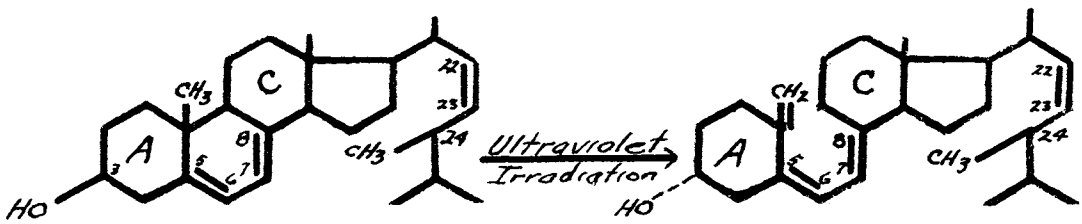
The study of the cancer-producing hydrocarbons (9) began with the isolation of naturally occurring substances found in coal tar. It developed through the detailed syntheses of all possible compounds with characteristic groupings. The investigation reached its climax when methylcholanthrene, the most potent agent yet discovered, was found (53) among the degradation products of desoxycholic acid. The structure of methylcholanthrene was confirmed (54) later by synthesis.



Desoxycholic Acid Methylcholanthrene

Antirachitic Factors

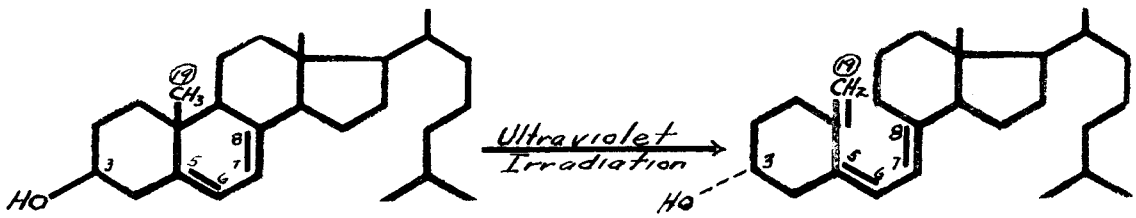
The search for the natural precursor or precursors of the antirachitic vitamin D has shifted from cholesterol to ergosterol and from ergosterol back to cholesterol. The artificial vitamin "calciferol" or vitamin D_2 was prepared (9) by proper irradiation of ergosterol:



Ergosterol Vitamin D_2 "Calciferol"

The production of the natural vitamin, D_3, by irradiating 7-dehydrocholesterol has clarified the exact nature of the structure (9a) necessary for antirachitic activity. All of the known 7-dehydrosterols, with the exception of the stigmasterol derivative, have acquired antirachitic properties upon irradiation although the products differ in potency. This evidence was final proof of the position of the nuclear double bonds in ergosterol and of its relation to cholesterol. The most essential feature of structure was the presence of a system of three conjugated double bonds extending between the original Rings A and C. The double bond in the side chain seemed to detract from the activity while differences in

groupings in the side chain affected the potency very little. The final conclusion reached was that a sterol, in order to function as provitamin D, must contain a diene system between #5 and #8 which can be utilized in the formation of a conjugated system upon irradiation.

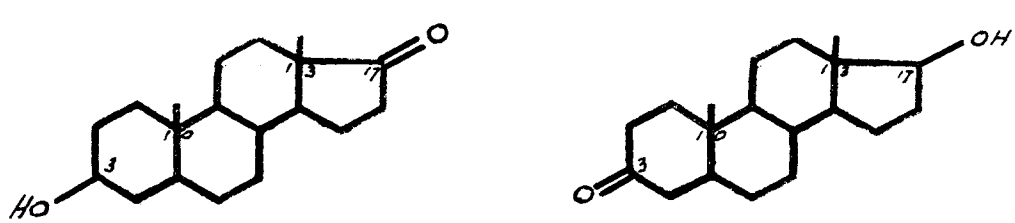


7-Dehydrocholesterol Vitamin D_3

Sex Hormones

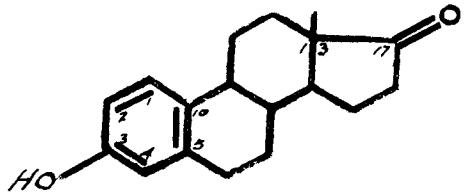
The chemistry of this group (9,55,56) has been strictly a modern problem, extending from 1929 to the present, and has proven to be one of the most spectacular achievements of organic chemistry. These hormones may be classified as male and female with the female hormones subdivided into follicular (oestrin) and corpus luteum (progestin) hormones. Examples of each type have been given below:

Male Hormones

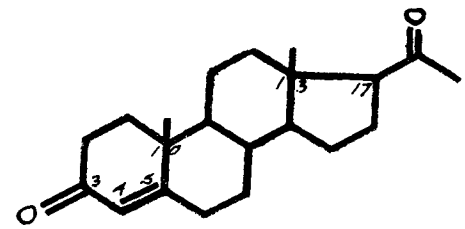


Androsterone Testosterone

Female Hormones

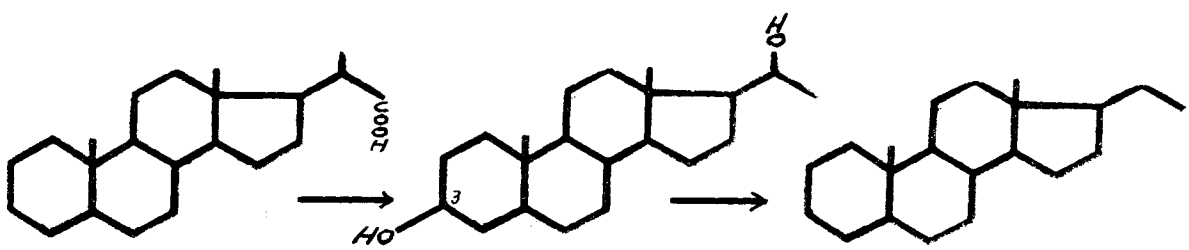


Oestrone



Progesterone

Pregnanediol, an inactive companion substance of the follicular hormones, was the first hormone to be correlated with the bile acid series. The occurrence and close structural relationship of this inactive alcohol with the active sex hormones suggested that it might represent an intermediate stage in the degradation of either the sterols or the bile acids. The saturated hydrocarbon, pregnane, was prepared by the degradation of bisnorcholanic acid:



Bisnorcholanic Acid Pregnanediol Pregnane

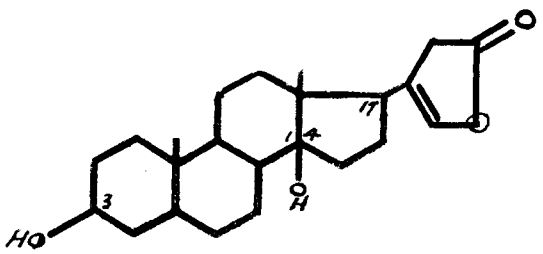
The preparation of oestrone from ergosterol has been accomplished. Progesterone has been synthesized from stigmasterol. An interesting hypothesis has arisen which correlates

oestrogenic and carcinogenic activity, namely, that certain female hormones may under conditions of deranged metabolism give rise to hydrocarbons that induce the formation of cancers and tumors. The male hormones have the same carbon skeleton as the female oestrogenic hormones except for the absence of the methyl group at #10. Androsterone has been correlated with progesterone, synthesized from cholesterol, and used to prepare testosterone. Many interrelationships have been indicated and various schemes have been proposed to explain the biogenetic evolution of these compounds on the basis of a general mechanism involving biological oxidation and reduction. The many structural relationships among the hormones and between them and the sterols have provided abundant circumstantial evidence that the hormones arise from cholesterol rather than by independent biosyntheses.

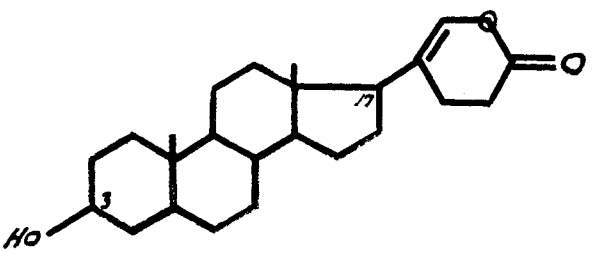
Animal and Vegetable Cardiac Poisons

Two groups of naturally occurring poisons, the glycosidic plant heart poisons and the toad venoms, have been found (9,55,56) to possess a similarity in chemical structure as well as in pharmacological action. These poisons all contained a steroid portion which was correlated with the specific physiological action on the heart muscle. This steroid portion termed "genin" was found linked with one or more molecules of sugar together with acetic acid in some cases in the plant aglycones, while the genin of the toad

venoms was found joined to suberyl arginine. The most characteristic chemical feature of both of these groups of compounds has been the unsaturated lactone ring at #17 which has been responsible also for the specific physiological action of these drugs. A typical example of each has been given below:



Digitoxigenin



Bufotalin

Saponins

The most striking property of these soap-like compounds (9,55) has been the hemolytic action on red blood corpuscles. The hemolytic effect has been explained as due to the formation of a stable molecular compound with cholesterol within the cell membrane. The saponins have been classified into two main groups, namely, the triterpenoid and the steroid sapogenins. The distinction was based on the formation of sapotalene or even an alkyl phenanthrene in the first group and Diels hydrocarbon in the second upon dehydrogenation. Although the cardiac poisons are actually saponins they have

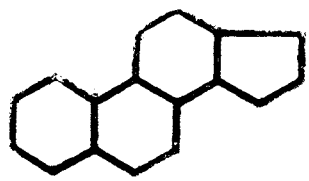
been discussed separately because of their specific action.

It may be seen from the above brief resume that structurally the sterols are related closely to exceedingly important groups of physiological substances.

THE PRESENT STATUS AND THE UNSOLVED PROBLEMS OF STEROL CHEMISTRY

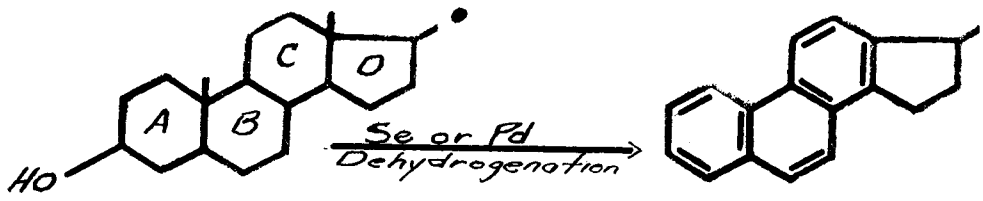
The situation with regard to sterol chemistry at the time the present research was carried on has been summarized herewith preliminary to a presentation of the Experimental Section. The revised chemical definition, methods of preparation, color tests, physical criteria and reactions have been set forth briefly because these served as the starting points in attacking the various problems of the present research. Modifications of procedures and new developments in methods have been described in the Experimental Section.

Structure: The term "steroid" (18) has come to be applied to those compounds which are related chemically to cholesterol and contain a hydrogenated cyclopentenophenanthrene ring system:



including therefore the sterols proper, bile acids, sex hormones, cardiac aglycones, toad poisons and saponins. All of these compounds are defined as "steroid" because they can be dehydrogenated with selenium or palladium charcoal to a certain aromatic hydrocarbon in which the original cyclic carbon skeleton is intact. The reaction serves as an ultimate test

for the reduced cyclopentenophenanthrene nucleus and therefore forms the basis for the inclusion of a compound in the steroid group.



Steroid Molecule
(• represents side chain)

Diels Hydrocarbon
$C_{18}H_{16}$

The Diels hydrocarbon has been established by synthesis as gamma-methylcyclopentenophenanthrene. The production of this unique hydrocarbon is final proof of the steroid nature of a substance. Many substances formerly considered to be sterols because of their occurrence and response to color tests fail to yield this hydrocarbon on dehydrogenation. The absence of the characteristic nucleus in these compounds and hence their non-steroid natures are indicated by such negative results. On the other hand the steroid relationship of an unknown compound is established frequently by the degradation or the transformation of a very small amount of material into a steroid compound of known structure. The new conclusions permit of much more satisfactory investigation of sterols than was possible before these formulae were established.

Preparation: The unsaponifiable portion (57) isolated from fatty material usually serves as the starting material in sterol investigations. The sterols (58) are separated from other naturally occurring substances by recrystallization from suitable solvents, treatment with decolorizing carbon, precipitation with digitonin, or combinations of these methods. Complete separation into individual sterols can not be brought about by ordinary fractionation methods. It is necessary to employ special methods involving the relative degree of unsaturation (16,59) of the sterols or the selective adsorption (60) of suitable adsorbent columns.

Color Tests: The color reactions (9) used for the detection of sterols are not always specific. Many other polynuclear hydroaromatic compounds which are unsaturated or potentially unsaturated behave in a similar manner. The color tests serve primarily to distinguish the unsaturated from the saturated sterols. These colorless, unsaturated sterols give rise to striking color displays when treated with strong acids under dehydrating conditions. The more common tests used are the Salkowski (58), Liebermann-Burchard (58), Whitby A and B (61), Lifschutz (61), Tschugajeff (61), Kahlenberg (62), and Rosenheim (63) reactions.

Physical Criteria: The melting point of a sterol sample or one of its derivatives is not always a good indication of the final purity of the preparation. This exception to the general rule is due to the probable component nature of a

given sterol portion. The specific rotation has been recommended (64) above the melting point as the best evidence as to whether a sample is a mixture of two components or whether it is homogeneous. In a general investigation the component nature is unknown, the amount of material available is small, and ultimate purity is the desired goal. Therefore the mixed melting point method is to be preferred for routine work wherever authentic samples are available for comparison. Photographs of absorption spectra (65) often show distinctive bands in the ultraviolet region and thus may serve as durable evidence of the purity and nature of a given sterol sample.

Reactions: Other typical reactions which may be carried out in an investigation of a sterol sample include many standard organo-chemical procedures together with a number of special procedures designed to utilize distinctive properties of the complex structures. Esterification of the secondary alcoholic group may be followed by saponification of the ester to regenerate the free sterol. Bromination or hydrogenation of the unsaturated linkages may be reversed by suitable reduction or dehydrogenation methods. Dehydration results in the formation of unsaturated linkages. The cyclization of dicarboxylic acids during pyrolysis also splits off the elements of water. Oxidative degradation and ozonization yield clues as to the structure of the sample. In addition to the ordinary reduction procedures involving catalytic hydrogenation, there are several special methods (9)

such as the Clemmensen and the Wolff-Kishner procedures for reducing carbonyl groups. Addition reactions involving the formation of digitonides (66) with digitonin and the diene synthesis with maleic anhydride (67) by the Diels-Alder reaction furnish evidence concerning structural relationships.

There remain other lines of research that require further consideration. The future may be expected to bring forth more information concerning complete synthesis, biogenetic relationships, and supplementary criteria.

Total Synthesis: The approach to this problem has been made plain through the establishment of the cholane structure. A beginning has been made in the synthesis of cyclopentenophenanthrene, the parent hydrocarbon of the cholane structure. The attack on the synthetic side of the problem is as promising of success now as it seemed doomed to failure a short number of years ago when the Nobel laureates, Wieland and Windaus, admitted that the peculiar ring system of their formulae defied attempts at synthesis. The most significant contribution to this problem will come probably from a complete mastery of stereochemical principles as applied to the complicated structures. An attractive feature of the synthetic approach is found in that, although a plan may not succeed in duplicating a natural product, it may result in a physiologically active substance which can be used for biological experimentation or may even prove to be

of therapeutic value.

Biogenetic Relationships: A second fundamental problem concerns the exact physiological and biogenetic relationships of all the compounds closely related to the sterols. The interconversions of all these compounds, the modes and sites of the transformations, together with the formation of other physiologically active compounds, all require rigid explanation. This information can be secured only by continuing the search for additional intermediates of metabolism from new or neglected sources.

Supplementary Criteria: A third problem, not so fundamental but important because it will furnish corroborative evidence to the above two problems, is the development of criteria in other branches of science such as physics and biology which will serve as convenient guide posts and time savers to the organic chemist.

TABULATIONS OF SOURCES AND PROPERTIES OF KNOWN STEROLS

The following tabulations[#] of characterizing information regarding the sterols are presented for the purpose of ready reference. Frequently the values are found to be misleading and irregular. It was believed formerly (16) that apparent discrepancies would be eliminated if phytosterol mixtures were to be separated into two closely related sterols, namely sitosterol and stigmasterol. More recent investigations (1) have proved the existence of an increasing number of isomeric and homologous sterols in mixtures obtained from original sources. Consequently the possibilities for uncertainity with regard to determined values have increased rather than decreased. Original references should be consulted in order to evaluate properly the information given in these tabulations. The last value listed under any heading is intended to be the most up to date.

[#] Tables listing further information with regard to phytosterols and substances of high melting points analagous to phytosterols may be found in Allen's "Commercial Organic Analysis" (68).

Table 1.

Sources and Properties of Some Zoosterols

Name	Source	Formula	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Cholesterol	Animal cells	$C_{27}H_{46}O$ (9)	148-150 (57) 148.4 to 150.8 (69) 150 (9)	-34.3 to -36.6 (57)
Metacholesterol	Contamination of cholesterol (69)			
Dihydrocholesterol	Companion of cholesterol	$C_{27}H_{48}O$ (9)	142 (9)	+28.8 (9)
Coprosterol	Faecal matter	$C_{27}H_{48}O$ (9)	95-96 (69) 98-100 (57) 99-100 (69) 102 (9)	+24 (57) +23.5 (9)
Isocholesterol	Wool fat	$C_{27}H_{46}O$ (57)	139-140 (57)	+60 (57)
Lanosterol	With agnosterol in wool fat	$C_{30}H_{48}O$ (57) $C_{30}H_{50}O$ (9)	141 (9) 140.5 (60)	+84 (57) +58 (9) +58.0 (60)
Agnosterol	With lanosterol in wool fat	$C_{30}H_{46}O$ (57) $C_{30}H_{48}O$ (9)	162 (9)	+70.6 (9)

Table 1. (Concluded)

Sources and Properties of Some Zoosterols

Name	Source	Formula	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Bombicesterol	Chrysalis oil Wax	$C_{27}H_{46}O$ (9a)	148 (57) 139-140 (9a)	-34.9 (57) -31.5 (9a)
Bombicysterol	Silkworm	85% cholesterol 15% sitosterols (18)		
Ostreasterol	Oysters Clams	$C_{29}H_{48}O$ (9)	143 (9)	-43.9 (9)
Actiniasterol	Sea anemone	$C_{27}H_{44}O$ (9a)		
Microciona-sterol	Microciona prolifera	$C_{27}H_{46}O$ (18)		

Table 2.

