THE STEROLS OF CERTAIN TROPICAL OILS

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Approved by 

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I. INTRODUCTION

The sterols are crystalline substances of highly complex alcoholic structure found in the unsaponifiable matter of all plants and animals. They belong to a much larger category of naturally occurring organic compounds, the steroids, which includes bile acids, cardiac and toad poisons, saponins, sex hormones, and certain vitamins. The molecular structure of these compounds and their diverse physiological action and function have held the keen interest of many chemists for well over a century.

It is not surprising that an immense volume of data, some of doubtful reliability, has been compiled on the chemical and biological nature of the sterols. Many of the problems particularly those concerning their biogenesis are yet to be solved.

The vegetable sterols occur in minute amounts in all parts of the plant but are found most concentrated in the oil-bearing seeds and pollen grains. The potential and perhaps immediate importance of the plant sterols resides in the fact that methods have been developed for their conversion to other steroids such as sex hormones. For example, stigmasterol, a fairly common plant sterol, has been converted to dehydroisoandrosterone. this in turn has been transformed by the Serini acetylene synthesis (1) into desoxycorticosterone, a compound of

great therapeutic value. Consequently, searches are in progress over the entire world for plants containing steroids or related compounds from which substances of therapeutic importance may be prepared.

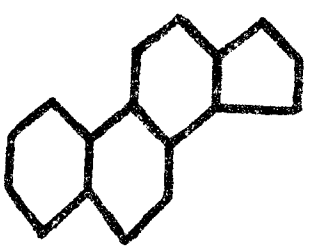
Although extensive research has been done on the sterols of vegetable origin, a survey of the literature shows that the tropical oils as sources of phytosterols have had relatively little attention. A study of five tropical oils was made by Westgate (2) in 1938. As sources of phytosterols he utilized avocado (Persea gratissima), kukui (Aleurites moluccana), china wood (Aleurites fordii), chaulmoogra (Taraktogenos kurzii), and cocoanut (Cocos nucifera). He established methods for isolating and purifying the sterols and he determined their physical characteristics and constants. He confirmed the earlier work of Anderson and coworkers (3-6) who demonstrated the closely related component nature of phytosterol preparations. Westgate emphasized the importance of separating the intimate mixtures of sterols into homogeneous fractions. Although he accomplished partial separation of the sterol mixtures into fractions, he did not determine the exact nature of the individual components of the mixtures.

The present research was a logical outgrowth in the same laboratory, of the work initiated by Westgate on tropical oils. The object was to continue the study of tropical oils as sources of phytosterols and to establish the chemical identities of the individual sterols present

in the intimate mixtures obtained in the early stages of the preparations. Four tropical oils namely, avocado, chaulmoogra, cocoanut, and macadamia were used as sources of sterols. Macadamia nut oil, derived from nuts which have become commercially valuable in the Hawaiian islands, was used in sterol studies for the first time. Recently, a report concerning the fatty acids present in this oil was published by Hilditch et al (7).

II. HISTORICAL SECTION

Fifty years of chemical investigation has resulted in the recognition of a large group of naturally occurring substances known as the steroids. For a long time the various substances within this group were ill-defined chemically but now they are classified as sterols, bile acids, heart poisons, the toad poisons, the saponins, the sex hormones, certain vitamins, and most recently the adrenal cortical hormones. The term steroid refers to any substance naturally occurring or synthetic which contains the cyclopentanoperhydrophenanthrene nucleus.



At the close of the eighteenth century, the first sterol, a well defined crystalline compound was found in bile stones by the French chemist Pouelletier (8). Chevreul (9) was the first to examine this product which he called "cholesterine", now known as cholesterol. This unusually interesting substance led to the discovery of other sterols. Coprosterol was found by Flint (10) in 1862 in human excrements, plant sterols were isolated from the Calabar bean in 1878 by Hesse (11), and in 1897 Burian obtained sterols from the germ

of cereal grains. Tanret (13) was the first to prepare ergosterol which he separated from ergot oil in 1889.

The recognition that sterols were present in higher plants and animals and also in lower forms of life such as yeasts, fungi, and algae, led to their classification in 1889 as phytosterols, zoosterols, and mycosterols. This taxonomic grouping proposed by Gerard (13) and based upon the occurrance of sterols, remains today. Chemically speaking they are found as free sterols or as esters. The ubiquitousness of the sterols is remarkable in that they are found in every cell of animal or plant origin having a well-defined nucleus. The most important of the zoosterols is cholesterol which seems to be the only protoplasmic sterol found in the vertebrate animals. It has been found usually mixed with other sterols in crustaceans, and gastropods, and other mollusks, and in insects. Dihydrocholesterol (14) has been isolated as a companion of cholesterol. Coprosterol (10) has been identified in fecal matter. Following the isolation of a sterol by Beneke (15) from leguminous seeds, a great many phyto-sterols were isolated from higher plants. The included brassicasterol, a C_{28} sterol of rape seed oil (16), campesterol from wheat germ oil (17), and "α"-spinasterol from spinach (18), senega root (19), and alfalfa (20). Sitosterol, the most widely distributed of plant sterols was found by Anderson and coworkers (3-5,21-23) to consist of mixtures of at least three monosaturated substances designated "α", "β", and "γ"-sitosterol, and of a dihydro- "β" -sitosterol. The

characterization of the sitosterols is complicated by unusual difficulties of purification and identification. "α"-sitosterol upon further processing yielded sterols designated "α_1", (24), "α_2", (24) and "α_3"-sitosterol. (24,25) This interesting and intricate group is found in cotton seed oil (26), calycanthus oil (27), tall oil (28), wheat germ oil (23), crepe rubber (29), rye germ oil (25), corn oil (23). The phytosterols of avocado oil, chaulmoogra oil, kukui nut oil, china-wood oil, and cocoanut oil were investigated between 1932 and 1937 by L. N. Bilger and coworkers (30-32).

The highly diverse sterols of marine invertebrates were investigated by W. Bergmann (33-42) and others (43,44) in the hope of discovering starting materials for the preparation of steroid hormones. It had been suggested that invertebrates may be dependent upon exogenous plant sterols and incapable of effecting the full synthesis of the sterols they may require. In 1945, Bergmann isolated cholesterol from sponges and from snails. He is still engaged at Yale University in the isolation and identification of the sterols in mollusks and sponges. He has advanced the interesting hypothesis that the greatest diversity of the sterols is found among the most primitive animals such as sponges and coelenterata (38). The process of evolution is accompanied by the use of a variety of sterols in favor of the exclusive use of cholesterol. Cholesterol accompanied by no other sterol is present in man.

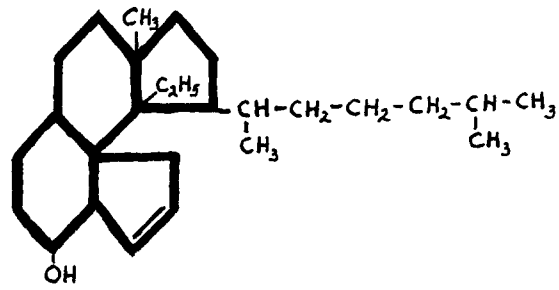
Heilbron (45-47) and Carter (48) have investigated the sterols of algae and have reported sitosterols to be present in chlorophyceae and fucosterol in phaeophyceae.

As was to be expected, running hand in hand historically with the discovery and isolation of sterols was the investigation of their chemical character and molecular structure, a magnificent effort extending from the analyses of Chevreul in 1823 through the researches of Diels, Wieland, and Windaus a century later. The gratifying results established not only the structure of the individual sterols but of the other steroids which included the vitally important bile acids, hormones, heart and toad poisons, saponins and certain vitamins. To all was assigned the cycopentanoperhydrophenanthrene nucleus.

Some of the greatest chemists of the past half century contributed to the clarification of these large and elusive molecules. In 1859 Berthelot (8) detected the characteristic hydroxyl group, and Diels and Abderhalden (49,50) in 1903 demonstrated the secondary character of this alcoholic group. Wislicenus and Moldenhauer (51) discovered the presence of a center of unsaturation in 1868 by the formation of the dibromide and Mauthner and Diels (51) saturated cholesterol with hydrogen.

Meanwhile intensive studies were pursued simultaneously by Windaus on the structure of sterols and by Wieland on the bile acids. The two apparently isolated fields converged in an amazing manner, and were demonstrated to be fundamentally one and the same from the standpoint

of chemical structure. By 1928 the researches of Wieland and Windaus, supported by those of Wislicenus, Moldenhauer, Mauthner, and Diels, culminated in the formulation of a structure for cholesterol.

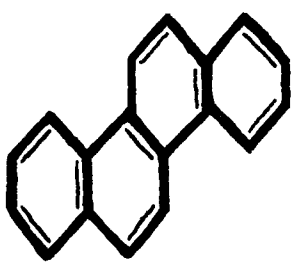


The men were awarded a divided Nobel Prize in 1928 for their work on the bile acids and the sterols and for the establishment of a structure for cholesterol presumed until 1932 to be correct and known in the literature as "Wieland's old formula".

In as much as this paper is concerned with sterols the historic definition given by the eminent Windaus (52) in 1911 is herewith quoted: "The sterols are unsaturated alcohols, possessing a high molecular weight. The ratio of carbon to hydrogen resembles the corresponding ratio in polyterpenes. They crystallize from ether in needles, from dilute alcohol in plates containing water of crystallization. They give fairly characteristic colour reactions with acetic anhydride, and concentrated sulfuric acid as well as with other reagents. They can detoxicate saponins and give insoluble addition products

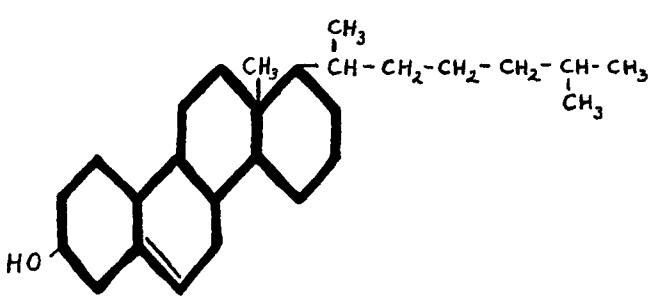
with digitonins".

It became increasingly important that an indisputably correct formula for cholesterol be established because of the bearing of this achievement upon the structure of other sterols and upon the clarification of the structure of compounds belonging to the overall steroid category. Ironically the structure advanced for cholesterol at the time of the Nobel award was shown to be erroneous. In 1927 Diels (53) had observed that cholesterol was converted to a hydrocarbon, chrysene, when dehydrogenated.

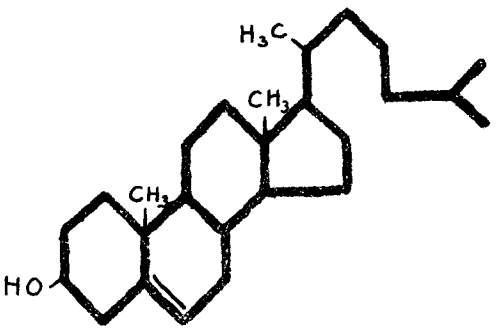


Further, Bernal (53) in 1932 demonstrated that Wieland's old formula was in disagreement with his X-ray investigations of sterols.

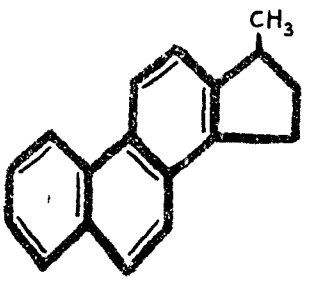
Making use of these two pieces of information, Rosenheim and King (55) in 1932 proposed their chrysene formula for cholesterol:



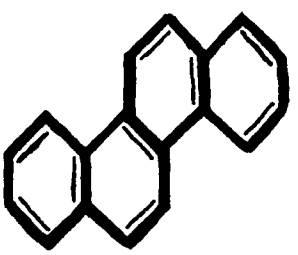
Researches by Wieland and Dane (56), reported in 1932, lead to the proposal of the formula known as "Wieland's new formula".



Strong support was given to this formula by the discovery of "Diel's hydrocarbon", γ-methyl-1:2-cyclopentenophenanthrene, in Diel's researches from 1927 to 1933:



Diels obtained this hydrocarbon when his dehydrogenation of cholesterol was carried out at 320°C rather than at 400°C when the product had been identified as chrysene.

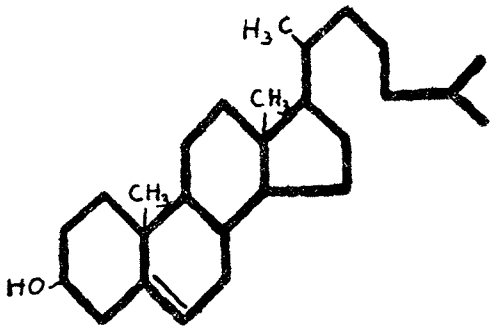


The accepted interpretation of the formation of these two hydrocarbons is that chrysene was formed by rearrangement of "Diel's hydrocarbon" when the higher temperature was used. "Wieland's new formula" was also in agreement with Bernal's X-ray measurements and is now universally accepted for cholesterol.

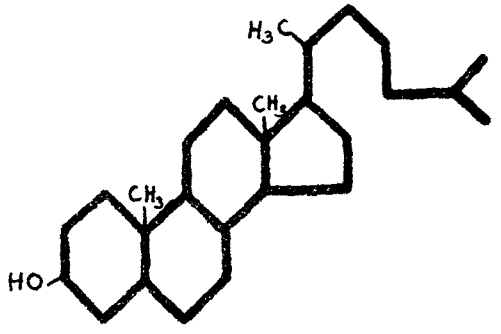
As a result of these tedious and extensive researches, the cyclopentanoperhydrophenanthrene nucleus is now assigned to all sterols and to all other steroids. During the past fifteen years sterol and steroid studies have been directed toward establishing the differences in structure and the interrelationships among the various classes of steroids and individual members of classes; the significance of their relationships to phenanthrene; their transformations within living organisms; and the stereochemistry of steroids.

Although a tremendous volume of data concerning the formulations of the sterols is present in the literature, it has become increasingly apparent that due to the unusual difficulties of purification and identification conclusions regarding identity or nonidentity are qualified. Nevertheless, structures of those sterols originating from various sources which seem to have been established without ambiguity are listed below:

Vertebrates:

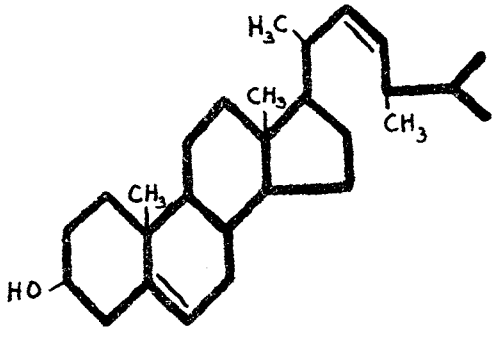


Cholesterol (57)

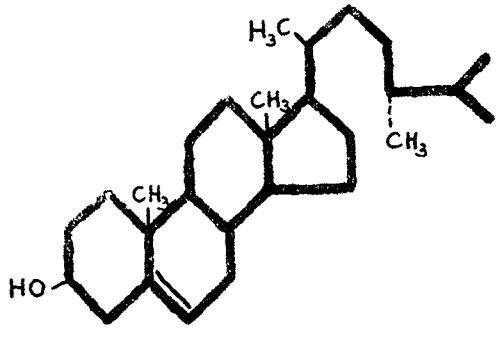


Coprostanol (14)

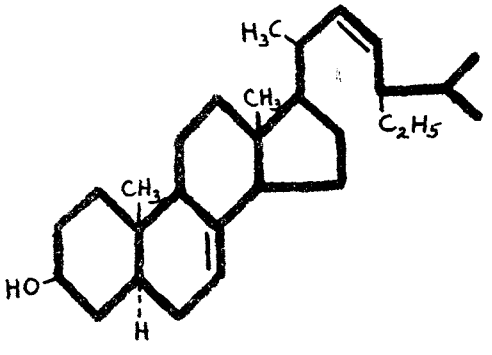
vegetables:



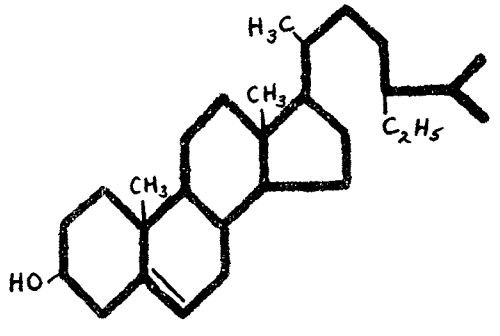
Brassicasterol (16)