Sources and Properties of Some Phytosterols

Name	Source	Formula	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Sitosterol	Higher plants	$C_{27}H_{44}O$ (70) $C_{27}H_{46}O$ (57) $C_{29}H_{50}O$ (69)	138-139 (57) 137.5 (59)	-36.69 (57) -34.0 (59)
$alpha_1$ "	" "	$C_{29}H_{48}O$ (9a)		
$alpha_2$ "	" "	$C_{30}H_{50}O$ (9a)		
beta "	" "	$C_{29}H_{50}O$ (9a)		
gamma "	" "	$C_{29}H_{50}O$ (9)	147-148 (71) 146 (9)	-42 (71) -42.4 (9)
Dihydrositosterol	Companion of sitosterol	$C_{27}H_{48}O$ (57) $C_{29}H_{52}O$ (72)	137 (73) 144 (74) 144-145 (57) 143-144 (57)	∠28 (74) ∠25.82 (57) ∠24 (57)
Stigmasterol	Calabar bean Soya bean	$C_{30}H_{48}O$ (75) $C_{30}H_{50}O$ (57) $C_{29}H_{48}O$ (9) $C_{29}H_{50}O$ (18) $C_{29}H_{52}O$ (18)	170 (9)	-45.01 (16) -44.67 (16) -45 (9)

Table 2. (Continued)

Sources and Properties of Some Phytosterols

Name	Source	Formula	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Brassicasterol	Rape oil	$C_{28}H_{46}O$ (57)	148 (57)	-64.25 (57) -63.31 (57) in ether
Fucosterol	Algae	$C_{29}H_{48}O$ (9)	124 (9)	-38.4 (9)
Mycosterol	Fungi Boletus granulatus	$C_{30}H_{46}(OH)_2$ (76)	159-160 (76) 161 (77)	-129.4 (76)
Fongisterol	Fungi	$C_{25}H_{40}O$ (75)	144 (75)	
Ergosterol	Ergot Yeast Lower plants	$C_{27}H_{42}O$ (57) $C_{28}H_{44}O$ (9)	165 (75) 168 (57) 160-162 (69) 163 (9)	165 (69) -132 (57) -133 (9)
Dihydroergosterol	Companion of ergosterol	$C_{28}H_{46}O$ (9)		
Zymosterol	Yeast	$C_{27}H_{44}O$ (9)	108-109 (57) 107-110 (69) 110 (9)	≠17.3 (57) ≠49.2 (69) ≠47.3 (9)

Table 2. (Concluded)

Sources and Properties of Some Phytosterols

Name	Source	Formula	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Kryptosterol	Yeast		138-140 (60)	+58.7 (60)
Cerevisterol	Yeast		240 (57)	-49 (57)
Hyposterol Faecosterol Ascosterol Episterol Anasterol	Yeast (69) Yeast (69) Yeast (69) Yeast (69) Yeast (69)			
Alpha-Typhasterol	Pollen		133-134 (69)	laevo (69)
Hygrosterol	Roots		194 (69)	dextro (69)
Chortosterol	Grasses	$C_{27}H_{54}O$ (78) $C_{27}H_{56}O$ (78)	135-137 (78)	
Cinchol	Cinchona bark	$C_{29}H_{50}O$ (9)	140 (9)	-34 (9)

Table 3.

Properties of Some Phytosterol Preparations

Source	Free Sterol		Acetyl Derivative	
	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Corn oil	137.5 (79)	-34.38 (79)	127 (79)	
Crude corn oil	138-139 (59)	-34.87 (59)	128-130 (59)	-38.81 (59)
Corn gluten	138-139 (59)	-36.69 (59)	130-131 (59)	-40.20 (59)
Corn pollen	137.5 (80)		131 (80)	
Cottonseed oil	139 (58)		125.5 (81)	
	134-135 (79)	-33.61 (79)	119 (79)	
	138-139 (79)	-34.19 (79)	124 (79)	
Linseed oil	134 (79)	-31.16 (79)	124 (79)	
	138 (79)	-34.22 (79)	129-130 (79)	
			131.5 (81)	
Wheat bran	142 (75)		137 (75)	
	140-141 (59)	-34.90 (59)	130-131 (59)	
Cocoanut			127 (82)	
			129.5 (81)	
Palm			131 (81)	
Palm kernel			127 (81)	
Stinging nettle	136-137 (83)	-34.1 (83)	127 (83)	
Scopolia root	137 (73)		127 (73)	

Table 3. (Continued)

Properties of Some Phytosterol Preparations

Source	Free Sterol		Acetyl Derivative	
	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Sunflower seed			119-119.5 (81) 115.5 (81) 119.25 (81)	
Sesame oil			129.5 (81)	
Arachis oil Hydrogenated arachis oil			129 . (81) 128.5 (81) 128.0 (81)	
Olive oil			123.5 (81)	
Soya bean 1st recryst. 15th recryst. 25th recryst. 35th recryst.	 137-138 (74) 139 (74) 140-141 (74) 142 (74)	 -33.9 (74) -29.1 (74) -14.4 (74) + 4.7 (74)		
Rape 1st recryst. 20th recryst.	148 (78) 142 (78) 141-142 (74) 141-142 (74) 141-142 (74)	 -34.4 (74) -34.1 (74) -29.1 (74)	 130 (74) 137-138 (74)	 -38.3 (74) -39.3 (74)
Beet	117 (75)			
Coffee	138 (84)			

Table 3. (Continued)

Properties of Some Phytosterol Preparations

Source	Free Sterol		Acetyl Derivative	
	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Chaulmoogra	132 (85)			
Cabbage seed	142 (78)		137 (78)	
Cabbage leaf	78-79 (78)			
	60-75 (78)			
Cabbage stalk	63-64 (78)			
	102 (78)			
Calabar bean	132-133 (75)		120 (75)	
Grass fruits	135-137 (78)		119-120 (78)	
Adonis vernalis	138-139 (86)	-36 (86)	127-128 (86)	-38.5 (86)
Viburnum prunifolium	186-187 (87)	+115 (87)	223-224 (87)	
General preparations	132-137 (75)			
	135 (58)			
	130-137 (68)		128 (68)	
Wheat and rye	137.5 (75)	-26.71 in ether (75)	127 (75)	
Dihydro-phytosterols	136-137 (88)	+22.97 (88)	134-135 (88)	
	137 (88)	+27.90 (88)	132 (88)	
	142-143 (88)	+24.16 (88)	138-139 (88)	

Table 3. (Continued)

Properties of Some Phytosterol Preparations

Source	Free Sterol		Acetyl Derivative	
	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Alpha-sitosterol				
Corn oil	135-136 (88)	-13.45 (88)		
Wheat germ oil	138-141 (88)	-23.41 (88)		
Beta-sitosterol				
Corn oil	139-140 (88)	-36.11 (88)		
Wheat germ oil	140 (88)	-35.06 (88)		
Gamma-sitosterol				
Corn oil	145-146 (88)	-42.43 (88)		
Wheat germ oil	146-147 (88)	-41.32 (88)		
	147-148 (88)	-42.47 (88)		
Average	147-148 (88)	-42 (88)	143-144 (88)	-45 (88)
Reduced sitosterols				
alpha- corn	139-140 (88)	≠23.53 (88)	137-138 (88)	≠13.64 (88)
alpha- wheat germ	137-138 (88)	≠23.20 (88)	136-137 (88)	≠13.69 (88)
beta- corn	140-141 (88)	≠24.91 (88)	136-137 (88)	≠14.32 (88)
beta- wheat germ	139-140 (88)	≠24.22 (88)	137-138 (88)	≠13.57 (88)
gamma- corn	144-145 (88)	≠17.82 (88)	143-144 (88)	≠ 8.98 (88)
gamma- wheat germ	144-145 (88)	≠17.88 (88)	144-145 (88)	≠10.11 (88)
	143-144 (88)	≠18.01 (88)	142-143 (88)	≠ 9.98 (88)

Table 3. (Concluded)

Properties of Some Phytosterol Preparations

Source	Free Sterol		Acetyl Derivative	
	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform	Melting Point °C.	Specific Rotation $(\alpha)_D$ in Chloroform
Dihydro-sitosterols				
Corn gluten	143-144 (89)	+24.23 (89)	137 (89)	+14.41 (89)
Corn bran	142-143 (89)	+24.05 (89)	139 (89)	
Corn oil	143-144 (89)	+25.18 (89)		
Wheat bran	144-145 (89)	+23.79 (89)	140 (89)	+12.78 (89)
Wheat germ	143-144 (89)	+23.61 (89)	141 (89)	+12.72 (89)
Rice bran	144-145 (89)	+23.90 (89)		

EXPERIMENTAL SECTION

The experimental work included:

1. studies of original oil sources,

2. the determination of percentages of unsaponifiable matter obtainable from each source,

3. the preparation of supplies of crude materials, i.e. unsaponifiable fractions,

4. the application of standard qualitative tests to prove the presence of sterols,

5. the isolation and purification of the sterol portions,

6. the determination of physical and chemical properties of these portions,

7. the investigation of the component nature of these portions, and

8. nutritional investigations on avocado oil and its sterol portion.

A flow sheet has been presented in Figure 1 to illustrate the various procedures available for isolating individual sterols from plant material.

FLOW SHEET

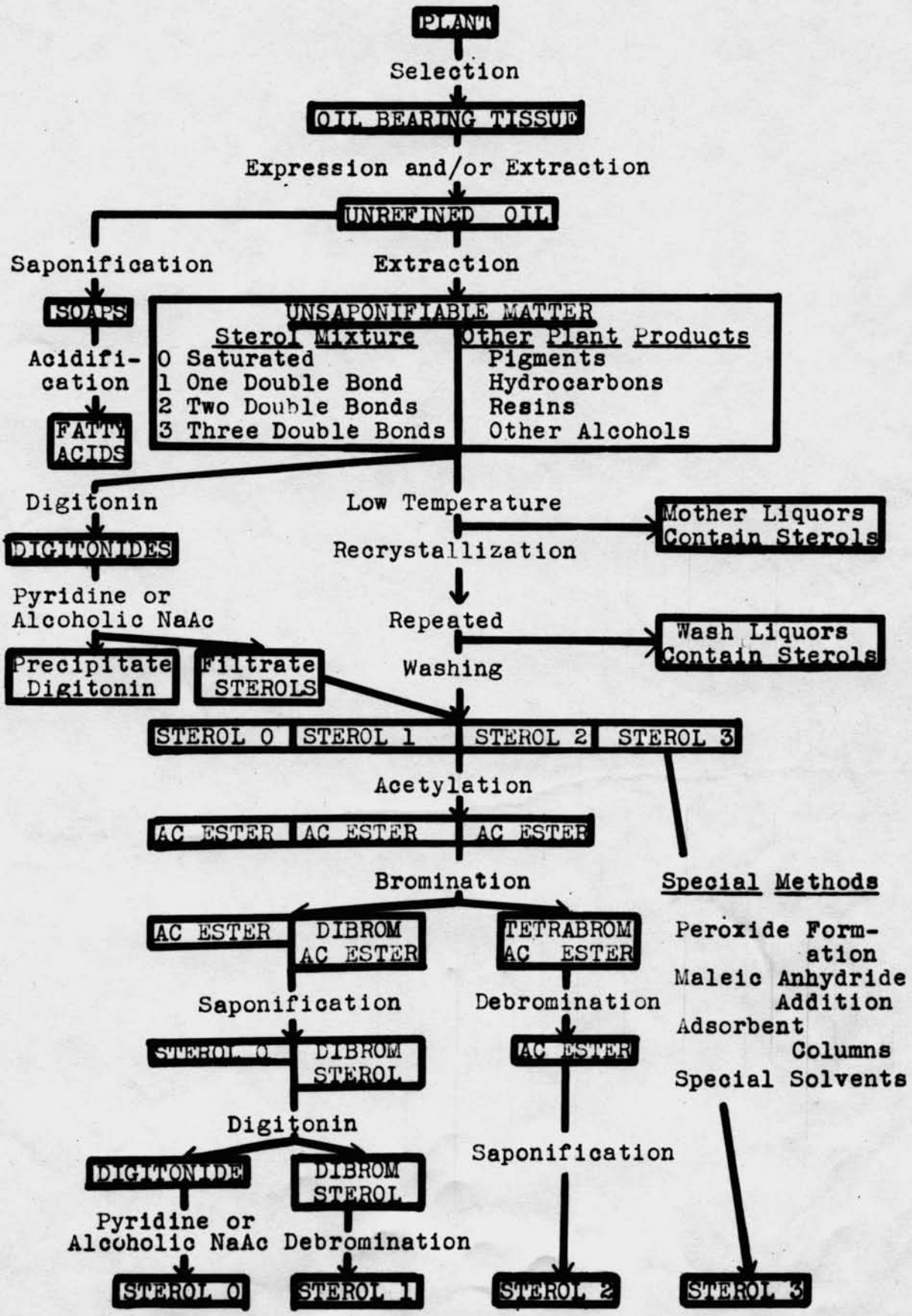


FIGURE 1

A. Materials

A preliminary study (90) showed conclusively that the percentage of unsaponifiable matter and consequently the sterol content of avocado oil depended to a great extent upon the purification process to which the oil had been subjected. Therefore whenever possible, available sources of various oils were used which had been subjected to no purification process that might have removed or destroyed sterols present in the original plant material. A good index of the sterol content of an oil could be obtained by the determination of the percentage of unsaponifiable material present.

Original Sources of Oils

Avocado (Persea gratissima): The oil was prepared by the Hawaiian Avocado Company from the pulp of ripe fruit. Hydraulic pressure in the cold, with no attempt at purification except to draw off the water layer, produced a crude grade of oil that contained a large amount of unsaponifiable matter.

Kukui (Aleurites moluccana): Commercial kukui-nut oil was not available. Fallen, weathered nuts were gathered, the shells cracked, and the kernels loosened and floated in a brine solution. These kernels were washed with water, ground coarsely, and the oil expressed in a manner similar to the avocado oil. Additional oil remaining in the press-cake was obtained by extraction with a mixture of three parts of petroleum ether to one part of ethyl ether. The total yield of

oil was 44.3% based on the weight of the kernels. It was noted that the expressed oil and the extracted oil contained almost exactly the same percentage of unsaponifiable matter.

China-wood (Aleurites fordii): The first sample of china-wood oil available for comparison with kukui-nut oil was a refined Fuller product. A preliminary analysis showed that the unsaponifiable matter was present in such quantity as to indicate a loss during the refining process. Three gallons of the crudest oil available at the Fuller laboratories in San Francisco were secured under the specification "wood-oil foots, 2.46% unsaponifiable matter" and bearing the label "Crude Tung Oil." Although a trial determination on this oil did not confirm the quoted value, the percentage of unsaponifiable matter was found to be more than twice that present in the refined oil.

Chaulmoogra (Taraktogenos kurzii): The first source investigated was the wash liquor from the preparation of chaulmoogric acid in the chemistry laboratories of the University of Hawaii. No sterols could be found but the fatty-acid fraction gave a fair yield of unsaponifiable matter which in turn gave positive qualitative tests for sterols. This evidence indicated that the sterols, although unsaponifiable, had dissolved in the fatty-acid layer rather than in the wash layer.

The second source investigated was the chaulmoogra oil which had been imported from P.K.Sen and Sons of India. The percentage of unsaponifiable matter in this sample approxi-

mated the amount isolated from the fatty-acid fraction mentioned above. A specified order to the Indian firm requesting the crudest grade of oil available and information concerning the process of manufacture resulted in the delivery of guaranteed oil that yielded still less unsaponifiable matter.

Approximately eighty pounds of chaulmoogra nuts were secured from Waiaole, Oahu, through the courtesy of Mr. Charles Judd of the Territorial Board of Agriculture and Foresty. Inasmuch as a brine solution would not float the kernels free from the thin shells, the nuts were cracked and shelled by hand and extracted with the same mixture of solvents used in the kukui-nut oil extraction. The yield of oil was only 10.6% based on the weight of the kernels. The percentage of unsaponifiable matter was so low that the oil prepared by hand showed no appreciable advantage over the oil imported from India. These two oil supplies were kept separate but the yields were combined.

Cocoanut (Cocos nucifera): A partially refined oil was purchased from Benson, Smith & Company, Honolulu, which gave a very small yield of unsaponifiable matter. A cruder grade of oil was secured in cans bearing the label "Cochin Brand" which gave a better yield but still proved to be a very poor source of unsaponifiable matter.

The results of these studies of oil sources have been summarized in Table 4. These results show that avocado oil was by far the best source of unsaponifiable matter and consequently of crude phytosterol.

Table 4.

Comparison of Oils as Sources of Unsaponifiable Matter

Oil and Source	Runs No.	Oil Used g.	Unsaponifiable Matter %
Avocado			
Hawaiian Avocado Co. 1932 supply	1	400	1.05
Hawaiian Avocado Co. 1935 supply	9	3650	1.58
Kukui			
Expressed from local nuts	1	400	1.01
Extracted from local nuts	1	400	1.03
China-wood			
Fuller Co. refined grade	2	800	0.27
Fuller Co. crude tung oil	2	800	0.64
Chaulmoogra			
Wash liquor from fatty acids	1	2000	Trace
Fatty-acid layer	1	200	0.25
Imported from India #1	2	600	0.27
Imported from India #2	2	800	0.21
Extracted from local nuts	1	3850	0.36
Cocoanut			
Benson,Smith & Co. partially refined grade	2	800	0.17
Benson,Smith & Co. cochin brand	2	800	0.24

Quantitative Analyses

The standard method (57) for determining the percentage of unsaponifiable matter in oil samples was modified in order to reduce the probable error for those oils possessing small amounts of unsaponifiable matter. The details of this modified method have been described and compared with other possible modifications of procedure in the section entitled, "Methods."

The analyses based on this modified method were carried out on the oil samples without difficulty. The results have been compiled in Table 5, and are in good agreement with the data given in Table 4.

Table 5.

Unsaponifiable Matter Analyses

Oil	Sample g.	Residue g.	Unsaponifiable Matter %
Avocado	10.04	0.1818	1.81
Kukui	10.00	0.0692	0.69
China-wood	10.02	0.0591	0.59
Chaulmoogra			
Local	10.00	0.0608	0.61
India	10.00	0.0518	0.52
Cocoanut	10.00	0.0254	0.25

Large-Scale Isolation

An improved method used to isolate the unsaponifiable matter from large quantities of oil differed in some respects from the modified method used to analyze small samples. The details of this large-scale isolation have been described in the section entitled, "Methods." While not strictly quantitative in the separation of saponifiable from unsaponifiable matter, this improved method accomplished the separation of the sterol portions without the consumption of very large quantities of ether in the formation of emulsions with the soapy solutions. Even with improved apparatus, partial recovery of solvent, careful manipulation, and admittedly incomplete extraction, over sixty gallons of ether were used in the isolation of the various unsaponifiable fractions according to this procedure.

The application of the improved method to large quantities of the various oils under investigation resulted in yields which are in fair agreement with the quantitative results presented in Table 5. These yields have been compiled in Table 6. Slight discrepancies between these two tables can be explained by the variations in method and the grouping of values obtained from various sources of the oils.

Table 6.

Large-Scale Isolation of Unsaponifiable Matter

Oil	Runs No.	Sample g.	Unsaponifiable Matter %	Unsaponifiable Matter Available g.
Avocado	9	3650	1.58	55.463
Kukui	6	4990	0.75	21.256
China-wood	4	4400	0.44	19.857
Chaulmoogra	9	6850	0.34	17.007
Cocoanut	4	1200	0.26	------#
Cocoanut	1	3600	----	------
Cocoanut	1	200	0.32	0.636

This yield was contaminated by the following yield. The formation of resins was suspected. A small yield from cocoanut oil was secured pending the investigation of the contaminated mixture.

Standard Qualitative Tests

Standard color reactions were applied to the unsaponifiable fractions in order to make certain that the unsaponifiable matter from the various oils contained phytosterols. The standard color reactions used in this investigation were the Whitby A and Whitby B reactions (61). These qualitative tests were designed to improve upon the older, more classical reactions such as the Salkowski (58) and Liebermann-Burchard (58) tests.

Ergosterol was used as the best positive check available. This sterol served to give a number of satisfactory comparisons although it was not entirely typical of the phytosterols as a class. A blank test was run also on the reagents. The Whitby A and Whitby B reactions were applicable to the crude sterol samples represented by the unsaponifiable fractions. The avocado sterol resembled the kukui sterol, the chaulmoogra sterol resembled the cocoanut sterol, but the china-wood sterol failed to give some of the color reactions until the tests were repeated on a purified, dried sample.

The success of all these color reactions depended upon the absence of moisture and the quick perception of some of the fleeting color changes. The tests were sufficiently positive to indicate an abundance of sterol-like material in the unsaponifiable matter from each of the five oils under investigation. The results of these qualitative tests have been compiled in Table 7.

Table 7.

Whitby A and Whitby B Color Reactions

Sample	Whitby A						Whitby B			
	Acid Layer		$CHCl_3$ Layer		Ac Anhydride		Acid Mixture		On Standing	
	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect
Blank	-	-	-	-	-	-	-	-	-	-
Ergosterol	+++	++	+	-	+	-	++++	+++	+++	++++
Avocado	+++	+++	+++	++	+++	+++	+++	++	++	+++
Kukui	+++	+++	++	+++	++	+++	++	+	+	++
Chaulmoogra	++	++	++	++	+	++	++	++	+++	+++
Cocoanut	+++	+++	++	++	+	++	++	++	+++	+++
China-wood	++	++	++	++	-	-	+++	+++	+++	++
China-wood (repeated)	+++	++	++++	++++	++++	++++	+++	++++	++++	++++

(-) Negative color display
(+) Slight color display
(++) Moderate color display
(+++) Strong color display
(++++) Intense color display

B. Methods

Comparison of Methods for Isolation of Unsaponifiable Matter

The difficulties encountered in separating the unsaponifiable matter from large quantities of oil made it necessary to develop a method which would facilitate the handling of large volumes of liquids, which would give satisfactory yields, and which would not require the use of excessive quantities of ether. A study of various methods was made in order to establish a consistent procedure for the isolation of total unsaponifiable matter. Cocoanut oil was used because it had been found to offer the greatest difficulties.

Procedure (a): 5 g. of crude cocoanut oil was weighed out in a tared flask, 30 cc. aldehyde-free 95% alcohol and 5 cc. of a 50% potassium hydroxide solution added with stirring, and the saponification mixture refluxed on a water bath for 20 minutes. After cooling, 50 cc. ether was added and the soap mixture transferred to a separatory funnel. The flask was rinsed with two 50 cc. portions of ether and these portions mixed with the ether-saturated soap solution in the separatory funnel. The resulting ether layer was drawn off and washed with three 100 cc. portions of a dilute solution of potassium hydroxide prepared by dissolving 11.2 g. of the solute in 1000 cc. water. The alkaline ether extract was washed with 30 cc. portions of distilled water until the last portion was colorless to phenolphthalein. The ether was

removed by distilling from a tared flask and the residue dried to constant weight in an oven at 100-105°. The percentage of unsaponifiable matter was calculated in the usual manner. This procedure was essentially the same as the standard method (57) used for examining oils and fats.

Procedure (b): 41 cc. of a cocoanut-soap solution equivalent to 5 g. of cocoanut oil was extracted with a total of 50 cc. ether in four operations, namely 20, 10, 10, and 10 cc. successively. The combined extracts were washed with five 10 cc. portions of water until free from alkali and dried over anhydrous sodium sulfate. The dried extract was filtered into a tared flask, the ether distilled, and the residue brought to constant weight over a water bath. This procedure was designed to adapt the method used in the investigation of the original sources of oils mentioned in the section entitled "Materials" to a smaller scale for analytical purposes.

Procedure (c): The procedure was similar to (b) except that the ether extract was washed first with three 10 cc. portions of dilute potassium hydroxide solution, then with water until free from alkali. This procedure was designed to liberate sterols from loosely formed esters or complexes with fatty acids that might have arisen during or after saponification and that were soluble in ether.

Procedure (d): The procedure was similar to (b) except

that the excess alkali in the soap solution was neutralized with hydrochloric acid and 30 cc. of the dilute potassium hydroxide solution was added before extraction with ether. This procedure was designed to facilitate extraction by first neutralizing the excess alkali in the viscous soap solution and then adding a known amount of alkali sufficient to repress any hydrolysis of the soaps or esterification of the sterols.

Procedure (e): The procedure was similar to (d) except that the ether extract from the neutralized soap solution was washed with 30 cc. of the dilute potassium hydroxide solution. This procedure was designed to insure the complete saponification of all fatty acids.

The results of these procedures have been compared in Table 8. Procedure (a) required too much ether to be practicable although it gave a true maximum value for the percentage of unsaponifiable matter present in the oil sample. Procedure (b) was practicable except for the possibility of the formation of ether-soluble sterol complexes or esters with fatty acids. The presence of these complexes could not be detected in the yields which were low because of incomplete extraction. Procedure (c) was an improvement upon procedure (b) because the sterol portion was freed from possible fatty acids. However, some ether with its share of sterols was removed during the additional washing with the dilute potassium hydroxide solution. The extraction process was simplified by

procedure (d) but fatty acids were released which contaminated the yield. Procedure (e) was successful in overcoming this latter difficulty but resulted in the loss of unsaponifiable matter.

Table 8.

Comparison of Isolation Procedures Using Cocoanut Oil

Procedure	Sample g.	Ether cc.	Ether Extract cc.	Residue g.	Unsaponified Matter %
(a)	5	150	25	0.0286	0.57
(b)	5	50	25	0.0198	0.39
(c)	5	50	20	0.0071	0.14
(d)	5	50	28	0.3654	7.30
(e)	5	50	20	0.0028	0.06

Adopted Methods: From the preceding study two methods were adopted:

1. a procedure referred to on page 61 as a modified method for the quantitative analysis of small samples of oils for their percentage of unsaponifiable matter, and

2. a procedure referred to on page 62 as an improved method for the large-scale isolation of unsaponifiable matter.

The modified method, based upon procedure (a), was devised in order to reduce the probable errors due to insufficient sample and incomplete extraction when careful analyses were to be made. The quantities referred to in procedure (a), page 66, were doubled in order to reduce the probable error for those oils possessing low percentages of unsaponifiable matter. The total volume of ether was divided into three equal parts and three separate extractions were made in order to increase the efficiency of the partition. The large volumes of ether in relation to the small volume of soap solution enabled the extractions to be carried out efficiently. The method consisted in the following operations: 10 g. of oil was weighed in a tared flask and 60 cc. of aldehyde-free alcohol and 10 cc. of a 50% potassium hydroxide solution were added with stirring. The saponification mixture was refluxed for 20 minutes on a water bath, cooled and transferred to a separatory funnel with 100 cc. of ether. The soap solution was saturated with the ether by rotating

the separatory funnel, the ether layer was drawn off and the extraction repeated with two 100 cc. portions of ether. The combined ether extracts were washed with three 200 cc. portions of dilute potassium hydroxide solution prepared by dissolving 11.2 g. of potassium hydroxide in 1000 cc. of water. The alkaline ether extract was washed with 60 cc. portions of distilled water until the last portion was free from alkali as indicated by phenolphthalein. The washed ether extract was distilled from a tared flask and the residue dried to constant weight in an oven at 100-105°. The percentage of unsaponifiable matter was calculated in the usual manner.

The improved method, based on procedure (b), page 67, was devised in order to isolate unsaponifiable matter on a large scale. The large volumes of ether required to extract still larger volumes of soap solution made it necessary to construct suitable apparatus and to develop a certain technique of operation. The method consisted in the following operations: 400 g. of oil was weighed in a 2-liter round-bottom flask and warmed on a water bath. Eight hundred cc. of a 20% alcoholic potassium hydroxide solution, prepared by dissolving 160 g. of U.S.P. potassium hydroxide in a minimum amount of water and then adding sufficient 95% aldehyde-free alcohol prepared by the silver oxide method to make the required 800 cc. volume, was added to the warm, swirling oil. The saponification mixture was refluxed for at least two hours on a water bath, and the excess alcohol was removed by replacing the reflux condenser with a distilling condenser

and later applying a gentle suction in order to draw a current of air through the soap solution. The thick soap solution was diluted with eight or more volumes of water to a consistency suitable for optimum extraction. The dilute soap solution was allowed to trickle through ether until both liquids were saturated with respect to each other. The dilute soap solution was extracted with several portions of fresh ether. It was estimated that a total of 8 liters of ether produced about 4 liters of fairly complete extract. The ether extract was washed carefully with tap water and then vigorously with distilled water until the last portion of wash water was free from alkali as indicated by phenolphthalein. The washed ether extract was dried over anhydrous sodium sulfate placed in several flat-bottom flasks. The dried ether extract was filtered into a distilling flask and the ether was recovered by distillation. The remaining concentrated solution was transferred with anhydrous ether to a tared beaker. The ether solution was evaporated to dryness and the last traces of ether were removed by evacuating carefully over a water bath. The percentage of unsaponifiable matter was calculated in the usual manner. Successive yields were collected in the same tared beaker. The combined yields from one source of oil were resaponified with a suitable quantity of 2% alcoholic potassium hydroxide solution and the unsaponifiable residue was isolated and reserved for further purification.

STEROL EXTRACTION APPARATUS

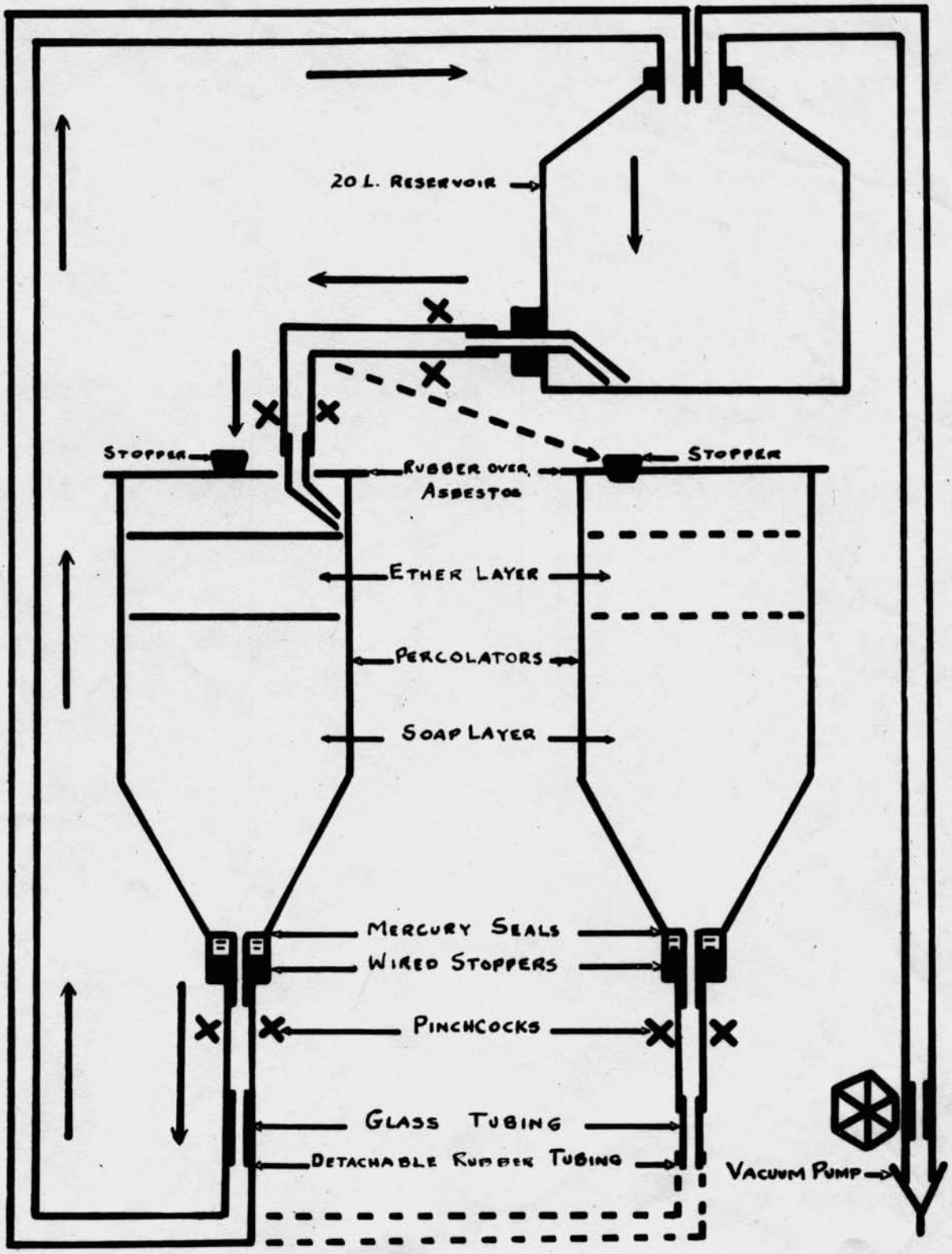


FIGURE 2

The isolation of unsaponifiable matter sufficient for purposes at hand involved such large volumes of liquids that it was deemed expedient to run the operations on a semi-factory scale and to recover solvents concurrently with the other manipulations. Fire and health hazards resulting from such a practice were reduced to a minimum by using proper precautions. Special apparatus was designed to handle the large volumes easily and safely, to prevent undue agitation during the transfer of such large volumes, and to minimize the loss of ether due to evaporation. A sketch of this apparatus has been presented in Figure 2.

The diluted soap solution referred to on page 72 was drawn into the reservoir by means of the vacuum pump (see Figure 2.). Ether was placed in the bottom of the percolator and the diluted soap solution was allowed to flow in along the edges and down through the ether. The two liquid phases were saturated in this manner without agitation. After complete saturation a stream of recovered alcohol was directed from a wash bottle so as to drain in a film down the edges of the percolator. A layer of ether extract began to form immediately. Its final thickness depended upon the tenacity of the particular soap solution and upon the rapidity of evaporation from the surface. The evaporation of the ether could not be suppressed completely but it could be diminished by fitting the top of the percolator with a stoppered asbestos cover tied down under a sheet of rubber.

The ether (upper) layer was separated by drawing off the

soap solution (lower) layer into the reservoir. The emulsion lying between these two layers was drawn off and placed in the second percolator where it broke up either spontaneously or with the aid of a film of alcohol. The removal of this layer greatly expedited the subsequent extractions. The combined extracts were placed in the percolator and water was poured in carefully along the sides and drawn off into the extracted soap solution in the reservoir. Opalescence in the wash water indicated the removal of soap. The partially washed ether extract was shaken thoroughly with small portions of tap water to remove the last traces of soap and alkali. The last washing was made with distilled water and was tested with phenolphthalein. A faint, persistent pink color actually served for the end point because continued washing with water tended to decrease the yields by dissolving an appreciable amount of ether together with its dissolved solute.

In the resaponification of the combined yields referred to on page 72, difficulties were encountered because of the formation of persistent emulsions involving the sterols which were present at this stage in high concentration. This difficulty was overcome by bringing the resaponification mixture as nearly as possible to dryness without excessive heat or protracted action of concentrated alkali. The semi-liquid mass was dissolved as completely as possible with ether, water was added cautiously, and the resulting opalescent soap solution was drawn off and re-extracted with fresh ether. This procedure was repeated as many times as necessary to separate the soap from the unsaponifiable matter. Agitation

of water in the presence of suitable amounts of soap, unsaponifiable matter, alkali, and alcohol formed emulsions which could be broken up only with great difficulty. The combined ether extracts were washed and dried in the manner prescribed and finally evaporated to constant weight. The unsaponifiable residue served as the supply of crude material from which sterol portions were isolated.

A method was devised incidentally for collecting one of the by-products, the expensive fatty acids which were present in the extracted soap solution referred to on page 75. Quantities of ether present in the soap solution were removed by distillation from a 20-liter flask. A gentle stream of air drawn through the soap helped to bring the ether to the surface, to break the bubbles, and to release the ether into the top of the still. Care was taken to prevent excessive frothing which would otherwise have contaminated the recovered ether with soap. This wet, recovered ether could be used for further extractions. The hot soap solution was poured out into a large evaporating dish and allowed to stand for some time in a draught to remove all traces of ether. The soap solution was brought to the boiling point and crude, concentrated hydrochloric acid was added with constant stirring until a strip of blue litmus turned definitely red. A white curd of fatty acids formed which could be liquefied by continued boiling. The entire contents were poured into a 5-gallon container and the water layer was drawn off with a siphon. The fatty-acid layer was washed with hot water until

the last portion of wash water was neutral to methyl orange. The fatty acids were stored after removal of water.