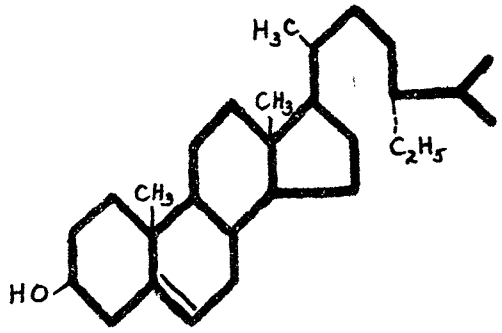
Campesterol (17)



"α"-Spinasterol (18)

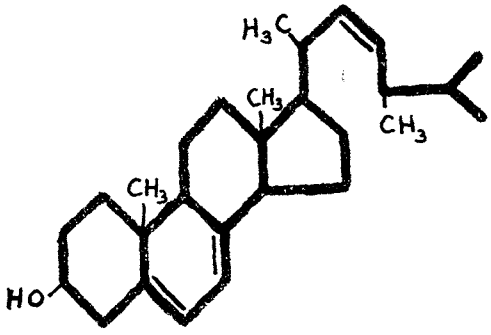


"β"-Sitosterol (58)

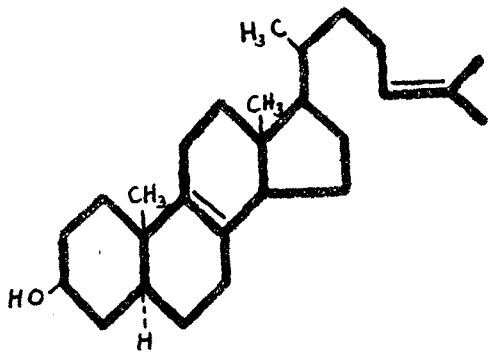


"γ"-Sitosterol (59,60)

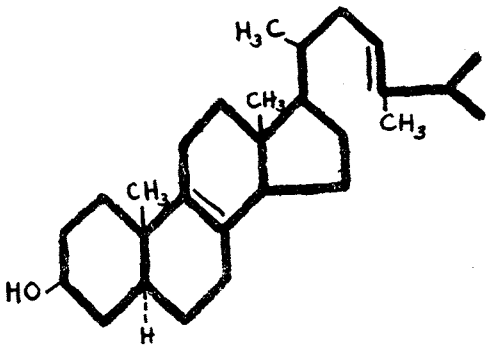
yeasts:



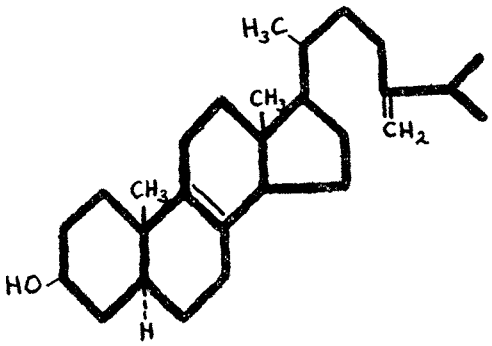
Ergosterol (13)



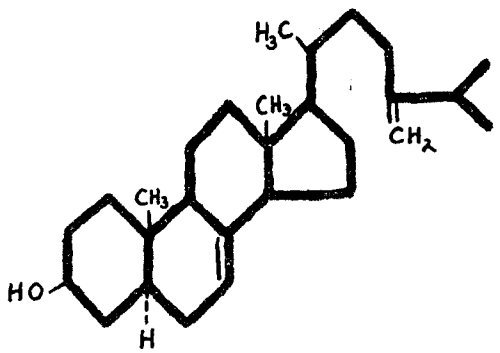
Zymosterol (61)



Ascosterol (62)

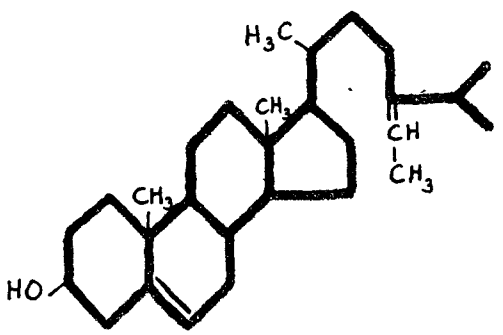


Fecosterol (62)



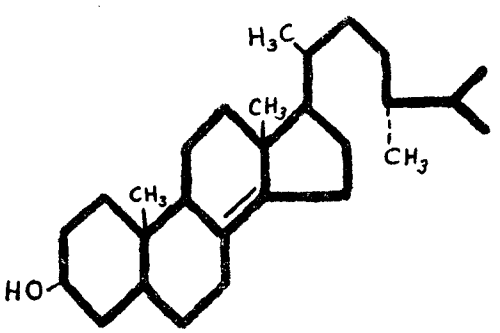
Episterol (62)

algae:



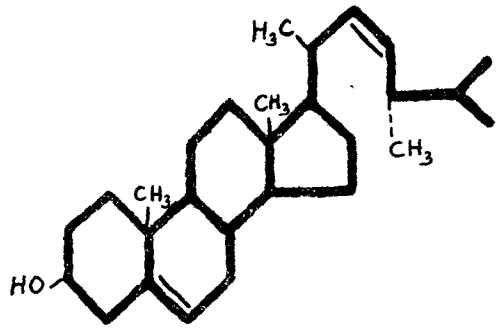
Fucosterol (46-48)

starfish:

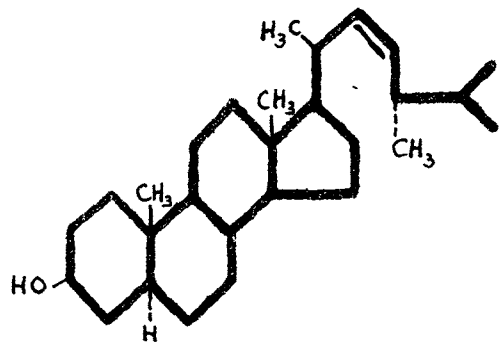


"α"-Stellastenol (40)

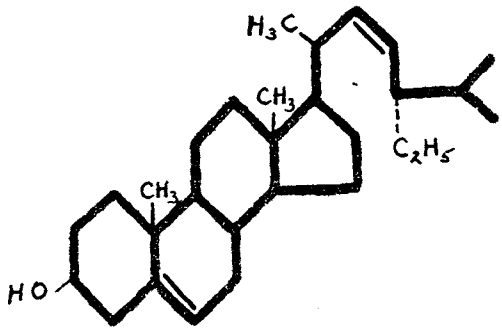
sponges:



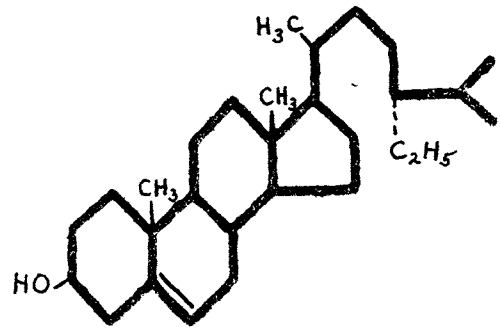
Chalinasterol (39)



Neospongosterol (36)



Poriferasterol (63)

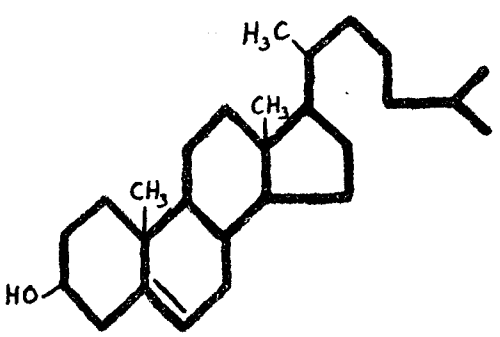


Clionasterol (63)

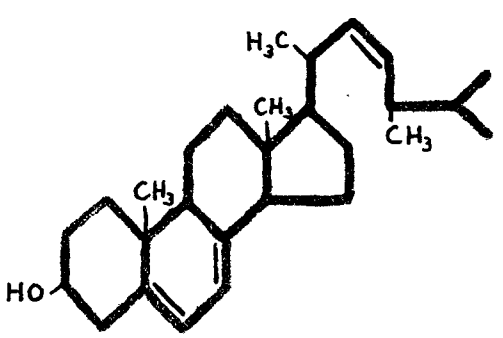
The history of the sterols has been distinguished by the productivity of many renowned chemists who have succeeded in elucidating gradually and painstakingly the chemical nature of the large complex molecules. However, the physiological functions and interrelationships of these substances which are so widely distributed are still obscure.