Isolation of Sterol Portions from Unsaponifiable Matter

It was anticipated that the conversion of crude unsaponifiable matter into acceptable sources of sterols would present difficulties. The usual procedure, purification by recrystallization until a constant melting point resulted, was carried out on all the unsaponifiable fractions but a great loss of material occurred in the mother liquors and washings. The solubilities of the sterols themselves and of the accompanying impurities[#] were so close that repeated recrystallization was necessary. In order to arrive at the optimum method of purification it was necessary to know:

1. the loss of sterol in the filtrate and washings, and
2. the gain in purity of the crystallized product as indicated by the rise in its melting point.

In the following series of experiments two methods were compared, namely, recrystallization from alcohol, and washing with petroleum ether. A third method of purification, involving a freezing-out process at low temperatures, was developed later.

[#] The unsaponifiable fraction of wheat-germ oil (91) was found to contain sitosterol, dihydrositosterol, ergosterol, dihydroergosterol, lutein, kryptoxanthin, beta-amyrin, squalene, a second squalene hydrocarbon, a liquid hydrocarbon, gamma-carotene or rubixanthene, and vitamin E.

Recrystallization from Alcohol: 0.4975 g. of avocado sterol, previously purifed to a melting point of 135-136°, was dissolved in a minimum volume of 15 cc. of boiling alcohol, cooled, stoppered and set in the refrigerator. The resulting semi-solid mass was filtered rapidly and the precipitate was washed with 10 cc. of chilled alcohol. The washed precipitate was redissolved in a stream of boiling alcohol and evaporated in a tared dish giving a residue weighing 0.5275 g. This weight was larger than that of the sample and indicated that some alcohol was trapped in the mass of sterol purified by recrystallization. Therefore the yield, 0.4170 g., was estimated by taking the difference between the weight of the sample, 0.4975 g., and the weight of the residue, 0.0805 g., obtained by evaporating the filtrate. The melting point, determined directly on the purified residue, was found to lie in the range 137.5° to 140.0°.

The same procedure was applied to 1.000 g. of crude avocado sterol of melting point 118-120°. A total of 40 cc. of solvent was used and the melting point of the product was found to lie in the range 126.5° to 129.5°.

Washing with Petroleum Ether: It was noted that petroleum ether readily dissolved the yellow coloring matter that accompanied the crude sterol supplies. This solvent dissolved the coloring matter from the surfaces first, leaving white, shiny flakes behind. It was though that an efficient purification might be effected by washing and filtering rapidly in the cold.

A 0.6305 g. sample of avocado sterol of melting point 135-136° was shaken with 5 cc. of cold petroleum ether, chilled for several minutes, filtered rapidly and the process repeated with a fresh 5 cc. portion of solvent. The combined filtrates were evaporated in a tared dish giving a residue of 0.0220 g. The washed portion was dissolved in ether and evaporated in a tared dish. A residue weighing 0.6000 g. and having a sharp melting point of 142.0-142.5° was obtained. The yield was good and the product comparatively pure.

The same procedure was applied to 1.000 g. of crude avocado sterol of melting point 118-120°. Forty cc. of petroleum ether were used in wash portions of 20, 10, 5, and 5 cc. respectively. The melting point of the product was 128.0-130.0°.

The results of this series of experiments, compiled in Tabled 9, indicated that petroleum ether might serve as a purification agent if it were used to wash quickly the large, crusty, relatively pure crystals of sterol material which retained most of the remaining impurity on the exposed surfaces. The solubility of the sterol portion was high when more complete solution was required to dissolve the impurity contained within semi-amorphous masses. Recrystallization of such masses from alcohol offered the better approach to final purity.

Table 9.

Comparison of Two Methods for Purifying Sterol Portions

Sample and Factors Involved	Recrystallization from Alcohol	Washing with Petroleum Ether
Avocado Sterol (135-136°)		
Loss in filtrate (g./cc.)	0.0037	0.0024
Loss in filtrate (%)	17.1	3.5
Loss in transfer (%)	----	1.3
Recovery yield (%)	83.9	95.2
Melting point (°C.)	137.5 - 140.0	142.0 - 142.5
Rise in melting point (°C.)	4.0	6.5
Crude Avocado Sterol (118-120°)		
Loss in filtrate (g./cc.)	0.0039	0.0076
Loss in filtrate (%)	15.5	30.5
Loss in transfer (%)	3.3	8.0
Recovery yield (%)	81.2	61.5
Melting point (°C.)	126.5 - 129.5	128.0 - 130.0
Rise in melting point (°C.)	9.5	10.0

Low-Temperature Method: In order to avoid the large loss of material incurred during repeated recrystallization from alcohol, a suggestion found in the literature (91) concerning the concentration of vitamin E was expanded to effect the recovery of sterols. The method consisted in placing the entire unsaponifiable residue in an Erlenmeyer flask, dissolving it in a minimum volume of methyl alcohol over a water bath, and freezing out the sterol portion by placing the solution in a bath at -60°. In order to adapt this suggestion to the purification of the sterol portions present in the crude unsaponifiable residues mentioned in Table 6, the appropriate solvent action had to be determined. Various organic solvents were tested to see whether they would:

1. dissolve all unsaponifiable matter at the temperature of the water bath,
2. remain liquid at -60°,
3. precipitate out most of the sterols,
4. retain most of the impurities in solution, and
5. allow rapid, efficient filtration.

Ethyl alcohol dissolved all unsaponifiable matter in a small volume and remained liquid at -60°. The sterol precipitate, however, retained most of the solvent and the dissolved impurities. No gain in purity could be effected unless an excessive amount of solvent was used for washing the precipitate.

Petroleum ether dissolved all unsaponifiable matter very easily but formed a colloidal precipitate on freezing which

could be separated only with difficulty in a centrifuge. The filtration was difficult and the temperature rose before any separation could be effected.

Methyl alcohol brought about complete solution but required the use of a larger volume of solvent and more prolonged heating. A coarse, grainy precipitate settled out quickly on freezing which could be filtered rapidly and washed easily. Concentration of the filtrate followed by refreezing showed that only a relatively small quantity of sterol remained in the saturated solution at the temperature of the freezing bath.

In order to combine the good features of the methyl alcohol and ethyl alcohol treatments, tests were run to determine what relative proportions of the two solvents should be used to bring about optimum solution of impurity together with optimum precipitation of sterol. It was found that the ratio should be varied for successive purifications depending upon the relative purity of each fraction. A convenient plan was to freeze out the crude sample from pure methyl alcohol, whereby most of the sterol was retained together with some impurity. Further purification was made with a mixture of one part ethyl alcohol to two parts methyl alcohol in order to dissolve most of the remaining impurity and at the same time to retain a large part of the sterol. In the third step in the purification a mixture of one part ethyl alcohol to three parts methyl alcohol was used, and so on, until comparative purity was attained. The final purification

was made either by recrystallizing from absolute alcohol or by washing with chilled petroleum ether. The recrystallization process produced beautiful crystalline structure from amorphous masses. The washing process could be used advantageously if the precipitates were grainy and porous.

The mother liquors and washings obtained from repeated purifications were evaporated and allowed to stand for several months. Beautiful, crystalline products were deposited which could be separated very easily by filtration from the dark-colored mother liquors. These bottom fractions melted at a higher temperature than did the purest yields obtained by recrystallization of the top fractions. These unexpected results have been studied in detail in the section entitled, "The Component Nature of the Sterol Portions", and the view that each sterol portion was actually a mixture of closely related sterols has been confirmed.

The conclusions that could be drawn concerning the best technique for purifying sterol portions were as follow:

1. Sterol portions from different oils required different procedures. It was necessary to experiment on small samples of each product before each purification stage. If a mixture of solvents was required, most of the material was dissolved in the solvent possessing the greatest solvent action. The second solvent was added then to complete the solution, to decrease the solubility of the sterol portion, and to furnish a convenient medium for the freezing-out process and subsequent filtration.

2. The freezing bath was constructed of a box packed with asbestos insulation in which was imbedded a beaker containing ether and methyl alcohol. Cakes of carbon dioxide ice, formed by releasing the gas from a cylinder into a mold, were added to this mixture until the desired temperature of -60° was attained. If absolutely pure carbon dioxide had been available, the ice could have been dropped directly into the alcoholic solution of unsaponifiable matter, the chilled solution filtered directly over a cake of ice, and the efficiency of the process thereby increased. Unfortunately, however, the carbon dioxide ice made in the laboratory contained traces of oil. It was necessary, therefore, to chill the filter plate during the filtration and to replace clogged filter papers in order to permit the filtrate to pass through at the low temperature.

3. The use of water to decrease the solubility of sterols in organic solvents is mentioned often in the literature, but it was found that the disadvantages more than outweighed the advantages of such a procedure. The admixture of water and sterols often caused emulsions and semi-solids to form which were difficult to handle. Also, the texture of the precipitated product was poor and the melting point was low. Unless the product was rendered anhydrous by thorough drying at the dangerously high temperature of $100\text{-}105^{\circ}$, sterol hydrates of varying degrees of hydration confused analytical data. Water could be used advantageously in careful extractions and washings, but it could not be used for subsequent purification by precipitation.

C. Properties of the Sterol Portions

Physical and chemical properties of the sterol portions as entities are presented in this section. It was pointed out in the preceding section that the sterol portions isolated by the low-temperature method were actually mixtures of closely related sterols. A discussion of the physical and chemical data pertinent to the component nature of the sterol portions has been reserved for the following section entitled, "The Component Nature of the Sterol Portions."

Physical Properties

The sterol portions could not be characterized completely by the usual criterion of a constant melting point, nor could they be characterized completely by the more definite criterion of a specific rotation. These two criteria were applicable to homogeneous fractions only.

Solubility: The sterol portions were soluble in most of the ordinary organic solvents. They were extremely soluble in ether, petroleum ether, chloroform, acetone, and hot absolute alcohol; soluble in cold 95% alcohol, benzene, and pyridine; sparingly soluble in methyl alcohol and glacial acetic acid; and formed colloidal semi-solids in water.

Crystalline Form: The sterol portions crystallized in silky, lustrous, white plates from absolute alcohol and in solid, needle-like, white tufts from anhydrous ether. These crystalline forms tended to revert to amorphous powders on long standing in a desiccator.

Molecular Weight: The molecular weights of the various sterol portions were determined by the camphor fusion method in order to conserve material. This method had been checked previously with the freezing point depression method and found to agree for avocado sterol (90) within the limits of probable error. A sample of ergosterol was used as a known to check these determinations. The available molecular weight for ergosterol was 382.33 at the time the determinations were made; later it was found that the present accepted value (9) for ergosterol is 396.35. The agreement between the determined and accepted values for ergosterol is excellent.

The molecular weights determined for the other sterol portions can be considered valid to within 5% of the probable true values. Assuming that the molecular weights of any two components would fall within the limits of probable error, no further error was introduced by making the determinations directly on unfractionated sterol portions from each oil under investigation. The results of the molecular weight determinations have been compiled in Table 10.

Table 10.

Molecular Weight Determinations by the Camphor Fusion Method

Sterol Portion	Camphor g.	Sterol g.	Depression in Fusion Point °C.	Molecular Weight
Ergosterol	0.1511	0.0143	9.5	398.4
Avocado	0.1527 0.1582	0.0153 0.0118	11.0 8.5	364.4 351.0
Kukui	0.1515	0.0170	12.3	364.9
China-wood	0.1527	0.0177	12.7	365.0
Chaulmoogra	0.1521	0.0150	10.0	394.4
Cocoanut	0.1554	0.0199	13.0	394.0

Chemical Properties

Formation of Acetyl Derivative: The sterol portions under investigation were purified still further and converted into the corresponding acetyl derivatives by refluxing with a slight excess of acetic anhydride, removing the excess reagent under vacuum, and recrystallizing the acetylation product from alcohol. The formation of the acetyl derivative of each sterol portion served three distinct purposes:

1. It gave a derivative for characterization distinct from the free sterol.

2. It furnished a value for the mean equivalent weight of the sterol portions which could be correlated with the mean molecular weight determined by other methods.

3. It made possible the determination of the number of hydroxyl groups present in each sterol molecule.

The melting points and specific rotations of the acetyl derivatives were determined in the usual manner. The corresponding values for sitosterol found in the literature and compiled in Table 2 and Table 3 varied considerably. The general range of values cited was 137-140° for the melting point of the free sterol, 127-130° for the melting point of the acetyl derivative, and -38° to -40° for the specific rotation of the acetyl derivative. It is evident that final purity was not attained and that repeated purification did not guarantee an approach to final purity.

Table 11.

Melting Points and Specific Rotations of the Acetyl Derivatives

Sterol Portion	Free Sterol Melting Point °C. (corr.)	Acetyl Derivative Melting Point °C. (corr.)	Acetyl Derivative Specific Rotation $(\alpha)_D^{26}$
Avocado	134.3	122.8	-27.77
Kukui	133.2	123.3	-22.72
China-wood	135.3	124.3	-26.14
Chaulmoogra	131.6	125.9 126.0# 126.9##	-29.14
Cocoanut	131.0	128.5	-31.20###

First recrystallization

Second recrystallization

The small amount of sample available for the determination made this value doubtful.

The equivalent weights of the various acetyl derivatives, and hence the number of hydroxyl groups present in the original sterols, were determined by standard methods (72). A weighed quantity of the purified acetyl derivative was hydrolyzed in each case with a calculated amount of standard alcoholic potassium hydroxide and the excess hydroxide ion was titrated with standard hydrochloric acid. The amount of acetate ion available, as determined indirectly by the titration, was used to calculate the equivalent weight of the acetyl derivative and hence the number of hydroxyl groups present in the free sterol. The results have been compiled in Table 12. The equivalent weight is usually slightly higher than the corresponding molecular weight calculated by adding the acetyl value of 42 to the molecular weight of the free sterol determined by the camphor fusion method. The mean of these two determinations, however, furnish values which are within 5% of the reported value (72) for sitosteryl acetate, 456.4. Therefore each sterol molecule must contain only one hydroxyl group. All of the sterol portions under investigation can be classified as "sitosterol" on the basis of these determinations.

Table 12.

Equivalent Weights of the Acetyl Derivatives

Sterol Portion	Acetyl Derivative g.	0.2520 N Acid cc.	Milli-equivalents #	Equivalent Weight	Molecular Weight	Hydroxyl Groups #
Avocado	0.0420	0.33	0.0832	505	415	1
Kukui	0.0517	0.45	0.1134	456	407	1
China-wood	0.0875	0.63	0.1588	551	407	1
Chaulmoogra	0.0776 0.0401	0.50 0.40	0.1260 0.1008	616 398	436 436	1 1
Cocoanut	0.0628	0.50	0.1260	499	436	1

Formation of Digitonide: It is known (25) that an alcoholic solution of digitonin precipitates as insoluble addition products all sterols conforming to the beta-configuration of the free hydroxyl group at position #3. This reaction is used to separate sterols out of mixtures in a quantitative manner (57,58) because the insoluble digitonides of definite composition can be collected and weighed. The problem of digitonin precipitation was investigated in detail in order to facilitate further investigations on sterol mixtures. This study included:

1. the preparation of the digitonin solution,
2. the precipitation of the cocoanut sterol portion from the contaminated unsaponifiable residue, and
3. the quantitative application of the standard method (57,58) to unsaponifiable residues from all of the oils.

The preparation of a 1% digitonin solution in 90% alcohol presented unexpected difficulties. One g. of digitonin would not dissolve completely in 100 cc. of 90% alcohol at the boiling point. Approximately 10 mg. samples of Merck's digitonin were tested with 5 cc. portions of various solvents. Water formed frothy suspensions, ether failed to exert any noticeable solvent action, 95% alcohol showed slight solvent action, while absolute alcohol dissolved the sample completely. Therefore 1.000 g. of digitonin was dissolved in 50 cc. of warm, absolute alcohol. Sufficient alcohol and water were added to make a clear, 90% alcoholic medium containing approximately 1% dissolved digitonin.

The precipitation, as digitonide, of the cocoanut sterol portion from the persistent contamination was attempted next in order to test the method and at the same time to secure an uncontaminated supply of cocoanut sterol. Preliminary tests on alcoholic solutions of cholesterol and uncontaminated cocoanut sterol were positive. The digitonin solution prepared as described above was added to the unsaponifiable residue. The mixture was warmed for one hour and placed in the refrigerator. A voluminous, somewhat amorphous precipitate was collected on a filter paper, washed with cold 90% alcohol and ether, and dried at 105° to constant weight. This precipitate of digitonide accounted for 0.995 g. of digitonin and 0.319 g. of sterol. This almost quantitative yield was divided into two parts and reserved for cleavage into sterol and digitonin.

The quantitative application of this method to the unsaponifiable residues from all the oils listed in Table 5 was justified by the above result. The precipitation of each sample was carried out as described in the above paragraph. The digitonide was collected in a Gooch crucible fitted with a double-thickness of filter paper. The calculations of quantities of reagents and solvents to be used were based on the assumption that the unsaponifiable matter in each sample was 100% sterol. It was found that the sterol portion could be separated quantitatively from other unsaponifiable matter in the form of its digitonide. The results of these studies have been compiled in Table 13. Avocado oil is shown to contain approximately twelve times as much sterol as cocoanut oil, with the other oils lying between these extremes.

Table 13.

Determination of Sterol Content by Digitonin Precipitation

10 g. Oil Sample	Unsaponifiable Matter g.	Digitonide g.	Sterol in Unsaponifiable Matter %	Sterol in Oil %
Avocado	0.1818	0.4310	57.63	1.05
Kukui	0.0692	0.1114	39.13	0.27
China-wood	0.0591	0.0907	37.30	0.22
Chaulmoogra				
Local	0.0608	0.1045	41.77	0.25
India	0.0518	0.0700	32.82	0.17
Cocoanut	0.0254	0.0367	35.13	0.09

Cleavage of Digitonide: Special methods were required for the quantitative recovery of the sterols and the expensive digitonin from the digitonides. The methods were tested in advance on various digitonides in order to establish the optimum procedure for the examination of the digitonides mentioned in Table 13. The entire study was intended to facilitate further investigations involving the use of digitonin as a precipitant for sterol mixtures. The methods tested on a quantitative basis involved the application of:

1. xylene,
2. pyridine,
3. acetic anhydride, and
4. alcoholic sodium acetate

to various available digitonides.

The xylene method (9,58) was applied to the digitonide prepared from avocado sterol. About 1.3 g. of the washed and dried precipitate was boiled with five 25 cc. portions of xylene. The hot xylene was decanted each time through a filter and the undissolved material on the filter paper was transferred back into the flask with fresh xylene. The combined extracts were distilled under a partial vacuum at a temperature never exceeding 85°. A light-yellow liquid remained instead of the white solid sterol expected. Acetylation with 20 cc. of acetic anhydride, followed by the removal of the excess reagent under a vacuum at the temperature of a water bath, resulted in an amber-colored liquid instead of a white, crystalline mass of sterol acetate. The residue from the xylene extraction was treated again with 125 cc. of xylene

under reflux for three hours with slight success. The solubility of the filtered material in alcohol was poor, indicating that considerable undissociated digitonide was present after repeated treatment with xylene. The filtrate was acetylated again, but the product indicated even greater decomposition. It was concluded that the method failed due to the excessive temperature of boiling xylene and the failure of this solvent to dissociate the digitonide into the original sterol and digitonin. The xylene method was abandoned in favor of the pyridine method.

The pyridine method (66) seemed to offer a solution to the difficulties encountered with xylene. The method was applied to:

1. the xylene residue,
2. a known sample of cholesterol digitonide,
3. a purified sample of avocado sterol digitonide, and
4. several samples of cocoanut sterol digitonide.

The undissolved and uncleaved digitonide residue from the xylene extraction was dissolved in 30 cc. of pyridine. An opaque suspension resulted when 50 cc. of ether was added to the pyridine solution. This suspension settled on standing and could be filtered free from the supernatant liquid presumably containing the free sterol. The precipitate was washed with ether and dried. The product weighed 0.699 g.; assuming this to be recovered digitonin and ignoring any dissociation that the xylene might have effected previously,

the recovery of digitonin amounted to 66.9%. The pyridine in the filtrate was evaporated with difficulty and the residue was recrystallized from alcohol. The formation of a dark-colored product indicated that the original sterol had undergone decomposition. The yield amounted to 32.4% or more, melted at 144-145° on a copper bar, melted at 140-142° in a tube, and did not resemble the original sterol in color, crystalline form, or solubility. It was concluded that the original method as outlined in the literature (66) should be checked thoroughly before risking limited supplies of sterol portions.

The pyridine method was checked using cholesterol rather than continuing to experiment on unknown sterol portions. A sample of cholesterol, Eastman Brand #909, melting point 147°, and weighing 0.1124 g. was dissolved in 10 cc. of absolute alcohol and treated with 30 cc. of the digitonin reagent. A definite precipitate formed almost immediately which differed to some extent in appearance and time of formation in comparison with the sterol digitonides under investigation. An additional 3 cc. of digitonin reagent caused no further precipitation. The precipitate was digested for one hour over a water bath, allowed to stand overnight in a refrigerator, filtered, washed with absolute alcohol and ether, and dried to constant weight at 105°. The yield of digitonide was 87.9%. More digitonide crystallized out from the filtrate on standing due to the use of absolute alcohol instead of 95% alcohol. This accounted for the low yield of 87.9% in comparison with the quantitative results claimed for the method.

The cholesterol digitonide was dissolved in a little over 2 cc. of warm, purified pyridine to give a slightly colored solution. A little over 20 cc. of anhydrous ether was added and after cooling, filtering, and washing the precipitate, it was treated with another 2 cc. portion of cold pyridine and 20 cc. of anhydrous ether. The precipitate only partially dissolved in absolute alcohol, indicating that some uncleaved digitonide was present. A clear filtrate of this alcoholic solution gave a positive test for digitonin by precipitating an alcoholic solution of cholesterol. There was no doubt that some digitonin was recovered but no yield could be estimated. The combined filtrates containing the free sterol portion were brought to dryness. The ether was removed over the water bath but the pyridine could be removed only by evacuation over concentrated sulfuric acid. A slight amount of color developed despite all precautions. The yield amounted to 78.6% and melted at 146.9° in a tube. Upon recrystallization from absolute alcohol the melting point of the product rose to almost 148° in comparison with the original sample which melted at 147°. These melting points indicated that the original sample had been purified to within 2° of the theoretical value for cholesterol. It was concluded from this rigid check of method that the pyridine method for the cleavage of cholesterol digitonide did not give the quantitative results claimed for it, although it was satisfactory in other respects. A slight yellow color developed during the process which could be removed by crystallization, whereupon the purity of the product exceeded that of the Eastman preparation and approached the theoretical stand-

ard. The digitonin could not be recovered completely but the recoverable portion could be used for reprecipitation of sterols. The pyridine method was feasible if precautions were taken to use a minimum amount of pyridine, to precipitate and recover the digitonin with anhydrous ether, and to avoid unnecessary heating.

The pyridine method was applied to a purified sample of avocado sterol using purified reagents and solvents and keeping the above precautions in mind. A sample of the purest available avocado sterol, melting point checked twice at 137.4°, and weighing 0.500 g. was converted into the digitonide in the prescribed manner. The dried digitonide was dissolved in a minimum amount of pyridine purified by distilling at 112-117° over potassium hydroxide, treated with an excess of anhydrous ether, and set in the refrigerator. A small amount of digitonide precipitated out at the colder temperature and was filtered with the digitonin that had separated. A quantity of digitonide also crystallized out on standing from the mother liquor of the digitonide precipitation. Both of these precipitates were collected together and cleaved with pyridine and ether in the prescribed manner. The yield of digitonin amounted to 72.3%. The combined filtrates and washings were concentrated to a light yellow-colored liquid over phosphorous pentoxide. It was necessary to set the desiccator on a water bath at 80° and evacuate for several hours in order to bring the solution to dryness. The intensity of the yellow color was not increased. The yield amounted to 52.1%, melted at 131.3° on a copper bar and at

134.3° in a tube, and was poor in quality. Crystallization from alcohol followed by repeated washing with the same solvent did not remove completely the odor of pyridine. The crystallized product amounted to about one-half of the original yield, melted at 133.3° on a copper bar and at 135.4° in a tube, and was not comparable with the original avocado sterol in melting point, color, or crystalline form. It was concluded that no purification had been effected by the pyridine treatment of avocado sterol digitonide, and that a loss of sterol had resulted instead.

Several portions of cocoanut sterol digitonide were subjected to the pyridine cleavage method. The pyridine used was purified by refluxing over potassium hydroxide for one hour and then distilling over fresh potassium hydroxide at 114° into a dry container. The products presumed to be digitonin were not soluble completely in absolute alcohol, and the products presumed to be sterols were yellow in color, poor in yield, and melted below 100°. It was evident that the pyridine method could not be applied despite the precautions taken.

The conclusions drawn from this study were as follow:

1. Although the pyridine method was applicable on a non-quantitative basis to cholesterol, it could not be applied to the phytosterols under investigation with any assurance.

2. Some other method, such as the acetic anhydride method, might cleave a digitonide into purified sterol and unaltered digitonin.

The acetic anhydride method (92) involved the acetylation of a sterol digitonide into an ether-soluble sterol acetate and an ether-insoluble digitonin portion. The study of this method covered the following procedures:

1. the original method was tried out on a sample of cocoanut sterol digitonide,

2. a modification of this method was worked out to avoid precipitation with water, and

3. the modified method was applied to the cocoanut sterol digitonide that had not been cleaved with pyridine, indicated on page 101 as being insoluble in absolute alcohol.

The original method was applied to 0.665 g. of cocoanut sterol digitonide. The prescribed 6.5 cc. of acetic anhydride failed to dissolve the sample completely after one-half hour of refluxing. The addition of 1 cc. of acetic anhydride and further refluxing resulted in a clear, yellow solution. The reaction product was poured into 104 cc. of warm[#] water but no filterable esters precipitated. Instead, a semi-solid formed which passed through doubled filter paper. The colloidal, foamy filtrate was extracted with ether and the ether extract was washed with water, dried over anhydrous sodium sulfate, and brought to dryness over a water bath. The residue

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[#] The prescribed method did not indicate the temperature of the water. When no immediate precipitate formed, it was thought that heating might bring the sterol acetate to the surface in the form of a layer which could be separated.

was presumed to be cocoanut sterol acetate and amounted to a yield of 41.2%. During the ether extraction a middle layer of colloidal material formed which was brought to dryness, dissolved completely in hot absolute alcohol, filtered, and evaporated once more to dryness. The product was presumed to be recovered digitonin and amounted to a yield of 73.6%.

A modification of the prescribed method was introduced to avoid pouring the acetate-digitonin mixture into water. A sample of cocoanut sterol digitonide weighing 0.369 g. was refluxed for one hour with 7 cc. of acetic anhydride. The excess reagent was removed under vacuum at 80°, the residue was extracted with ether, filtered to remove insoluble digitonin, and evaporated to dryness. A product weighing 0.466 g. and presumed to be cocoanut sterol acetate was in excess of 100% recovery due to the presence of digitonin which had passed through the filter. The complete recovery of digitonin could not be effected because a quantity of it had passed through the filter paper along with the sterol acetate. The contaminated cocoanut sterol acetate was dissolved in 25 cc. of a 1% alcoholic potassium hydroxide solution and refluxed for one hour. The reaction product was a yellow solution having a sweet odor and forming a white precipitate on treatment with ether. The saponification process had converted the ether-soluble form of digitonin back into the ether-insoluble form. The reaction product was brought to dryness, partially dissolved with 50 cc. of ether, and diluted with 100 cc. of water. The foamy mixture was extracted with ether and the residue was obtained in the usual manner. The

product amounted to a yield of 132.5% which indicated that material other than sterol was present. The semi-crystalline, yellow product was crystallized from equal volumes of ethyl alcohol and methyl alcohol. The crystalline product amounted to a yield of 46.2%, melted at 123.8°, and was presumed to be cocoanut sterol in an impure form. During the ether extraction an insoluble layer of semi-colloidal material separated out between the ether and the aqueous layers. This insoluble layer was drawn off and allowed to separate into three layers. The upper, ether layer contained cocoanut sterol which was precipitated by adding digitonin. It was calculated from the weight of the digitonide which was formed that the former yield of sterol amounting to 46.2% should have been 51.6%. The middle, semi-solid layer did not resemble digitonin but was presumed to be a degradation form of digitonin. The lower, aqueous layer was filtered and tested for the presence of potassium acetate by heating with concentrated sulfuric acid. This negative test confirmed the opinion that the ether-soluble substance that contaminated the recovered cocoanut sterol was a decomposition product of digitonin.

A further modification of the prescribed method was introduced to prevent foaming during the ether extraction. It had been concluded that acetylation decomposed digitonin into an ether-soluble substance that still retained the saponin property of foaming when agitated with water. It was necessary, therefore, to dissolve the residue representing

the acetylation product in an excess of ether and then to wash the decomposition product of digitonin out of the mixture with small portions of water saturated with ether. The washed ether solution was dried over anhydrous sodium sulfate and brought to dryness in the usual manner. The residue was crystallized from absolute alcohol in white, shiny plates. The crystalline product amounted to a yield of 23.9%, melted sharply at 131.0°, and was considered tc be pure cocoanut sterol acetate.

The conclusions drawn from this series of experiments were as follow:

1. The acetic anhydride method for cleaving a sterol digitonide resulted in a decomposition product of digitonin that was ether-soluble.

2. This decomposition product could be removed from the mixture of acetylation products by careful washing with water.

3. The acetyl derivative of the cocoanut sterol portion could be isolated in pure form but in low yield.

4. The loss of expensive digitonin and the poor recovery of the sterol portion in the form of its acetyl derivative made it inadvisable to proceed with this method.

The alcoholic sodium acetate method (93) was studied near the end of the entire investigation and was found to offer a convenient way of cleaving a sterol digitonide with assurance that both the sterol portion and the digitonin portion could be recovered unchanged.

The alcoholic sodium acetate method (93) was checked on cholesterol digitonide and on cocoanut sterol digitonide. A dried sample of cholesterol digitonide weighing 0.131 g. was ground in a mortar with 1.3 g. of crystalline sodium acetate and transferred to a small flask with 13 cc. of alcohol. The mixture was refluxed for one-half hour over a water bath and the warm solution was mixed with 58 cc. of ether while connected to the reflux condenser. The precipitate was freed from sterol by decanting the supernatant liquid through a filter and washing the residue with ether. The residue was washed with boiling absolute alcohol to dissolve digitonin and with water to remove sodium acetate. A slight residue remained after these three washings that gave a positive sterol test with the Whitby A color reaction. This evidence showed that a small amount of digitonide had not dissociated in the alcoholic sodium acetate medium. The alcoholic washings were tested for digitonin by treating a portion of the clear filtrate with an alcoholic solution of avocado sterol. A heavy precipitate of avocado sterol digitonide proved that the original digitonin was available as a precipitating reagent. The alcoholic washings were tested for acetate ion by attempting to form the characteristic red color of ferric subacetate. The qualitative test was sufficiently negative to show that there was no danger of contaminating the alcoholic digitonin with excessive sodium acetate that might otherwise impair its precipitating power. Concentration of the ether filtrate produced good crystalline plates of

cholesterol. The success of the entire method was assured by this series of desired results.

The successful method was repeated with cocoanut sterol digitonide. A sample weighing 0.251 g. was treated in an identical manner except that the insoluble residue was dissociated a second time in fresh alcoholic sodium acetate. The sterol portion was recovered in a yield of 64.3%. The product could be precipitated once more with the alcoholic solution of recovered digitonin. The product also gave very sensitive results with the Whitby A and Whitby B color reactions. The recovered sterol portion was crystallized from absolute alcohol in silky crystals that melted sharply at 135.0°. A second crop, melting at 133.7°, was obtained by diluting the mother liquor with a small amount of water. Both of these values were higher than had been secured previously.

The conclusions drawn from this series of experiments were as follow:

1. The alcoholic sodium acetate method led to the recovery of digitonin in a form that could be used over and over again.

2. The sterol portion could be recovered from its digitonide in a fair yield by the alcoholic sodium acetate method.

3. The recovered sterol portion could be purified to a degree of purity approximating that of "sitosterol."

It was felt that the use of larger samples and improved technique would have improved the yields.

D. The Component Nature of the Sterol Portions

It was pointed out in preceding sections that atypical systems were present in the sterol portions isolated from the various oils. The possibility that a sterol portion might contain two or more closely related sterols has been recognized for some time (16), but the importance of determining the exact nature of each of the components in a sterol portion before assigning definite properties and formulae has been emphasized only comparatively recently (60,83,94,95,96, 97). The major emphasis in the literature concerning sterols has been laid primarily upon characterizing new sterols found in sterol mixtures rather than upon establishing with certainty the homogeneity of sterols already characterized.

It was realized that the presence of a component mixture of closely related sterols would result in ambiguous physical and chemical data concerning the sterol portion isolated from a given oil. Therefore the entire problem connected with the separation of a given sterol portion into homogeneous fractions was studied. This series of investigations covered:

1. the bromination and subsequent debromination of sterol acetates,

2. the application of the Liebermann-Burchard reaction to the separation of saturated from unsaturated sterols,

3. the fractionation of sterol mixtures by recrystallization, and

4. the correlation of the melting points and specific rotations with ultimate quantitative analyses of these fractions.

Methods

The classical method (16) for separating stigmasterol from sitosterol involves the bromination of their respective acetyl derivatives in a mixture of glacial acetic acid and ether. The insoluble tetrabromacetate corresponding to stigmasterol is separated by filtration from the soluble dibromacetate corresponding to sitosterol. The bromine atoms are removed by reduction with zinc dust and acetic acid and the original sterols are regenerated by saponification with alcoholic potassium hydroxide. It was thought that bromination would bring about some similar separation of the phytosterol mixtures under investigation based on varying degrees of unsaturation[#] of the component sterols. The study involved the bromination of avocado sterol acetate and the subsequent debromination by the following methods:

1. the classical method (16) using zinc dust and acetic acid,

2. the recommended method (98) using sodium iodide, and

3. the modified classical method (94) in an alcoholic medium.

[#] The reader is referred to the Flow Sheet given in Figure 1.

Bromination of Acetyl Derivative: The bromination of avocado sterol acetate followed the preparation of this acetyl derivative from 0.975 g. of avocado sterol melting at 120°. The product, weighing 1.225 g., was dissolved in 12.5 cc. of ether and brominated with 15.7 cc. of a brominating mixture prepared by dissolving 5 g. of bromine in 100 cc. of glacial acetic acid. A white, amorphous solid settled out of the orange colored solution upon cooling and evaporating the bromination product. This white solid, which was filtered off and presumed to be a bromacetyl derivative, turned black upon drying in an oven at 90°. An unsuccessful attempt was made to purify the charred mass by dissolving it in hot chloroform and precipitating with cold alcohol. The decomposed product could not be clarified with Norite. It was doubtful whether the precipitated bromination product was actually stigmasteryl tetrabromacetate which was reported (16) to decompose at 211-212°. The orange filtrate was assumed to contain the soluble dibromacetate corresponding to sitosterol.

Debromination of Bromacetyl Derivative: The classical method for debromination (16) was applied to the orange filtrate which was assumed to contain a maximum of one gram of dibromacetate. One gram of zinc dust and 40 cc. of glacial acetic acid were added to the solution and refluxed for 3 hours. The dark-brown solution which resulted was filtered and precipitated with 10 cc. of water. The flaky product did not crystallize well from absolute alcohol. The amorphous product was dark in color and poor in yield. The

first attempt at clarification with Norite produced a product melting at 108°, a second attempt increased the value to 122°. The final product was assumed to be the acetyl derivative of avocado sterol. Several samples of the original acetyl derivative were purified by different methods. The product obtained by extracting with ether a water suspension of the acetyl derivative, drying the residue, and recrystallizing from absolute alcohol melted at 115°. The product obtained by evaporating the excess acetic anhydride and crystallizing the residue from 5 parts of acetone to 1 part of methyl alcohol melted at 111°. The same product recrystallized from absolute alcohol melted at 122.8°. A comparison of these melting points showed that the debromination product agreed with the acetyl derivative prepared free from contact with water. The conclusions that could be drawn were as follow:

1. The classical method for debromination resulted in a discolored product in low yield that was comparable with a previously identified sample.