III CHEMISTRY OF THE STEROLS

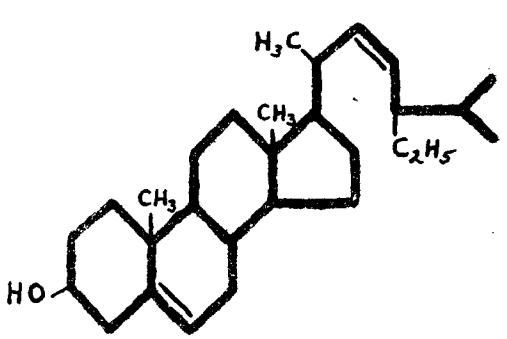
A brief review of the established chemical reactions of the sterols is given here because of their bearing upon the present research in the establishment of the structure and identity of the sterols of the four tropical oils investigated. Conceding that sterols are alike in that they contain the cyclopentanoperhydrophenanthrene nucleus, structural differences among the sterols are to be sought in specific parts of the large molecules. It is obvious from a consideration of the formulas below for cholesterol, ergosterol, and stigmasterol, for example, that they are identical in the principal nucleus and they differ in the number and position of double bonds and in the length of the chain in position 17.



Cholesterol

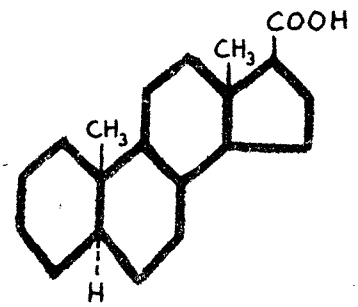


Ergosterol



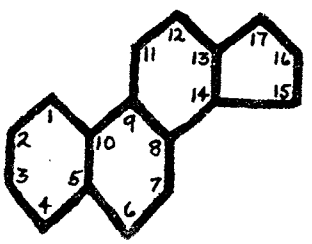
Stigmasterol

In a chemical study of sterols their close relationship is shown by degrading them to one and the same substance. Cholesterol, ergosterol and stigmasterol may all be converted to completely reduced hydrocarbons by saturation of the double bonds and reduction of the hydroxyl groups. A further series of reactions involving oxidation, esterification, and the Grignard reaction, result in the degradation of the long side chain of all three sterols and their conversion to aetio-allo-cholanic acid:



Aetio-allo-cholanic acid

Thereby the essential hydrocarbon nucleus is shown to be the same for cholesterol, ergosterol, and stigmasterol:



Cyclopentanoperhydrophenanthrene

The chemical reactions shown by the sterols include many standard organic reactions and a number of reactions which are fairly specific for the complex sterol molecules.

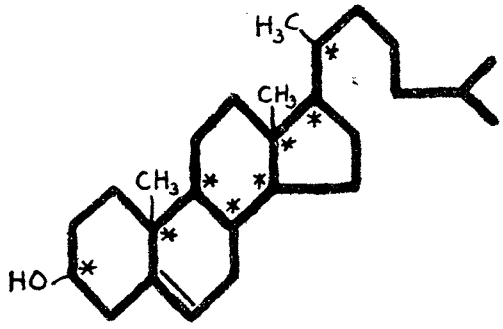
Many sterols show characteristic color reactions which are of diagnostic value in determining the presence or absence of certain types of sterols.

Functional hydroxyl groups are readily attacked and acetates, benzoates, dinitrobenzoates, are easily prepared. Vigorous oxidation with dichromate in sulfuric acid results in the degradation and removal of the side chain at position C-17. The Oppenauer oxidation, utilizing aluminum ter-butoxide and acetone, causes migration of a double bond. Digitonides are prepared by reaction of digitonin at hydroxyl groups in certain positions. The diene reaction takes place when sterols containing conjugated systems are treated with maleic anhydride.

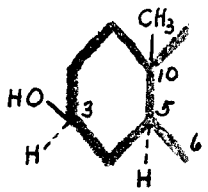
The monounsaturated sterols can be separated from the doubly unsaturated forms by bromination of the acetates in ether. Insoluble tetrabromides and soluble dibromides are produced. Reactions at double bonds also occur when sterols are hydrogenated, or when treated with perbenzoic acid or with ozone.

The reactions of sterols, summarized briefly above, are utilized extensively in sterol research for purposes of determining structure, degrading and synthesizing sterols and related substances, and in the study of biogenetic relationships.

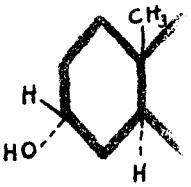
Although the major problems of structure determination and chemical behavior of the sterols were well clarified by 1935, the elucidation of spatial configurations, that is the stereochemistry, has been the principal field of intensive research during the past fifteen years. The essential features of these investigations and the present status of the problem are illustrated very briefly by utilizing cholesterol as an example. Cholesterol contains eight asymmetric carbon atoms seven of which are nuclear.



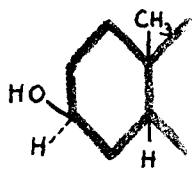
The hydrogenation of cholesterol generates a new nuclear center of asymmetry at position C-5. The two isomers that are formed as a result are known as cholestanol and coprostanol. By reversing the positions of the hydrogen atom and the hydroxyl group at C-3 without changing the configurations of any of the other centers of asymmetry, the epimers of cholestanol and coprostanol may be obtained.



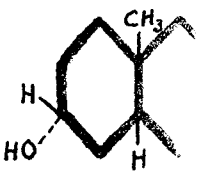
Cholestanol



epi-Cholestanol



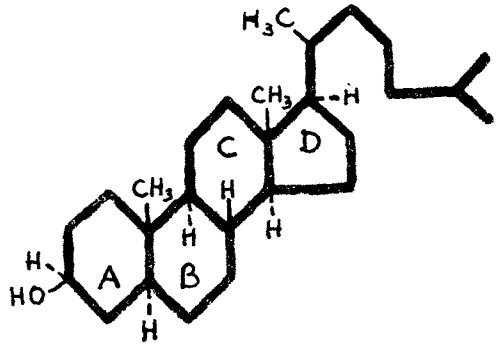
Coprostanol



epi-Coprostanol

The convention for the indication of configurations in formulas is to utilize a solid line for a group that is assumed to project above the plane of the nucleus and a dotted line for a group projecting below the plane. Arbitrarily the configuration of the C-3 hydroxyl of cholesterol has been assigned as β. Any hydroxyl group, angular methyl group, C-17 side chain, or tertiary hydrogen that lies on the same side of the ring plane as the C-3 hydroxyl of cholesterol is described as β-oriented and the carbon atom to which the group is joined by definition has the β-configuration. The opposite orientations and configurations are designated α. The complete configuration

for β cholestanol or simply cholestanol is shown in the formula below:



It is possible to define the configuration of the hydroxyl group of the four isomeric stanols, the formulas of which appear above with respect to the hydrogen at C-5. In cholestanol the hydroxyl is trans in respect to the hydrogen and in epi-cholestanol it is cis but defined with respect to the angular methyl group at C-10 it is in the cis position in cholestanol and in the trans position in epi-cholestanol. In coprostanol the hydroxyl group is in the cis position and epi-coprostanol in the trans position, both in respect to the hydrogen at C-5 and the methyl group at C-10. A clear formulation of the configurations of these compounds is possible by employing the methyl group as a standard of reference. In cholestanol and coprostanol the hydroxyl group at C-3 is in the cis position and in their epi derivatives in the trans position both with respect to the methyl group at C-10. It is important to note that the cis derivatives, as defined above, give insoluble molecular compounds with digitonin.

The cholestane series which includes the sterols has been shown conclusively to possess trans arrangement of the A/B ring fusion and clear proof has been advanced for the cis arrangement of the A/B ring fusion of the coprostane series which includes the bile acids. Investigations culminating within the last ten years have established beyond reasonable doubt the fact that the configurations of the B/C and the C/D ring fusion of both the sterols and bile acids are both trans.

The stereochemistry of the various sterols and of other steroid classes has been developed in a manner similar to the above described procedure.

IV EXPERIMENTAL SECTION

Four tropical oils, avocado, chaulmoogra, cocoanut, and macadamia, were utilized in the isolation, study of properties and identification of sterols. The experimental work comprised eight main procedures, namely:

(1) The oils were prepared from the raw products in cases where purchase was not feasible.