2. The addition of bromine had to be carried out in the dark and in the cold to prevent substitution, and that the excess bromine had to be removed with the same precautions.

3. The formation of a precipitate upon bromination did not establish the presence of stigmasterol or any other doubly unsaturated sterol (94).

The recommended method for debromination (98) was attempted because of the partial success obtained with the classical method. The mother liquor from the water precipitation referred to on page 110 was presumed to be a solution of dibromacetate. This brown solution was treated with a

solution of 2 g. of sodium iodide in 10 cc. of absolute alcohol and refluxed for 2 hours. No appreciable decolorization occurred. The solution was treated with a 10% solution of sodium bisulfite mixed with dilute sodium hydroxide in place of the unavailable sodium sulfite. No reaction with free iodine was noticed. The solution was washed with water, dried over sodium sulfate, and brought to dryness over a water bath. A dark-brown, tarry residue was secured that could not be clarified with Norite and could not be crystallized from absolute alcohol, dilute alcohol, or a mixture of acetone and methyl alcohol. A brown scum unfit for filtration and characterization was the only product that was obtained. A modification of the classical method was investigated because of the failure of the recommended method.

The modified method (94) consisted in carrying out the classical method in an alcoholic medium. A fresh supply of the brominated acetyl derivative was prepared by acetylating and brominating 2.507 g. of avocado sterol melting at 120°. The orange solution was debrominated by the action of zinc dust and glacial acetic acid in an alcoholic medium. The progress of the reaction was evidenced by the fading of the orange color. A light yellow color (95) persisted after 2 hours of refluxing in the presence of excess reagents. An unsuccessful attempt was made to secure the debrominated acetyl derivative by concentrating a portion of the filtered solution. A second portion of the filtrate was saponified with an excess of 5% alcoholic potassium hydroxide, diluted

with water, and extracted with ether. An ether-insoluble, white precipitate thrown down by the addition of water was tested for zinc. The formation of a green spot on glowing charcoal moistened with cobalt nitrate showed that the white precipitate contained zinc. The ether extract produced nothing that could be purified and characterized as a sterol. The remainder of the filtered solution was precipitated with water to produce brown, viscous residues. The combined residues were dissolved in alcohol, clarified several times with Norite, and precipitated with water. The product was saponified and extracted as described before, and the ether extract produced a residue that was purified by treatment with Norite and crystallized from diluted alcohol. The final product melted at 134.3°. The yield was too small to be determined, but the melting point showed that the original sterol sample had been purified to within a few degrees of the melting point of "sitosterol" by the chain of processes involving acetylation, bromination, debromination, precipitation with water, clarification with Norite, saponification, further clarification with Norite, and final crystallization from diluted alcohol. The modified method offered possibilities, but a simpler procedure for separating saturated sterols from unsaturated sterols was found in the application of a color reaction (99) to the sterol mixtures.

The Liebermann-Burchard Reaction: The suggestion (99) made for separating dihydrositosterol from sitosterol by causing the latter to form an insoluble, colored compound

with concentrated sulfuric acid and acetic anhydride was applied to the present problem. A sample of avocado sterol, crystallized from a mixture of acetone and methyl alcohol, melting at 131.2°, weighing 0.315 g., and assumed to be a mixture of saturated and unsaturated sterols, was dissolved in 5 cc. of carbon tetrachloride and treated with 1.5 cc. each of concentrated sulfuric acid and acetic anhydride. The addition of more carbon tetrachloride caused the green mass to separate into two layers. The carbon tetrachloride layer was treated with water to wash it, but instead a white semi-solid and a yellow emulsion formed. These were collected together and presumed to be the sulfate (97) of dihydrosterol forms. This sulfate product was saponified with 20% methyl-alcoholic potassium hydroxide, diluted with water, extracted with ether, dried over anhydrous sodium sulfate, and evaporated slowly to give needles of a crystalline product melting at 131.2°. The yield was too small to be determined but the melting point was identical with that of the original sample. The reaction did not agree exactly with the literature (99) but it did seem to furnish possibilities of successful application.

The conclusions drawn from these studies were as follow:

1. Some substitution by bromine could not be avoided during bromination of unsaturated linkages.

2. Debromination was applicable only to true addition compounds by using zinc dust and acetic acid in alcohol.

3. The Liebermann-Burchard reaction served to separate saturated from unsaturated sterols in a non-quantitative manner.

Fractionation of Sterol Mixtures by Recrystallization: In view of the slight success encountered in the study of appropriate methods for the quantitative separation of the components of a sterol mixture, it was decided to fractionate the sterol mixtures as completely as possible by repeated recrystallization and to study the physical and chemical properties of each fraction separately. A preliminary study was made on avocado sterol in order to determine the best drying procedure and then the top and bottom fractions from each sterol portion were fractionated still further according to the conclusions referred to on page 84.

The drying technique required for the products obtained by recrystallization was determined by studying the behaviour of avocado sterol. Evidence was secured that the mother liquors contained substances melting at higher temperatures than did the recrystallized products. It was noted also that the recrystallized products melted at lower temperatures after comparative whiteness was attained. In order to determine whether the melting point decreased due to the rearrangement of the component nature or whether the effect was due to heating at 100-105°, comparative samples were dried in the oven and under vacuum. It was found that heating in an oven produced a wider softening range and a lower final value of the melting point than did drying under vacuum. On the other hand, vacuum drying was slower than oven drying. However, the time required for drying the product could be reduced by warming it before placing it under vacuum. The results obtained in this study have been compiled in Table 14.

Table 14.

Fractionation and Drying of Avocado Sterol

Fractionation Stage	Drying Operation	Melting Point °C. (corr.)
Crude	oven	132.2
First Crop	oven	137.5
Second Crop	oven	141.6
Crude	oven	132.2
First Crop	oven	134.3
Recrystallized	oven	<134.3
Second Crop	oven	141.6
Recrystallized	oven	<141.6
Third Crop	oven	143.8
Crude	oven	132.2
First Crop	vacuum	136.5
Redried	vacuum	>136.5
Redried	oven	<135.5
Second Crop	vacuum	>141.6
Redried	vacuum	>142.6
Redried	oven	140.6

Purification of the top and bottom fractions, referred to on page 84, by the low-temperature method, referred to on page 82, brought about a partial separation of the various sterol mixtures. The operations used and the results obtained have been compiled in Table 15. These results indicate that the higher-melting components were more soluble and were concentrated in the mother liquors and washings. These higher-melting components could be crystallized, washed, and filtered more easily than could the lower-melting components present in the recrystallized products. It is postulated that atypical systems were present that did not lend themselves to standard methods of purification. The purification of a single substance mixed with impurities should have resulted in a series of recrystallized products increasing in melting point up to a fixed temperature regardless of the solvent used for the crystallizing medium. The material retained in the mother liquor should have had its melting point depressed by the increasing concentration of impurities. In this investigation the melting points of the recrystallized products rose during a certain number of recrystallizations, remained the same or dropped back on further recrystallization from the same solvent or mixtures of other solvents, and the material obtained from the mother liquors melted at a higher temperature than did the recrystallized product. This erratic behaviour of the sterol portions under investigation indicated the presence of two or more sterols varying slightly in their solubilities and melting points, but impossible of complete separation by ordinary fractionation.

Table 15.

Fractionation of Sterol Portions by Recrystallization

Sample	Operation	Yield g.	Melting Point °C. (corr.)
Avocado	Ordinary crystallization		
	Pure ethyl alcohol		121.7
	Redried		125.9
	Pure ethyl alcohol	1.924	131.7
	Mother liquors from above operations		
	Low-temperature method		
	Pure methyl alcohol		94
	1 methyl plus 1 ethyl alcohol		123.8
	Methyl plus trace ethyl alcohol		132.7
	Pure methyl alcohol		137.4
	2 methyl plus 1 ethyl alcohol	0.562	137.4
	Slow crystallization		
	Petroleum ether wash	0.355	137.4

Table 15. (Continued)

Fractionation of Sterol Portions by Recrystallization

Sample	Operation	Yield g.	Melting Point °C. (corr.)
Kukui	Low-temperature method		
	Pure methyl alcohol		103
	2 methyl plus 1 ethyl alcohol		121.7
	2 methyl plus 1 ethyl alcohol		130.1
	2 methyl plus 1 ethyl alcohol		126.9
	5 methyl plus 1 ethyl alcohol		126.9
	Pure ethyl alcohol	2.322	130.1
	Second crop from filtrate		130.1
	Mother liquors from above operations		
	Slow crystallization		
	Petroleum ether wash	2.694	131.7
	Second crop from filtrate	0.481	
	Petroleum ether wash		130.0

Table 15. (Continued)

Fractionation of Sterol Portions by Recrystallization

Sample	Operation	Yield g.	Melting Point °C. (corr.)
China-wood	Low-temperature method		
	Pure methyl alcohol		119.9
	2 methyl plus 1 ethyl alcohol		123.8
	3 methyl plus 1 ethyl alcohol		125.9
	4 methyl plus 1 ethyl alcohol		120.4
	Pure ethyl alcohol	2.022	131.2
	Pure ethyl alcohol		131.3
	Mother liquors from above operations		
	Slow crystallization		
	Petroleum ether wash	1.532	138.4
	Second crop from filtrate	0.411	
	Petroleum ether wash		121.7

Table 15. (Concluded)

Fractionation of Sterol Portions by Recrystallization

Sample	Operation	Yield g.	Melting Point °C. (corr.)
Chaulmoogra	Low-temperature method		
	Pure methyl alcohol		85
	2 methyl plus 1 ethyl alcohol		121.7
	1 methyl alcohol plus 1 ethyl ether		117.5
	3 methyl plus 1 ethyl alcohol		121.7
	3 methyl plus 1 ethyl alcohol		121.7
	Pure ethyl alcohol	2.797	121.7
	Mother liquors from above operations		
	Slow crystallization		
	Petroleum ether wash	0.786	140.0
	Second crop from filtrate	0.310	129.6
Cocoanut	Ordinary crystallization		
	Pure ethyl alcohol	<0.636	106.0
	Low-temperature method	-----	-----

Physical and Chemical Properties of Sterol Fractions

It was necessary to establish a criterion of final purity before preparing the sterol fractions for ultimate analyses. The literature (59) recommended specific rotation in preference to constant melting point for determining the relative purity of a component mixture. The presence of a small amount of saturated sterol might fail to influence the melting point but its dextro rotation would decrease the laevo rotation of the unsaturated sterol to a considerable extent. Another criterion that might have been used was the mixed melting point, but no pure samples of known phytosterols were available.

The melting point determination was much simpler to perform and was much less wasteful of material than the determination of specific rotation. The calculation of corrected melting points was facilitated by calibrating a thermometer against a standard, correcting each point for emergent-stem error, and constructing a smooth curve through these points. The specific rotation of a given sample was determined by dissolving the sample in chloroform and determining the value at a given temperature with the aid of a saccharimeter.

Preliminary Study on Avocado Sterol: A preliminary study was made on avocado sterol to determine relationships between the purification stage, the melting point, and the specific rotation. Samples were secured from fractions as widely separated as possible by recrystallization from alcohol and yet in as pure a condition as the removal of

non-sterol material would allow. Specific rotations could not be determined accurately after the second crop due to the scarcity of material. The recrystallizations were performed from this point on by semi-micro methods. It was found that as the melting point increased the laevo rotation also increased. These results have been compiled in Table 16.

Table 16.

Melting Points and Specific Rotations of Avocado Sterol Fractions

Fractionation Stage	Melting Point °C. (corr.)	Specific Rotation $(\alpha)_D^{20}$
Crude	132.2	-20.71
First Recrystallization	137.4	-25.21
Second Recrystallization	137.2	-21.63
Third Recrystallization	135.3	------
Crude	132.2	-20.71
First Crop	137.5	-21.26
First recrystallization	134.3	-19.76
Second Crop	141.6	-24.27
First recrystallization	141.6	-24.59
Third Crop	143.8	------
First recrystallization	145.0	------
Fourth Crop	146.0	------
Fifth Crop	142.7	------
First recrystallization	141.6	------

Characterization of the Sterol Fractions: Widely separated fractions were prepared from the sterol supplies listed in Table 15 and were purified as far as possible. The same procedures were followed as those described in the previous study except that the specific rotations of much smaller samples were determined. Each sample was weighed directly into a 1.5 decimeter tube of small bore, dissolved with chloroform added directly from a burette, and the readings were taken at room temperature rather than at 20° to avoid loss of visibility of the balance point caused by the condensation of moisture on the cool ends of the tube. The results of this study have been compiled in Table 17.

A correlation between the gain in melting point and the increase in laevo rotation was attempted. It could be shown that a mean gain of 3.7° in the melting point was accompanied by a mean increase of 3.83° in laevo rotation. Therefore it was concluded that a gain of 1° in the melting point was accompanied by an increase of approximately 1° in laevo rotation. This correlation shows that the sterol portion of each oil under investigation contained at least two components, and that the more soluble component or components melted at a higher temperature and exhibited a larger laevo rotation. The accumulation of such substances in the mother liquor seemed unusual until it was found in the literature (64) that the unsaturated form of phytosterol, namely sitosterol, accumulates in the bottom fractions while the saturated form, namely dihydrositosterol, remains in the top fractions.

Table 17.

Melting Points and Specific Rotations of Sterol Fractions

Sterol Portion	Sterol Fraction	Melting Point °C. (corr.)	Specific Rotation $(\alpha)_D^{26}$
Avocado	Top	137.5	-24.39 -24.53
	Bottom	142.6	-27.58 -30.24
Kukui	Top	130.1	-21.97
	Bottom	131.7	-24.80 -25.00
China-wood	Top	134.3	-19.17
	Bottom	136.0	-22.71
Chaulmoogra	Top	136.4	-21.83
	Bottom	140.0	-27.83
Cocoanut	Top	123.8	-21.21
	Bottom	-----	------

Combustion Analyses of the Sterol Fractions: Samples of the fractions listed in Table 17, together with a sample of the acetyl derivative of cocoanut sterol, were sent to the German firm of Dr. Ing. A. Schoeller in Berlin for micro-combustion analyses of carbon and hydrogen. The analyses obtained from this firm checked well with macro-combustion analyses performed on avocado sterol (90) and its acetyl derivative. The micro-analyses have been presented in Table 18.

These analyses are not in agreement[#] with the literature concerning sitosterol and dihydrositosterol. The percentages of hydrogen were in agreement with the theoretical values but the percentages of carbon were low by one or two units. The fractions secured from the mother liquors showed a definite decrease in carbon content wherever analyses were available, whereas the hydrogen content held constant. This evidence is not in agreement with the statement referred to on page 125 that the unsaturated form of phytosterol accumulates in the mother liquor. An unsaturated phytosterol is considered by inference to contain a higher percentage of carbon and a lower precentage of hydrogen than its saturated form.

[#] Discrepancies may be explained by the fact that the samples were not thoroughly dry. The presence of occluded solvents tends to lower ~~combustion~~ combustion values (100).

Table 18.

Micro-Combustion Analyses of Sterol Fractions and an Acetyl Derivative

Sterol Fraction	Carbon %	Hydrogen %	Remarks
Avocado			
Top	82.89 82.98	12.53 12.56	Assumed to be a check.
Bottom	81.89	12.12	Repeat samples returned opened and unanalyzed.
Kukui			
Top	82.20	12.24	
Bottom	81.93	12.27	
China-wood			
Top	82.03	12.16	Glass shards in residue.
Bottom	81.86	11.99	
Chaulmoogra			
Top	81.84	12.18	
Bottom	-----	-----	Broken in transit.
Cocoanut			
Top	77.54	11.48	
Bottom	-----	-----	Insufficient for analysis.
Acetyl Derivative	80.31	11.17	

Conclusions

Definite conclusions could not be drawn as to the identities of the various sterol preparations due to the lack of comparable standards and definite criteria. Methods designed to separate quantitatively the individual components of a sterol mixture could not be applied to the sterol portions under investigation.

The methods described in the literature for separating component mixtures into homogeneous fractions could not be applied satisfactorily. Bromination invariably brought about some substitution of bromine. Attempted debromination resulted in the formation of tarry residues that could not be clarified or crystallized successfully. The formation of an insoluble bromination product did not prove the presence of stigmasterol or other doubly unsaturated sterols. The modified classical method was the only one attempted that brought about successful debromination of bromine addition compounds. The Liebermann-Burchard color reaction separated the saturated sterols from the unsaturated sterols, but caused the former to be recovered in low yield and the latter to be destroyed. Fractionation of sterol mixtures by recrystallization at low temperatures was the only method studied that produced sufficient samples for characterization.

Difficulties were encountered in the working up of these recrystallized samples into samples suitable for characterization. First, the component mixtures of sterols were contaminated with non-sterol impurities that were difficult to remove

without sacrificing the major part of each sample. Second, the sterol mixtures retained solvents and made it difficult to dry samples thoroughly without using heat. Third, the use of moderate heat for drying samples resulted in products that softened over a wide range and melted at a lower temperature.

The purest top and bottom fractions could not be considered homogeneous preparations but merely fractions enriched by one or more of the components. The top fractions melted at lower temperatures, exhibited smaller laevo rotations, and contained more carbon. The bottom fractions melted at higher temperatures, exhibited large laevo rotations, and contained less carbon. The first two criteria, namely melting point and specific rotation, indicated that the top fractions were enriched by saturated forms of phytosterol and the bottom fractions by unsaturated forms. The third criterion, namely the carbon content, reversed this hypothesis and excluded the accepted range of formulae from $C_{25}H_{40}O$ to $C_{29}H_{52}O$.

The only conclusion that could be reached was that atypical systems were present in the sterol portions under investigation that resembled mixtures of sitosterol and dihydrositosterol. The discrepancies were not surprising when it may be seen from Table 2 that "sitosterol" itself has been resolved into several components.

E. Nutritional Investigations on Avocado Oil

It is recognized (101) that ergosterol, under suitable irradiation with ultraviolet light, can be converted into a substance having marked antirachitic activity. However, this substance has been found to differ (102) from the naturally occurring vitamin D. Therefore the ability to acquire antirachitic activity on irradiation is not confined to any particular sterol.