(2) Mixtures of sterols were obtained by saponification of oils and extraction of unsaponifiable portions with ether.

(3) Sterol mixtures were purified by recrystallization from petroleum ether and recrystallization of the product from anhydrous ethyl alcohol.

(For an understanding of the fact that sterol mixtures were used for certain studies in the experimental work, an explanatory statement is desirable here. The historical review indicated that much of the earlier research on sterols was done upon extremely intimate mixtures of closely related and difficultly separable sterols. Much of the information on sterols recorded in the literature referred to such mixtures. They were also used in the present work for certain important studies of the sterols isolated from the four oils. Following these studies extensive work was done on the separation of the intimate mixtures into separate sterols and these were investigated individually.)

(4) Physical properties of sterol mixtures including solubilities, crystalline structure, optical rotation, and melting points, were determined.

(5) Sterol mixtures were subjected to the usual color reactions for sterols.

(6) An extensive study of chemical reations was conducted on the sterol mixtures.

(7) Sterol mixtures were separated by chromatographic analysis into individual sterols and the same studies were applied to these as to the sterol mixtures.

(8) Carbon-hydrogen determinations were made on the individual sterols. Prior to analysis the sterols were dried with anhydrone for several hours at 100°C in vacuo, a highly essential procedure in view of their recrystallization from ethyl alcohol.

A. Avocado Oil

Sources of Oils:

The avocado oil from which sterols were isolated was obtained earlier by Westgate from the Hawaiian Avocado Company where it was prepared from the pulp of raw fruit, (*Persea gratissima*). Hydraulic pressure at room temperature was applied and the water layer was removed.

Saponification of Oils and Extraction of Sterols:

Isolation of the sterol portion of the oil was carried out by a modification of the method used by Westgate (2). A weighed quantity of the oil was saponified by heating it with a calculated amount of 20% alcoholic potassium hydroxide solution for several hours. The bulk of the alcohol was removed by distillation and the very viscous residue thinned down with water. The soap solution was extracted with ether, the ether thoroughly washed with distilled water, and the washed ether extract dried with anhydrous sodium sulfate. Finally the ether was distilled off and the unsaponifiable fraction which contained the sterols was obtained as a light yellow solid.

This process was carried out on amounts of oil varying from ten to eight hundred grams. Four hundred grams was selected as a suitable amount for convenient saponification and extraction with ether.

The oil was placed in a large crock and heated cautiously until liquefaction took place. The dark green-

ish liquid was stirred several minutes until homogeneous and the resulting oil was poured into appropriate jars and labeled.

A jar to be sampled was heated in a water bath until the oil liquefied. The oil was poured into a tared two liter round-bottom flask until the required four hundred grams had been added. A 20% alcoholic potassium hydroxide solution was prepared by dissolving one hundred sixty grams of U.S.P. potassium hydroxide in a minimum amount of distilled water and adding sufficient 95% aldehyde-free alcohol to give a volume of 800 cc. The aldehyde-free alcohol was employed to prevent oxidation of the sterols. The mixture of sterol and alcoholic potassium hydroxide was swirled by hand and refluxed over a hot plate from two to five hours. The time of heating after the minimum of two hours did not prove to be critical. As the refluxing progressed, the reaction mixture became darker and at a certain point the reaction seemed to be exothermic, when caution became necessary. The excess alcohol was removed as rapidly as possible by distillation which was discontinued when frothing began. The thick soap solution was diluted four or five times and a very thick viscous gel was formed. The gel was extracted with ether without complications. Hence it was not necessary to dilute the soap solution with eight or more volumes of water as recommended by Westgate. (2).

The soap solution was extracted in batches

with relatively small portions of fresh ether in a separatory funnel. Frequently during the first extraction an emulsion formed. This was broken up by the addition of ethyl alcohol and by allowing to stand about ten minutes. Each batch of soap solution was extracted three times with successive portions of several hundred cubic centimeters of fresh ether. The three or so liters of ether extract were washed first with distilled water containing a few cubic centimeters of diluted sulfuric acid and then with distilled water until the washings were neutral to phenolphthalein. The washed ethereal extract was dried over anhydrous sodium sulfate, usually overnight. The decanted ether solution was concentrated by distillation to a small volume. At this point the ether was allowed to evaporate spontaneously. The crude yellow solids which were thus obtained were ready for purifications. Several attempts to dry the solids at 80°C in vacuo had resulted in considerable darkening and decomposition. The crude mixture was allowed to stand in a vacuum desiccator for several days prior to weighing. The yields of the unsaponifiable fraction varied from 0.5% to 1.0% and in no case exceeded the latter figure. Westgate reported higher yields at this point than were obtained in the present research.

The unsaponifiable fraction was purified by crystallization from low boiling petroleum ether followed by recrystallization from anhydrous ethyl alcohol.

Purification of the Sterol Mixture:

The yellow solids isolated in the manner described above were dissolved in a minimum amount of low boiling petroleum ether. The filtered yellow solution was kept in the ice box for several hours. A definitely crystalline white solid separated and was filtered off. A second crop of white crystals was obtained by similar treatment of the yellow filtrate.

Physical Properties of the Sterol Mixture:

The white solids, subsequently referred to as the sterol mixture, were recrystallized from anhydrous ethyl alcohol from which beautiful white needles were obtained having a melting point of 132-33°C* and a specific rotation, $[\alpha]_D^{25}$, of -20.0.

All compounds whose specific rotations were determined in this research were dried for several hours with anhydrone at 100°C in vacuo. The specific rotation was determined by dissolving a weighed amount of the sterol mixture in dry chloroform at 25°C. Observations were made in a 1 d.m. tube by means of a polarimeter using the D line of sodium. The specific rotation was calculated in the usual way.

Solubility tests showed that several mg. of the sterol mixture was soluble in 2-3 cc. of any of the common organic solvents, ether, chloroform, acetone, hot anhydrous alcohol, ethyl acetate, hot low boiling petroleum ether,

* melting points are uncorrected

benzene, and pyridine. They were slightly soluble in methyl alcohol and diluted ethyl alcohol.

Color Reactions of the Sterol Mixture:

Colorless sterols when treated with strong acids under dehydrating conditions are known to give color reactions. This behavior forms the basis of a number of standard color tests which are of value for qualitative identifications and in the diagnosis of structure.

Liebermann-Burchard Color Test (64).

A few milligrams of the sterol mixture was dissolved in a solution made up of 2 cc. chloroform, 20 drops of acetic anhydride, and 2 drops of sulfuric acid. A reddish-violet color, changing to bluish-green resulted, showing the presence of unsaturated forms.

Whitby Color Test.

The following solution was prepared according to the directions of Whitby (65). The reagent consisted of a solution of concentrated sulfuric acid and formalin in the proportion by volume 50:1. To 2cc. of a chloroform solution, containing 1-2 mg. of sterol, 2 cc. of the sulfuric acid-formalin reagent was added. When the layers separated, the upper chloroform layer was cherry red and the lower layer brownish-red with a definite green fluorescence. When the upper layer was poured off in a dry test tube and 2-3 drops of

acetic anhydride added, a blue color was produced, showing the presence of sterols.

Rosenheim Color Test (66).

The Rosenheim test is designed to detect the presence of a conjugated system. The reagent was prepared by mixing nine parts of trichloracetic acid with one part of water. A few milligrams of the sterol mixture dissolved in 1 cc. of chloroform was added to 1 cc. of freshly prepared reagent. No color was developed which indicated the absence of a conjugated system.

Tortelli-Jaffe Color Test.

This test was devised by Tortelli-Jaffe (67) and was made more sensitive by Heilbron and Spring (68). It is specific for compounds which contain a ditertiary double bond between "bridge-heads" or which are easily isomerized to such substances. A crystal of the sterol mixture was dissolved in 5 cc. glacial acetic acid in a dry test tube. One cubic centimeter of a 2% solution of bromine in chloroform was introduced by means of a pipette. A green ring, which would have appeared at the surface of contact of the two solutions if the test had been positive, was not formed.

Chemical Behavior of the Sterol Mixture:

The sterol mixture was subjected to certain standard organic reactions. These included acetylation, benzoylation, tests for unsaturation, formation of the digitonide, bromination, the Diels-Alder reaction, and catalytic hydrogenation.

Acetylation.

One hundred milligrams of the sterol mixture was acetylated by treatment with an excess of acetic anhydride at the boiling point of acetic anhydride for forty-five minutes. Water was added to the cooled mixture, the precipitated solid was collected by filtration and recrystallized from ethyl alcohol. The product melted at 117-122°C and showed $[\alpha]_D^{25} = -25.5$. The weight of the crystals obtained was 95 mg.

Fifty milligrams of the recrystallized sterol acetate mixture was hydrolyzed with an excess of 5% alcoholic potassium hydroxide solution. The mixture was refluxed for one hour over a hot plate. The sterols were obtained by extraction of the mixture with ether. The solids that were obtained from the concentration of the ether were recrystallized from anhydrous ethyl alcohol and melted at 135-136°C. The weight of the leaf-like crystals was 32.5 mg.

Benzoylation.

One hundred milligrams of the sterol mixture

was benzoylated by dissolving the solids in 2-3 cc. of pyridine which had been distilled and stored over solid potassium hydroxide. To this solution was added an excess of benzoyl chloride with a medicine dropper. The reaction mixture was heated at 100°C for one hour over a hot plate. The cooled mixture was treated with water and the gummy brown solids were collected and washed with 5% sodium carbonate. The solids were recrystallized from a small amount of anhydrous acetone. The weight of the colorless plates varied from 100 to 125 mgs. Their melting point was 145-146°C.

Fifty milligrams of the sterol benzoates was hydrolyzed with an excess of 5% alcoholic potassium hydroxide. The sterol was obtained according to the procedure described above. Three-fifths of the original weight of sterol benzoates was obtained as sterol. The melting point was 134-135°C.

Tests for Unsaturation.

A few milligrams of the avocado sterol acetates was dissolved in a small amount of acetone. A very weak solution of aqueous permanganate was added slowly to this solution. The discharge of the purple color was evidence of a center or centers of unsaturation in the sterol nucleus or side chain.

A few milligrams of the sterol acetates was dissolved in about 2 cc. of ether. A brominating mixture was prepared by dissolving 1 g. of bromine in

20 cc. of glacial acetic acid. The brominating mixture was added dropwise to the ethereal solution of the sterol acetates. The first five or six drops of the orange solution were instantaneously decolorized. This was confirmatory proof for the existence of unsaturation in the sterol mixture.

Formation and Cleavage of the Digitonide.

A 1% alcoholic solution of digitonin was prepared according to the recommendation of Westgate (2). One gram of digitonin was dissolved in 50 cc. of warm absolute alcohol. To this solution were added required amounts of alcohol and water to make a 1% solution of digitonin in 90% ethyl alcohol.

The digitonin solution was added to 0.5 g. of the unpurified, unsaponifiable fraction from the saponification of the oil. The mixture was heated for an hour and placed in the refrigerator over night. The voluminous precipitate was collected on a Gooch filter, washed with alcohol and ether, and dried in vacuo at 80°C. The digitonide weighed 1.1 g.

The same test was carried out on a purified sample of avocado sterols. The sterol mixture reacted almost quantitatively with digitonin. Correlation of this with the result obtained above made it possible to calculate the percentage of sterol in the unsaponifiable fraction. This amounted to 55%. The formation of a digitonide demonstrated the beta configuration of the

3-hydroxyl group since a sterol possessing this particular configuration is known to form an insoluble digitonide whereas the alpha configuration does not. (51).

The avocado sterol digitonides were cleaved according to the method used by Westgate (2). A dried sample of the digitonides was ground with 2 g. of crystalline sodium acetate and the mixture was transferred to an Erlenmeyer flask with 20 cc. of ethyl alcohol. The mixture was refluxed for thirty minutes and 70 cc. of diethyl ether was added through the top of the condenser. The precipitate was washed first with hot alcohol and then with water. The sterol mixture was recovered upon concentration of the ether. The solid so obtained was recrystallized from ethyl alcohol and showed a melting point of 134-135°C and $[\alpha]_D^{25} = -21.5$.

Bromination.

The treatments of the sterol mixture with bromine and digitonin were combined by Schoenheimer (14). This procedure was based on the fact that the unsaturated sterols present in a mixture, when brominated, did not form digitonides whereas the saturated sterols, which could not be brominated, formed insoluble digitonides. Consequently, a separation of the saturated from the unsaturated sterols was possible.

One gram of the mixture was dissolved in hot ethyl alcohol. The solution was placed in a

freezing mixture of ice and water and when the temperature of the solution had reached 20°C a cold normal alcoholic solution of bromine was added dropwise. The addition was continued accompanied by shaking until a permanent yellow color was obtained. After several hours an excess of a 1% alcoholic solution of digitonin was added. The reaction flask was kept in the dark for two days. Occasionally small amounts of the alcoholic solution of bromine were added to keep the liquid faintly yellow. No precipitate of digitonide which would have been formed by the saturated forms if present, was obtained. From the solution the original sterol mixture was obtained after debromination with zinc in glacial acetic acid. The melting point was 134-135°C.

Diels-Alder Reaction.

The sterol mixture was treated with maleic anhydride after the fashion of Windaus (69) to detect the presence of a conjugated system.

Five hundred milligrams of the sterol mixture was dissolved in 10 cc. of xylene. To this solution was added 210 mg. of maleic anhydride. The reaction mixture was heated for eight hours at 135°C. The xylene was removed by distillation and the residue was refluxed for 2.5 hours with 5 cc. of an alcoholic potassium hydroxide solution, made from 1 g. potassium hydroxide and 12 cc. of methyl alcohol. Water was added and the reaction mixture shaken three times

with low boiling petroleum ether. The petroleum ether extract was washed with water, dried, and the petroleum ether evaporated. The resulting solid was recrystallized from anhydrous ethyl alcohol. The melting point was 132-133°C.

The alkaline solution was acidified with dilute sulfuric acid. The precipitated acid was extracted with ether and recrystallized from water. The melting point was 129-129.5°C. The melting point of maleic acid recorded in the literature is 130.5°C (70).

The failure of maleic anhydride to react demonstrated that a conjugated system was not present in the sterol mixture.

Catalytic hydrogenation.

Platinum oxide catalyst was prepared according to the method of Bruce (71). Five cc. of chlorplatinic acid was treated with an excess of ammonium hydroxide. The ammonium chlorplatinate was collected and dried. Three grams of the ammonium chlorplatinate was used for conversion to the catalyst. The ammonium salt was mixed with thirty grams of sodium nitrate in a casserole. Heat was applied, gently at first, until the rapid evolution of gas slackened and thereupon more strongly until a temperature of 500°C was reached. This operation required about fifteen minutes and there was no splattering. The temperature was maintained at 500-520°C for thirty minutes and the mix-

ture permitted to cool. The catalyst was collected on a Buchner funnel and washed with water until the soluble salts had been extracted. The dried catalyst amounted to 1.5 g. and was chocolate brown in appearance.

The efficacy of the catalyst was first tested by the reduction of maleic acid. 11.6 g. of pure maleic acid, m.p. 129-130°C, dissolved in 150 cc. of 95% alcohol was hydrogenated in an Adams low pressure hydrogenation apparatus at room temperature using 100 mg. platinum oxide catalyst. The solution and the catalyst were introduced in the apparatus which was first evacuated. Hydrogen at a pressure of 35 pounds was allowed to enter. The catalyst was reduced in a few minutes. After thirty minutes the reduction was presumed to be complete. The solvent was evaporated and the residue recrystallized from 15 cc. of boiling water. The yield of succinic acid was 10.5 g. and its melting point was 182-83°C (recorded 185°C).

One hundred milligrams of avocado sterol mixture was dissolved in 50 cc. of pure ethyl acetate. 50 mg. of platinum oxide catalyst was added and the mixture was reduced at 35 pounds pressure for several hours. The product was isolated after the platinum had been removed by filtration by allowing the ethyl acetate to evaporate. The product was recrystallized from ethyl alcohol. 85 mg. of plates was obtained

which melted at 138-140°C and showed an $[\alpha]_D^{25} = +23.0$.

Analysis:* Calcd. for $C_{29}H_{52}O$: C, 83.57; H, 12.57.

Found : C, 83.20; H, 12.16.

One hundred milligrams of the avocado sterol acetate mixture was reduced in 50 cc. of ethyl acetate with 25 mg. platinum oxide catalyst. The product was obtained by the method described above. The melting point of the plates crystallized from ethyl alcohol was 133.5-134.5°C and $[\alpha]_D^{25} = +15.0$.