In view of these conclusions the vitamin value of the sterol portion of avocado oil was investigated in order to determine whether the sterols would function as vitamins by producing similar physiological responses. Claims have been made (103) that avocados are an excellent source of a highly nutritious oil for the diet of humans. Vitamin studies have been made on avocados by Santos (104) who used dried oil-free pulp, Dickey (105) who used avocado mash, and Weatherby (106) who fed fresh ripe fruit to his animal subjects. A close connection (107) between the phytosterols from vegetable oils and the vitamins, in particular vitamin D, has been demonstrated.

Of the five oils studied in this research, avocado oil was shown by Table 13 to contain the highest percentage of phytosterol. In order to determine the vitamin value of the relatively large sterol portion of avocado oil, two series of nutritional experiments were carried out on white rats at the Nutrition Laboratory of the University of Hawaii.

Comparative Oil Studies: A total of 38 rats were fed on a maintainance diet consisting of 80 parts graham flour, 10 parts gluten flour, and distilled water ad libitum, to which was added 10 parts of the various oil samples under investigation. Eight of these rats were fed a common edible oil, namely a brand of cottonseed oil known as Wesson oil, to serve as comparative controls. Five rats were fed on irradiated Wesson oil, 7 rats on unirradiated avocado oil, 8 rats on irradiated avocado oil, 5 rats on irradiated avocado oil which had been extracted previously with boiling alcohol to remove sterols, and 5 rats on Wesson oil which contained irradiated avocado sterol amounting to 0.025% of the total diet. A record of the weight and food consumption of each rat was kept each week and an autopsy was performed either upon the death of the rat or at the end of the eighth week. The average results with regard to growth have been set forth graphically in Figure 3.

This general procedure could not demonstrate the presence of a particular vitamin, but the autopsies indicated that irradiation of the sterol portion of an oil resulted in the improved health of the animals. Avocado oil proved to offer no advantages over Wesson oil in these respects.

COMPARATIVE OIL STUDIES

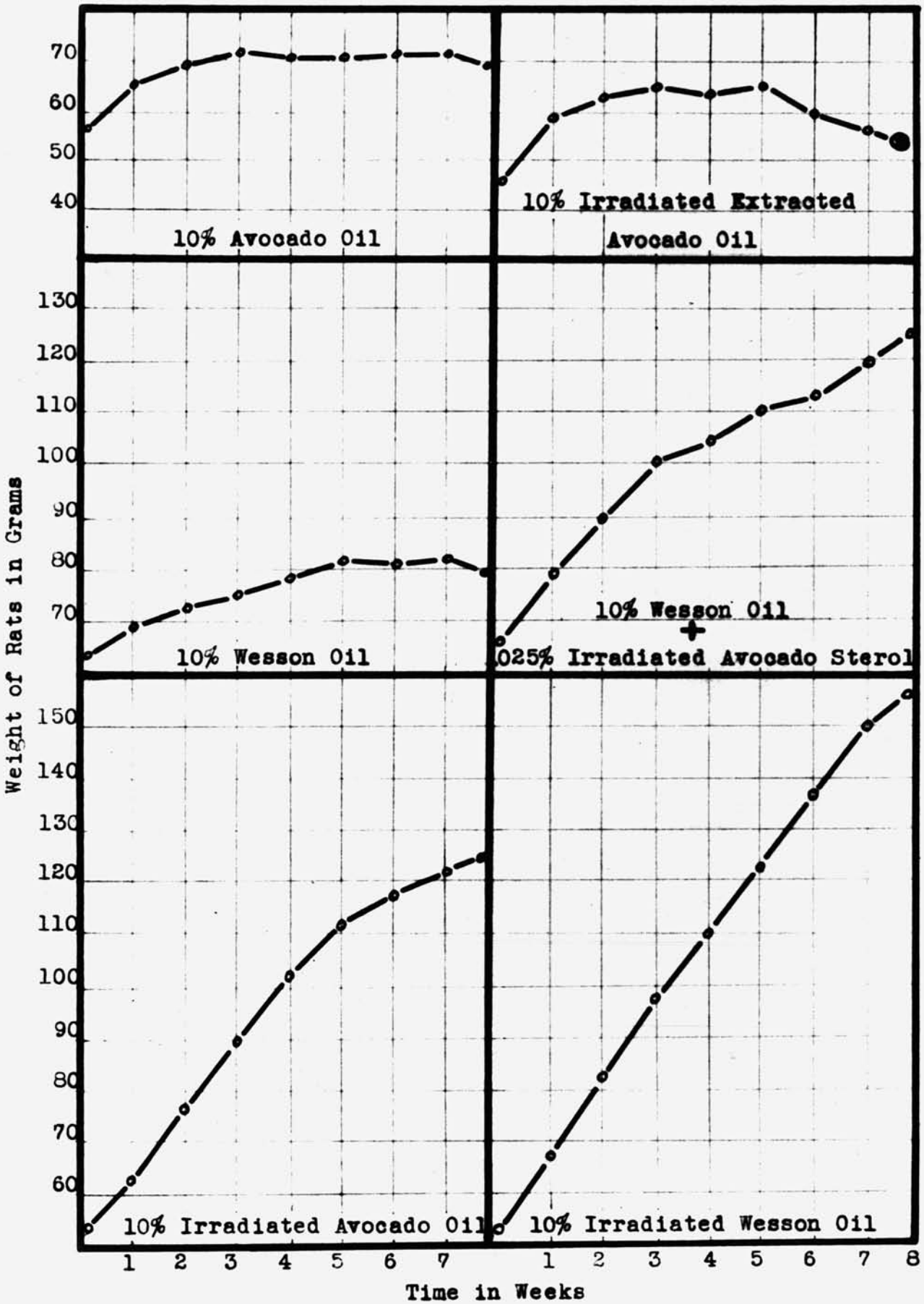


FIGURE 3

Vitamin D Studies: It is known that irradiation of oils (108) or their sterol portions (107) induces the formation of an antirachitic agent. The increased growth and improved health of the animals fed on irradiated avocado sterol in the comparative oil studies referred to on page 132, indicated the presence of a vitamin similar to the antirachitic agent known as vitamin D. As a result, it was decided to conduct a comparative series of experiments using Wesson oil, avocado oil, and the sterol portion from avocado oil in order to evaluate the antirachitic potency of irradiated and unirradiated samples.

A total of 61 rats were placed on the following rickets-producing diet (108):

Finely ground corn meal --	76 parts
Gluten flour ----------	20 parts
Calcium carbonate --------	3 parts
Sodium chloride ----------	1 part
Total ---	100 parts
Distilled water ad libitum	

At the end of 21 days positive and negative controls were chosen and the remainder of the rats were fed on their respective supplements for 8 days. The dosages were supplied from micro-pipettes calibrated to deliver 0.3 cc. of avocado oil weighing 0.269 g., and 0.3 cc. of a Wesson oil solution containing 0.0005 g. of avocado sterol. At the end of the experimental period each rat was killed and subjected to the "line test". This standard procedure (109) consisted in removing the tibia from the hind leg, splitting the tibia with a razor blade, soaking the exposed section in a weak

solution of formaldehyde, washing off the section with distilled water, applying a few drops of a silver nitrate solution to the exposed surface, irradiating the section in direct sunlight or under a Cooper-Hewitt mercury-arc lamp, and finally examining the section for evidences of rachitic healing. Absence of any visible sign of healing was given the designation, -----. Evidence of complete healing in a positive control fed on cod-liver oil was given the designation, ++++. Signs of slight healing, +, of moderate healing, ++, and of pronounced healing, +++, were evaluated qualitatively from the varying amounts of blackened, freshly deposited calcium in the marginal zone of calcification. The results of this series of line tests have been summarized in Tables 19, 20, and 21.

The conclusions drawn from these studies were as follow:

1. Wesson oil direct from the commercial can possessed no antirachitic activity and could be used with safety as an inert medium for administering sterol samples to rats.

2. Irradiated Wesson oil was definitely antirachitic.

3. Unirradiated avocado oil, carefully prepared so as to prevent exposure to direct sunlight, possessed no antirachitic activity.

4. Irradiation of this same avocado oil developed antirachitic potency even greater than that found in irradiated Wesson oil.

5. Unirradiated avocado sterol, in the amount of 0.0005 g. per day, failed to promote any signs of antirachitic healing, whereas the same amount of irradiated avocado sterol produced healing comparable with the positive controls.

Table 19.

Antirachitic Potency of Irradiated and Unirradiated Wesson Oil

Rat No.	Supplement Description	Line Test Evaluation
2742	0.3 cc. unirradiated Wesson oil	----
2743	0.3 cc. irradiated Wesson oil	++ or +++
2744	0.3 cc. unirradiated Wesson oil	----
2745	29 day negative control	----
2746	0.3 cc. irradiated Wesson oil	+++ or ++++
2747	Cod-liver oil positive control	++++
2748	0.3 cc. unirradiated Wesson oil	----
2749	0.3 cc. unirradiated Wesson oil	----
2750	0.3 cc. irradiated Wesson oil	++
2751	0.3 cc. irradiated Wesson oil	++++
2752	21 day negative control	---- or +
2753	0.3 cc. unirradiated Wesson oil	----
2754	0.3 cc. irradiated Wesson oil	++ or +++
2770	0.3 cc. irradiated Wesson oil	++ or +++
2771	0.3 cc. unirradiated Wesson oil	----
2772	21 day negative control	----
2773	29 day negative control	----
2774	0.3 cc. unirradiated Wesson oil	----
2775	0.3 cc. irradiated Wesson oil	++
2776	Cod-liver oil positive control	++++
2777	0.3 cc. unirradiated Wesson oil	----
2778	0.3 cc. irradiated Wesson oil	+++

Table 20.

Antirachitic Potency of Irradiated and Unirradiated Avocado Oil

Rat No.	Supplement Description	Line Test Evaluation
2786	Cod-liver oil positive control	++++
2787	0.3 cc. irradiated avocado oil	+++
2788	0.3 cc. unirradiated avocado oil	----
2789	21 day negative control	----
2790	0.3 cc. unirradiated avocado oil	----
2791	0.3 cc. irradiated avocado oil	+++ or ++++
2792	0.3 cc. irradiated avocado oil	++++
2793	0.3 cc. unirradiated avocado oil	----
2794	0.3 cc. irradiated avocado oil	++++
2795	Cod-liver oil positive control	++++
2796	0.3 cc. irradiated avocado oil	+++ or ++++
2797	0.3 cc. unirradiated avocado oil	----
2798	0.3 cc. unirradiated avocado oil	----
2799	29 day negative control	----
2800	21 day negative control	----
2801	0.3 cc. unirradiated avocado oil	----
2802	0.3 cc. irradiated avocado oil	++++

Table 21.

Antirachitic Potency of Irradiated and Unirradiated Avocado Sterol

Rat No.	Supplement Description	Line Test Evaluation
2900	0.0005 g. irradiated avocado sterol	++++
2901	0.0005 g. unirradiated avocado sterol	----
2902	0.0005 g. unirradiated avocado sterol	----
2903	Cod-liver oil positive control	++++
2904	0.0005 g. irradiated avocado sterol	++++
2905	0.0005 g. unirradiated avocado sterol	----
2906	0.0005 g. irradiated avocado sterol	++++ or +++
2907	0.0005 g. irradiated avocado sterol	+++ or ++++
2908	0.0005 g. unirradiated avocado sterol	----
2909	21 day negative control	----
2910	29 day negative control	----
2911	0.0005 g. irradiated avocado sterol	++++ or +++
2912	0.0005 g. unirradiated avocado sterol	----
2913	0.0005 g. irradiated avocado sterol	++++
2914	0.0005 g. unirradiated avocado sterol	----
2915	Cod-liver oil positive control	++++
2916	0.0005 g. irradiated avocado sterol	++++
2917	0.0005 g. unirradiated avocado sterol	----
2918	0.0005 g. irradiated avocado sterol	++++
2919	21 day negative control	----
2920	0.0005 g. irradiated avocado sterol	++++
2921	0.0005 g. unirradiated avocado sterol	---- or + #

Rat No.2921 was the only rat fed on unirradiated avocado sterol that failed to gain weight during the test period. Such a condition has been known to bring about spontaneous healing.

F. Summary and Discussion of Results

Five tropical oils, namely avocado, kukui, china-wood, chaulmoogra, and cocoanut were secured in crude form either by purchase or by manufacture. The source of a given oil was found to influence the percentage of unsaponifiable matter and hence the sterol content of that oil. The refining processes such as settling, decolorizing, and deodorizing carried out on commercial grades of oil remove or destroy a considerable part of the unsaponifiable matter. It was necessary to prepare oil in the laboratory or obtain supplies of oil under rigid specifications in order to guarantee the maximum amount of available sterol.

The oils were saponified with alcoholic potassium hydroxide and the resulting soaps were extracted with ether. Improved apparatus and technique were devised in order to extract large volumes of soap solutions quickly and safely. The unsaponifiable residues thus isolated were considered to be crude sources of sterols contaminated with other plant products. This supposition was checked by applying the Whitby A and Whitby B color reactions to the unsaponifiable residues.

The sterol portions were separated from the rest of the unsaponifiable matter by recrystallization from mixtures of methyl and ethyl alcohol at low temperatures. A temperature

of -60° was obtained by placing cakes of carbon dioxide ice in a well-insulated solution of methyl alcohol and ether. These sterol portions were purified as far as possible by decolorizing with Norite in alcohol, crystallizing from absolute alcohol, and washing with petroleum ether whenever the crystallized product was grainy and porous. Data secured from these purified sterol portions can not be considered characteristic of homogeneous preparations. Such data can be considered merely as composite values applying to closely related mixtures.

These purified sterol portions were characterized as far as possible by their melting points and by their specific rotations. The average molecular weights of the sterol portions were determined by the camphor fusion method. The average equivalent weights of the same sterol portions were determined by saponifying their acetyl derivatives and titrating the excess alkali. The average number of one hydroxyl group per sterol molecule was deduced from a comparison of the equivalent and molecular weights thus determined. The properties of these purified, unfractionated sterol portions have been compiled in Table 22.

Table 22.

Properties of Unfractionated Sterol Portions

Oil	Whitby Color Reaction	Sterol in Oil %	Melting Point °C.(corr.)	Molecular Weight ± 20	Acetyl Derivative		
					Equivalent Weight ± 50	Melting Point °C.(corr.)	Specific Rotation $(\alpha)_D^{26}$
Avocado	+	1.05	134.3	358	505	122.8	-27.77
Kukui	+	0.27	133.2	365	456	123.3	-22.72
China-wood	+	0.22	135.3	365	551	124.3	-26.14
Chaulmoogra	+	0.21	131.6	394	398	126.9	-29.14
Cocoanut	+	0.09	135.0	394	499	128.5	-31.20

The unfractionated sterol portions were investigated as component mixtures of closely related sterols. Attempts were made to separate the sterol mixtures into their component sterols by brominating their acetyl derivatives in a mixture of glacial acetic acid and ether. Studies of method were made in order to determine the best procedures for debrominating the bromacetyl derivatives, and for precipitating and recovering pure sterols from reaction mixtures by forming addition products with digitonin. The most promising debromination method involved the classical zinc dust and acetic acid reaction carried out in an alcoholic medium. The most promising dissociating reagent for cleaving a digitonide into undecomposed sterol and unaltered digitonin was found to be alcoholic sodium acetate rather than pyridine. The failure of many alternative methods to give satisfactory, quantitative results made it necessary to fractionate the sterol mixtures into top and bottom fractions by recrystallization at low temperatures. These fractions were characterized as far as possible by determining their melting points and specific rotations. The carbon and hydrogen content of these same fractions were determined by micro-combustion analyses performed in Germany. The properties of the fractionated sterol portions have been compiled in Table 23.

It was reported (101) that the sterol portion of cottonseed oil consisted of over 99% beta-sitosterol and less than 1% of saturated phytosterol. Beta-sitosterol (102) is considered to be equivalent to 22-dihydrostigmasterol. Alpha-sitosterol (100) as isolated from wheat-germ oil is considered

Table 23.

Properties of Fractionated Sterol Portions

Sterol Portion	Sterol Fraction	Melting Point °C. (corr.)	Specific Rotation $(\alpha)_D^{26}$	Carbon %	Hydrogen %
Avocado	Top	137.5	-24.46	82.94	12.55
	Bottom	142.6	-28.91	81.89	12.13
Kukui	Top	130.1	-21.97	82.20	12.24
	Bottom	131.7	-24.90	81.93	12.27
China-wood	Top	134.3	-19.17	82.03	12.16
	Bottom	136.0	-22.71	81.86	11.99
Chaulmoogra	Top	136.4	-21.83	81.84	12.18
	Bottom	140.0	-27.83	-----	-----
Cocoanut	Top	123.8	-21.21	77.54	11.48
	Bottom	-----	------	-----	-----

to be a mixture of two components. Alpha$_1$-sitosterol, $C_{29}H_{48}O$, is considered to be an isomer of the doubly unsaturated stigmasterol. Alpha$_2$-sitosterol, $C_{30}H_{50}O$, is considered to be a homologue of alpha$_1$-sitosterol.

The purest top and bottom fractions listed in Table 23 can not be considered homogeneous preparations, but merely fractions enriched by one or more of the many possible saturated and unsaturated forms of "sitosterol." A correlation of the melting points and the specific rotations of these fractions indicate that the top fractions were enriched by saturated forms, while the bottom fractions were enriched by unsaturated forms. The carbon content of these fractions did not agree with possible theoretical values. The discrepancies were probably due to the presence of occluded solvent (100) in the recrystallized samples sent away for analysis.

The nutritional significance of the sterols contained in avocado oil was determined by feeding experiments conducted on white rats. Although no new vitamin activity could be identified by the general method used, evidence was secured that irradiation of the sterol portion of avocado oil brought about a state of improved health in the rats comparable with that secured by irradiating cottonseed (Wesson) oil. A high degree of vitamin D potency was determined for irradiated avocado oil and for the irradiated sterol portion from unirradiated avocado oil by applying the line test to sectioned tibias of rachitic rats fed on these supplements.

CONCLUSIONS

1. The variable component nature of the phytosterol preparations from several tropical oils was established.

2. The importance of separating component mixtures into homogeneous fractions before characterizing the phytosterol preparations was demonstrated.