Saturated sterols and saturated acetates of these sterols, which correspond in properties to stigmastanol and its acetate, were obtained. The properties are compared in the following tabulation:

Substance	Reduction Product		Stigmastanol (72)	
	M.P.	$[\alpha]_D^{25}$	M.P.	$[\alpha]_D$
Sterol mixture	138-140°C	+23.0	140-141°C	+23.5
Acetate of sterol mixture	133.5-134.5°C	+15.0	135-136°C	+15.3

This concludes the experimental study of the intimate sterol mixture from avocado oil. The next step was an investigation of methods for the separation of the mixtures into individual sterols.

* carbon and hydrogen analyses in this research were conducted by the Clark Microanalytical Laboratory, Urbana, Illinois.

Individual Sterols from Sterol Mixtures.

Methods.

Two types of procedure were followed in the separation of the individual sterols which comprised the sterol mixture originally obtained from avocado oil. First, a modification by Bergmann (34) of the classical Windaus (73) method involved a bromination which resulted in the precipitation of tetrabromides while dibromides remained in solution. Second, chromatographic separation involved adsorption of individual sterols upon activated alumina.

In the use of the Bergmann modification of the Windaus procedure it was found essential to use a more concentrated ether solution than Windaus and to cool to 0°C before brominating. In the process, acetates of the sterol mixture were brominated, acetates were regenerated, and sterols were obtained from the regenerated acetates.

One gram of the sterol acetate mixture was dissolved in 7 cc. of anhydrous diethyl ether and cooled by an ice water mixture to 0°C. The cold solution was brominated by 15.7 cc. of a brominating mixture made by dissolving 5 g. of bromine in 100 cc. glacial acetic acid. The solid sterol tetrabromide which separated upon standing was collected. The melting point was 205-208°C. The bromide was treated with an excess of zinc dust and glacial acetic acid

for three hours with refluxing. After filtration the hot solution was treated with water until cloudy. The crystals were filtered and recrystallized from anhydrous alcohol. 55 mg. was obtained. The compound was designated as sterol acetate I. The melting point of sterol acetate I was 138-139°C and the $[\alpha]_D^{25} = -52.5$.

The sterol acetate I was hydrolyzed with an excess of 5% alcoholic potassium hydroxide according to the method described above. The white solid that was obtained was recrystallized from anhydrous ethyl alcohol. It was designated as sterol I. Its melting point was 168-170°C and the $[\alpha]_D^{25} = -43.8$.

Analysis: Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73.

Found : C, 84.27; H, 11.61.

Data are summarized below and compared with properties of stigmasterol (74).

Substance	M.P. °C	$[\alpha]_D$
Sterol I	168-169°	-43.8
Sterol Acetate I	138-139°	-53.5
Stigmasterol	170°	-44.7
Stigmasterol Acetate	140-141°	-55.6

The filtrate from the precipitation of the solid tetrabromide was treated with one gram of zinc dust and 40 cc. of glacial acetic acid for three hours

at the refluxing temperature. The mixture was filtered and the filtrate extracted with several portions of ether. A solid residue was obtained upon removal of the ether and recrystallized from anhydrous ethyl alcohol. The product was designated as sterol acetate II. The melting point of the white crystals which weighed 250 mg. was 126-127°C and the $[\alpha]_D^{25} = -40.3$.

The acetate was hydrolyzed by 5% alcoholic potassium hydroxide. The reaction mixture was refluxed for one hour over a hot plate. The product was obtained by extraction with ether followed by the evaporation of the dried ethereal extract. The crystals were purified by recrystallization from anhydrous ethyl alcohol and designated as sterol II. The melting point of sterol II was 136.5-137.5°C and the $[\alpha]_D^{25} = -35.5$.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15.

Found : C, 83.65; H, 11.92.

Fifty milligrams of sterol II was dissolved in 3 cc. of dry pyridine and treated with an excess of benzoyl chloride. The mixture was heated over a hot plate at a temperature of 100°C for one hour. The dark red solution was poured into water. The reddish brown gummy solid was chilled in a freezing mixture until the mass was sufficiently solid to be filtered. The product which weighed 55 mg. was washed with 5% sodium carbonate, the dried solid

recrystallized from anhydrous acetone and designated sterol benzoate II. The sterol benzoate crystals melted at 146-147°C and the $[\alpha]_D^{25} = -13.0$. Data are summarized below and compared with the properties of β-sitosterol (26).

Substance	M.P.	$[\alpha]_D^{25}$
Sterol II	136.5-137.5°	-35.5
Sterol acetate II	126-127°	-40.3
Sterol benzoate II	146-147°	-13.0
β- sitosterol	136-137°	-36.6
β- sitosterol acetate	125-126°	-41.0
β- sitosterol benzoate	146-147°	-13.8

The second and most productive procedure for separating the avocado sterol mixtures into individual sterols was the Chromatographic Method. Three attempts at chromatographic separation were unsuccessful. In these cases columns were used having dimensions 60 cm. x 1 cm.; the adsorbent was activated alumina from the Aluminum Ore Company; benzene petroleum ether and pure benzene were used as developers; benzene-petroleum ether and ether-benzene-alcohol were applied in elution; the columns were sectioned and sections extracted with ether; and columns were irradiated by ultraviolet light. From these procedures

there was no evidence of separation of sterol mixtures into individual sterols.

Chromatographic separations were attempted under entirely new procedures and proved to be successful. Fischer Alumina purchased from the Fischer Scientific Company especially for adsorption work, mesh 80-200 mm, was used.

A column with dimensions 18 in. x 1 in. was constructed. The end was drawn out and a stopcock attached. The column was packed in the following manner (75): At the bottom was a layer of glass wool. This was covered by a porcelain filter plate disc of the proper size over which was laid an accurately cut circle of filter paper. Since it was not possible to employ an ordinary lubricant for the stopcock, a satisfactory substitute was improvised by making a thick paste from Duz soap powder and a small amount of water. The paste when applied as a thin film to the stopcock worked admirably as a lubricant. The tube with the tap closed was filled completely with benzene. When all the air bubbles had been eliminated, purified sand was poured into the tube in a slow and steady stream until a layer approximately 1 in. in thickness was formed. The layer was covered with another accurately cut circle of filter paper. Alumina was introduced at this point in a fine stream and allowed to settle, a process promoted by gentle tapping until a layer of alumina about 5 in. in

depth was formed. The tap was opened and the benzene allowed to run out. It was poured repeatedly through the column until the alumina was firmly compacted. The column of adsorbent was covered with a third circle of filter paper upon which was placed a small wad of glass wool to prevent disturbance of the column by swirling. Finally, the benzene was allowed to run out until the alumina just remained covered with the solvent: at this point the column, Figure 1, was ready for use.

One gram of the sterol mixture, m.p. 132-133°C and $[\alpha]_D^{25} = -20.0$, was dissolved in 100 cc. of dry benzene. This solution was poured on the column and the solution was allowed to percolate through. The stopcock was opened slightly in order to maintain a flow of 30 drops per minute. Fourteen separate eluates were collected, eluants removed by evaporation, and residues, designated as sterol fractions 1-14, were obtained. The results of the process are compiled in the following tabulation.