3. The melting points and specific rotations of the purest top fractions obtainable were: avocado, 137.5°, -24.46°; kukui, 130.1°, -21.97°; china-wood, 134.3°, -19.17°; chaulmoogra, 136.4°, -21.83°; and cocoanut, 123.8°, -21.21°.

4. The melting points and specific rotations of the purest bottom fractions obtainable were: avocado, 142.6°, -28.91°; kukui, 131.7°, -24.90°; china-wood, 136.0°, -22.71°; and chaulmoogra, 140.0°, -27.83°.

5. The exact component nature of the various sterol fractions could not be determined. It was assumed that varying mixtures of saturated and unsaturated sterols were present.

6. The sources of the oils, the processing methods used to purify the oils, and the purification methods used to isolate the sterols from the oils influenced the amounts and the component natures of the sterol portions obtainable.

7. Irradiation of the sterol portion of avocado oil resulted in the improved health of white rats comparable to that effected by the irradiation of cottonseed oil.

8. The presence of a sterol corresponding to provitamin D was established in the sterol portion of avocado oil.

APPENDIX

A. Nomenclature and Symbols

The nomenclature and conventional symbols used in connection with the sterols and bile acids have undergone change along with the development of the field. In order to bring up-to-date the terminology used to describe the complex structures involved, the following glossary is presented as a convenience to the reader.

Aetio---prefix meaning fundamental or basic.

Allo---prefix signifying a stereochemical series in which Ring A / Ring B : trans decalin type.

Allomerization---form of isomerization involving allo- and normal configurations.

Alpha---prefix signifying a stereochemical series in which (OH) #3 / H #5 : cis position. More commonly termed epi-.

Andro---prefix meaning male.

Apo---prefix signifying a shift of the double bond.

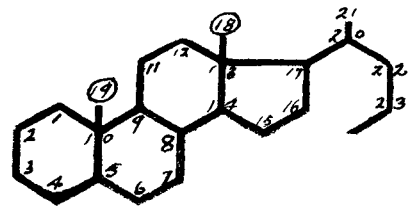
Beta---prefix signifying a stereochemical series in which (OH) #3 / H #5 : trans position.

Bile Acid---acid fraction occurring in bile; found in conjugation with glycine or taurine in the form of the water-soluble sodium salt; structure similar to that of a sterol.

Bilianic---adjective signifying a tricarboxylic acid derived from a bile acid by opening one ring.

Biloidanic---adjective signifying a hexacarboxylic acid derived from choloidanic acid in which three rings have been opened; also termed norsolannelic.

Cholane---adjective describing the structural formula:



Chole---prefix meaning bile.

Cholic---adjective applying to bile acids in general; most abundant acid secured from ox bile.

Choloidanic---adjective signifying a pentacarboxylic acid derived from a bile acid in which two unconnected rings have been opened.

Cilianic---adjective signifying the acid derived from a bilianic acid.

Ciloidanic---adjective signifying the acid derived from a cilianic acid.

Cyclo---prefix indicating atoms grouped in a ring.

Dehydro---prefix indicating a double bond formed by the subtraction of two hydrogen atoms; used to describe a ketone derived from a secondary alcohol.

Desoxy---prefix meaning one hydroxyl group less than.

Digitonide---insoluble addition compound of one molecule

of a sterol with one molecule of digitonin.

Dihydro---prefix indicating a double bond saturated by the addition of two hydrogen atoms; used to describe the reduced from of an unsaturated sterol.

Epi---prefix commonly used in preference to alpha.

Epimerization---form of isomerization involving epi- and beta configurations.

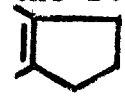
Hormone---substance produced by a specific gland for a specific purpose at a removed location; literally a chemical messenger.

Nor---prefix meaning one carbon atom less than.

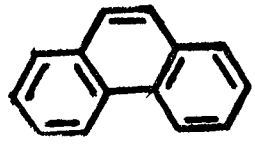
Normal---prefix signifying a stereochemical series in which Ring A / Ring B : cis decalin type.

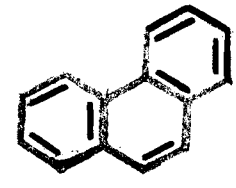
Oestro---prefix pertaining to the heat cycle in females.

Penteno---prefix signifying the structure:



Phenanthrene---a three-ringed hydrocarbon of the structure:





Progest---prefix pertaining to the corpus luteum of the ovary.

Pseudo---old term used to describe an allomerization product.

Pyro---prefix indicating thermal decomposition.

Steroid---adjective signifying the appropriate structural framework to yield the Diels hydrocarbon on dehydrogenation with selenium; applied to compounds chemically related to cholesterol.

Sterol---naturally occurring compound found in the unsaponifiable portion of the lipids; literally a solid alcohol.

Solannelic---adjective signifying a bile acid degradation product in which one ring has been left intact.

Tachy---prefix meaning rapid or swift.

Testo---prefix pertaining to the testes in males.

Sterols, bile acids, and related compounds are named usually from their sources by the use of prefixes. Examples are:

Anthropo---man	Bombice---wax
Brassica---rape	Bufo---toad
Cheno---goose	Chorto---grass
Copro---faeces	Ergo---ergot
Fuco---algae	Hyo---hog
Litho---gall stone	Myco---bacteria
Ostrea---oyster	Phyto---higher plants
Scymno---shark	Sito---cereals
Stigma---Physostigma	Urso---bear
Zoo---higher animals	Zymo---yeast

B. Selected Bibliography

"Chemistry of Natural Products Related to Phenanthrene"
Monograph Series #70, American Chemical Society
Reinhold Publishing Corporation, New York
2nd Edition (1937)
Chap. IV. pp. 111-186; pp. 357-382.
Fieser, L.F.

Annual Review of Biochemistry 1, 109 (1932)
"The Chemistry of the Sterols, Bile Acids, and other Cyclic Constituents of Natural Fats and Oils."
Windaus, A.

Annual Review of Biochemistry 3, 87 (1934)
"The Chemistry of the Sterols, Bile Acids, and other Cyclic Constituents of Natural Fats and Oils."
Rosenheim, O. and H. King

Annual Review of Biochemistry 6, 139 (1937)
"The Chemistry of the Steroids."
Schoenheimer, R., and E.A.Evans Jr.

Physiological Review 15, 1 (1935)
"Physiology of the Sterols, Including Vitamin D."
Bills, C.E.

"Recent Advances in Organic Chemistry"
Longmans, Green and Company (1936)
Volume II, Part II, Chap. XVIII.
Stewart, A.W. and H. Graham

"The Biochemistry of the Lipids"
Mimeographed Pamphlet (1935)
Page 44 on
Bull, H.B.

"Vegetable Fats and Oils"
Monograph Series #58, American Chemical Society
Reinhold Publishing Corporation, New York
Page 307 on
Jamieson, G.S.

"Chemical Technology and Analysis of Oils, Fats, and Waxes"
6th Edition (1921)
Volume I, pp. 268, 596, 607, 613, 617.
Lewkowitsch, J.I.

"Biochemical Laboratory Methods"
Pp. 267-271
Morrow, C.A.

"Outlines of Biochemistry"
Pp. 647-658
Gortner, R.A.

"Lehrbuch der Organischen Chemie"
George Thieme, Publisher
4th Edition (1936)
Page 710 on
Karrer, P.

"Principles of Biochemistry"
William Wood & Company
Reprinted (1937)
Page 180 on
Mathews, A.P.

REFERENCE LIST

(1) Physiol. Rev. 15, 1 (1935)
Bills, C.E.

(2) Ber. 36, 3752 (1903)
Windaus, A.

(3) Z.physiol.Chem. 80, 287 (1912)
Wieland, H. and F.J.Weil

(4) Chemistry and Industry 51, 464, 954 (1932)
Rosenheim, O. and H. King

(5) Ann. 192, 175 (1878)
Hesse, O.

(6) Monatsh. 18, 551 (1897)
Burian, R.

(7) Ann.chim.phys. series 5, 17, 493 (1879)
series 6, 20, 289 (1890)
Tanret, C.

(8) Compt.rend. 114, 1544 (1892)
121, 723 (1895)
126, 909 (1898)
Gerard, E.

(9) "Chemistry of Natural Products Related to Phenanthrene"
Monograph Series #70, American Chemical Society
Reinhold Publishing Corporation, New York (1937)
Fieser, L.F.

(9a) Ibid.
Appendix, 2nd Edition (1937)

(10) Monatsh. 9, 421 (1888)
Reinitzer, F.

(11) Annual Review of Biochemistry 3, 87 (1934)
Rosenheim, O. and H. King

(12) Ber. 36, 3177 (1903)
37, 3092 (1904)
Diels, O. and E. Abderhalden

This reference list includes only those references made directly from this thesis.

(13) Ann. 146, 175 (1868)
Wislicenus, J. and W. Moldenhauer

(14) Monatsh. 28, 1113 (1907)
30, 635 (1909)
Mauthner

(15) Ber. 41, 544 (1908)
Diels, O. and K. Linn

(16) Ber. 39, 4378 (1906)
Windaus, A. and A. Hauth

(17) Monatsh. 29, 395 (1908)
Abstracted in J.Chem.Soc.(London) 94, 636 (1908)
Schrotter, H. and R. Weitzenbock

(18) Annual Review of Biochemistry 6, 139 (1937)
Schoenheimer, R. and E.A.Evans, Jr.

(19) Ber. 52, 1915 (1919)
Windaus, A. and K. Neukirchen

(20) Ber. 60, 140 (1927)
Diels, O. and W. Gadke

(21) Compt.rend. 144, 1356 (1907)
Blanc

(22) Z.physiol.Chem. 210, 268 (1932)
Wieland, H. and E. Dane

(23) Z.physiol.Chem. 213, 147 (1932)
Windaus, A.

(24) Z.physiol.Chem. 65, 110 (1910)
Windaus, A.

(25) Z.physiol.Chem. 232, 97 (1935)
Fernholz, E.

(26) Ber. 59, 2064 (1926)
Wieland, H. and R. Jacobi

(27) Ber. 46, 1246 (1913)
Windaus, A. and C. Resau

(28) Z.physiol.Chem. 108, 295 (1920)
Wieland, H. and A. Kulenkampff

(29) Ber. 52, 162 (1919)
Windaus, A. and O. Dalmer

(30) Z.physiol.Chem. 114, 167 (1921)
Wieland, H. and W. Schulenberg

(31) Ber. 60, 723 (1927)
Borsche, W. and R. Frank

(32) Z.physiol.Chem. 108, 306 (1920)
Wieland, H.

(33) Z.angew.Chem. 42, 421 (1929)
Wieland, H.

(34) Z.physiol.Chem. 134, 276 (1924)
Wieland, H. and O. Schlichting

(35) Z.physiol.Chem. 161, 80 (1926)
Wieland, H., Schlichting, O. and R. Jacobi

(36) Z.physiol.Chem. 191, 69 (1930)
Wieland, H. and F. Vocke

(37) Z.physiol.Chem. 206, 243 (1932)
Wieland, H. and E. Dane

(38) Ann. 498, 185 (1932)
Tschesche, R.

(39) Z.physiol.Chem. 177, 68 (1928)
Wieland, H. and F. Vocke

(40) Z.physiol.Chem. 197, 17 (1931)
Wieland, H. and T. Posternak

(41) Z.physiol.Chem. 216, 91 (1933)
Wieland, H. and E. Dane

(42) Ber. 66, 601 (1933)
Butenandt, A., Weidlich, H.A. and H. Thompson

(43) Ann. 447, 233 (1926)
Windaus, A.

(44) Helv.chim.Acta 17, 1407 (1934)
Ruzicka, Brungger, Eichenberger, and J. Meyer

(45) Nature 129, 277 (1932)
Bernal, J.D.

(46) Ann. 507, 128 (1933)
Fernholz, E.

(47) Ann. 508, 215 (1933)
Fernholz, E.

(48) Z.physiol.Chem. 214, 89 (1933)
Guiteras, A.

(49) Z.physiol.Chem. 237, 46 (1935)
Bengtsson, B.E.

(50) Ber. 67, 2021 (1934)
Fernholz, E. and P.N.Chakrovorty

(51) Ann. 493, 181 (1932)
Reindel and Kipphan

(52) Ber. 64, 850 (1931)
Windaus, A. and A. Luttringhaus

(53) Z.physiol.Chem. 219, 240 (1933)
Wieland, H. and E. Dane

(54) J.Am.Chem.Soc. 57, 228, 942 (1935)
Fieser, L.F. and Seligman

(55) "Lehrbuch der Organischen Chemie"
George Thieme, Publisher
4th Edition, (1936)
Karrer, P.

(56) "Recent Advances in Organic Chemistry"
Longmans, Green and Company
Volume 2, (1936)
Stewart, A.W. and H. Graham

(57) "Vegetable Fats and Oils"
Monograph Series #58, American Chemical Society
Reinhold Publishing Corporation, New York (1932)
Jamieson, G.S.

(58) "Chemical Technology and Analysis of Oils, Fats and Waxes"
6th Edition, (1921)
Volume 1
Lewkowitsch, I.

(59) J.Am.Chem.Soc. 46, 2113 (1924)
Anderson, R.J. and F.P.Nabenhauer

(60) Ann. 529, 68 (1937) X
Wieland, H., Pasedach, H. and A. Ballauf

(61) Biochem.J. 17, 5 (1923)
Whitby, G.S.

(62) J.Biol.Chem. 52, 217 (1922)
Kahlenberg, L.

(63) Biochem.J. 23, 47 (1929)
Rosenheim, O.

(64) J.Am.Chem.Soc. 46, 1450 (1924)
Anderson, R.J.

(65) "Absorption Spectra of Vitamins and Hormones"
Morton, R.A.

(66) Z.physiol.Chem. 215, 59 (1933)
Schoenheimer, R. and H. Dam

(67) Ann. 486, 191 (1931)
Diels, O. and Alder

(68) "Commercial Organic Analysis"
5th Edition, (1924)
Volume 2
Allen, A.H.

(69) Annual Review of Biochemistry 1, 109 (1932)
Windaus, A.

(70) "The Biochemistry of the Lipids"
Mimeographed Pamphlet (1935)
Bull, H.B.

(71) J.Am.Chem.Soc. 48, 2987 (1926)
Anderson, R.J., Shriner, R.L. and C.O.Burr

(72) Ber. 64, 2167 (1931)
Sandquist, H. and E. Bengtsson

(73) Z.physiol.Chem. 140, 109 (1924)
Windaus, A. and J. Brunken

(74) Z.physiol.Chem. 176, 269 (1928)
Bonstedt, K.

(75) Biochem.J. 12, 160 (1918)
Ellis, M.T.

(76) J.Biol.Chem. 40, 175 (1919)
Ikeguchi, T.

(77) Australian J.Exptl.Biol.Med.Sci. 1, 53 (1924)
C.A. 18, 3626 (1924)
Marston, H.R.

(78) Biochem.J. 12, 154 (1918)
Ellis, M.T.

(79) J.Am.Chem.Soc. 45, 1944 (1923)
Anderson, R.J. and M.G.Moore

(80) J.Biochem.(Japan) 2, 27 (1922)
C.A. 17, 1820 (1923)
Miyake, S.

(81) J.Soc.Chem.Ind. 46, 433 (1927)
Allen, J. and C.W.Moore

(82) Analyst 48, 155 (1932)
Steuart, D.W.

(83) Z.physiol.Chem. 183, 74 (1929)
Zechmeister, L. and P. Tuzson

(84) University of Hawaii Occasional Papers #19
Dillingham, F.T. and R.T.Thompson

(85) J.Chem.Soc.(London) 85, 838 (1904)
Power, F.B. and Gornall, F.H.

(86) J.Am.Chem.Soc. 40, 436 (1918)
Heyl, F.W., Hart, M.C., and J.M.Schmidt

(87) J.Am.Chem.Soc. 42, 1744 (1920)
Heyl, F.W. and C. Barkenbus

(88) J.Biol.Chem. 71, 401 (1927)
Anderson, R.J. and R.L.Shriner

(89) J.Biol.Chem. 71, 389 (1927)
Anderson, R.J., Nabenhauer, F.P., and R.L.Shriner

(90) "Sterol Content and Vitamin Value of Avocado Oil"
University of Hawaii, M.S.Thesis (1933)
Westgate, M.

(91) Biochem.J. 29, 456 (1935)
Drummond, J.C., Singer, E. and R.J.MacWalter

(92) Z.physiol.Chem. 101, 89 (1918)
Abstracted in J.Chem.Soc.(London) 114, 179 (1918)
Lifschutz, I.

(93) Biochem.Z. 282, 441 (1935)
Lifschutz, J.

(94) J.Am.Chem.Soc. 48, 2976 (1926)
Anderson, R.J. and R.L.Shriner

(95) J.Am.Chem.Soc. 48, 2972 (1926)
Nabenhauer, F.P., and R.J.Anderson

(96) J.Am.Chem.Soc. 46, 1450 (1924)
Anderson, R.J.

(97) J.Am.Chem.Soc. 46, 1717 (1924)
Anderson, R.J., and F.P.Nabenhauer

(98) J.Biol.Chem. 110, 461 (1935)
Schoenheimer, R.

(99) J.Am.Chem.Soc. 46, 1957 (1924)
Anderson, R.J., and F.P.Nabenhauer

(100) J.Am.Chem.Soc. 58, 2446 (1936)
Wallis, E.S. and Fernholz, E.

(101) J.Org.Chem. 2, 335 (1937)
Wallis, E.S. and P.N.Chakravorty

(102) J.Org.Chem. 2, 341 (1937)
Bernstein, S. and Wallis, E.S.

(103) Avocado Year Book 93, (1916)
California Avocado Association
Mattill, H.A.

(104) Am.Jour.Physiol. 59, 310 (1922)
Santos, F.O.

(105) Science 65, 15 (1927)
Dickey, L.B.

(106) Avocado Year Book 58, (1928)
100, (1930)
Weatherby, L.S. and others.

(107) Proc.Soc.Exptl.Biol.Med. 22, 227 (1925)
Hess, A.F., Winstock, M., and F.D.Helman

(108) J.Biol.Chem. 64, 263 (1925)
Steenbock, H. and A. Black

(109) J.Biol.Chem. 51, 41 (1922)
McCollum, E.V., Simmonds, N., Shipley, P.G. and E.A.Park

(110) "Principles of Biochemistry"
William Wood & Company (1937)
Mathews, A.P.