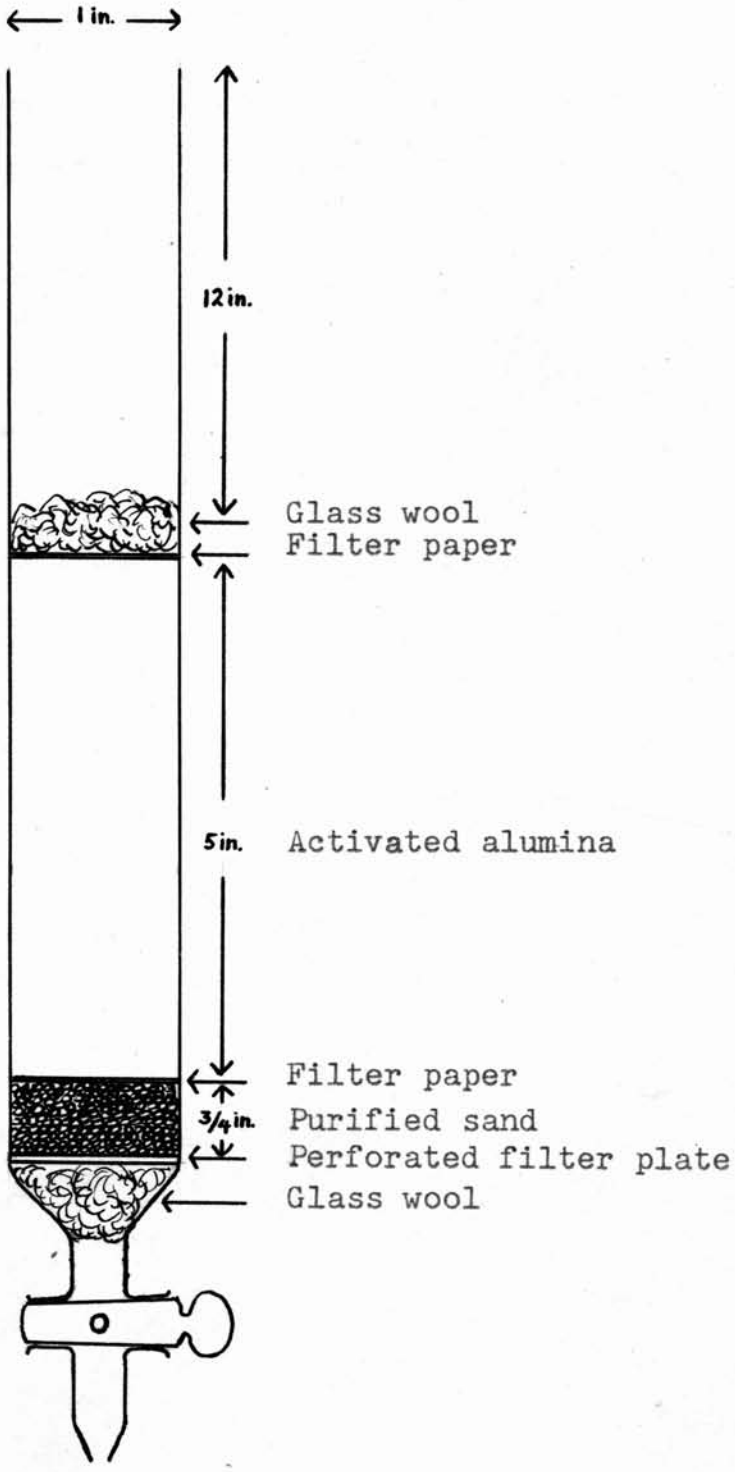


Figure 1. Chromatographic Column.

	Eluate	mg.	m.p.	$[\alpha]_D^{25}$
1.	100 cc. benzene	----	----	----
2.	90 cc. benzene-10 cc. Et_2O	----	----	----
3.	90 cc. benzene-10 cc. Et_2O	----	----	----
4.	90 cc. benzene-10 cc. Et_2O	40	----	----
5.	90 cc. benzene-10 cc. Et_2O	----	----	----
6.	50 cc. benzene-50 cc. Et_2O	----	----	----
7.	25 cc. benzene-75 cc. Et_2O	140	137-38	-35.0
8.	25 cc. benzene-75 cc. Et_2O	640	137-38	-35.5
9.	25 cc. benzene-75 cc. Et_2O	25	124-25	-36.7
10.	100 cc. Et_2O	70	168-69	-43.5
11.	97 cc. Et_2O-3 cc. MeOH	20	168-69	-44.0
12.	97 cc. Et_2O-3 cc. MeOH	----	----	----
13.	90 cc. Et_2O-10 cc. MeOH	----	----	----
14.	25 cc. Et_2O-75 cc. MeOH	----	----	----
		935 mg.		

The six sterol fractions obtained were investigated separately.

Fraction 4 gave a negative Whitby color test and apparently was not a sterol. It was not considered further.

Fractions 7 and 8 were recrystallized from anhydrous ethyl alcohol and well developed crystals were

obtained. Each fraction melted at 137-38°C. The specific rotations were $[\alpha]_D^{25} = -35.0$ and $[\alpha]_D^{25} = -35.5$, respectively. At this point it seemed obvious, as was proven later, that fractions 7 and 8 were the same. Carbon and hydrogen were determined on a combination of the two fractions.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15.

Found : C, 83.38; H, 12.12

Fractions 7 and 8 were acetylated and benzoylated separately and gave identical products.

Fifty milligrams of fraction 7 was acetylated with an excess of acetic anhydride. The acetate when recrystallized from ethyl alcohol yielded 32 mg. of white crystals which melted at 126-127°C and had a specific rotation, $[\alpha]_D^{25} = -40.0$.

Fifty milligrams of fraction 7 was benzoylated with benzoyl chloride in dry pyridine. The product was recrystallized from anhydrous acetone. 55 mg. of crystals in the form of plates was obtained which melted at 147-147.5°C and $[\alpha]_D^{25} = -12.7$. Data are summarized below and the properties compared with β-sitosterol.(26).

Substance	M.P. °C	$[\alpha]_D^{25}$
Fraction 7 sterol	137-38°	-35.0
Fraction 7 sterol acetate	126-27°	-40.0
Fraction 7 sterol benzoate	147-47.5°	-12.7
β-sitosterol	136-37°	-36.6
β-sitosterol acetate	125-26°	-41.0
β-sitosterol benzoate	146-47°	-13.0

Fifty milligrams of fraction 8 was acetylated with an excess of acetic anhydride. The acetate which was obtained after the addition of water to the reaction mixture was recrystallized from anhydrous ethyl alcohol. The product weighed 40 mg. and had a melting point of 126-127°C and a $[\alpha]_D^{25} = -40.2$.

Fifty milligrams of fraction 8 was benzoylated with benzoyl chloride in the manner described above. The benzoate was recrystallized from acetone. The crystals weighed 55 mg.. The product melted at 146.5-147.5°C and a $[\alpha]_D^{25} = -13.1$. Data are summarized below and the properties compared with β-sitosterol.

Substance	M.P. °C	$[\alpha]_D^{25}$
Fraction 9 sterol	137-38°	-35.5
Fraction 8 acetate	126-27°	-40.2
Fraction 8 benzoate	146.5-47.5°	-13.1
β-sitosterol	136-37°	-36.6
β-sitosterol acetate	125-26°	-41.0
β-sitosterol benzoate	146-47°	-13.8

Fraction 9 was recrystallized from absolute alcohol and had a melting point of 124-25°C and a $[\alpha]_D^{25} = -36.7$. The compound gave a positive Whitby test and formed a digitonide with an alcoholic solution of digitonin.

Analysis: Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73.

Found : C, 84.18; H, 11.56

Twenty milligrams of fraction 9 was benzoylated with benzoyl chloride in dry pyridine. 22 mg. of crystals was obtained which when recrystallized from anhydrous acetone melted at 118-119°C. Data are summarized below and the properties compared with fucosterol (46-48).

Substance	M.P. °C	$[\alpha]_D^{25}$
Fraction 9 sterol	124-25°	-36.7
Fraction 9 benzoate	118-19°	----
Fucosterol	124-25°	-38.4
Fucosterol benzoate	119-20°	----

Fractions 10 and 11 which amounted to 90 mg. were recrystallized from ethyl alcohol. The melting point of each fraction and of a mixture of the fractions was 138-39°C. The $[\alpha]_D^{25}$ of fraction 10 was -43.5 and of fraction 11 -44.0. Assuming the two to be the same sterol, they were combined for acetylation.

Analysis: Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73.

Found : C, 84.25; H, 11.48.

The acetate of the combined fractions, 10 and 11, was prepared with acetic anhydride. When recrystallized from ethyl alcohol the crystals melted at 138-39°C and the specific rotation, $[\alpha]_D^{25} = -51.8$. When mixed with a sample of the acetate prepared from Sterol I (see p. 40) no depression of m.p. was observed. Fractions 10 and 11 and Sterol I were shown to be the same. Data are summarized below and the properties compared with stigmasterol (74).

Substance	M.P. °C	$[\alpha]_D^{25}$
Fractions 10 and 11 sterols	168-69°	-43.5
Fractions 10 and 11 acetate	138-39°	-51.8
Stigmasterol	170°	-44.7
Stigmasterol acetate	140-41	-55.6

The sterol mixture from avocado oil was resolved into three individual sterols.

In Table 1 are compiled data for carbon-hydrogen values, melting points, and specific rotations for the individual sterols which were isolated from avocado oil.

Table 1.

Avocado Oil - Individual Sterols

Sterols	Isolated on page	M.P. °C	$[\alpha]_D^{25}$	Carbon		Hydrogen	
				calc.	found	calc.	found
"β"-sitosterol	41	136.5-37.5	-35.5	83.98	83.65	12.15	11.92
stigmasterol	40	168-70	-43.8	84.38	84.27	11.73	11.61
stigmastanol	38	138-40	+23.0	83.57	83.20	12.57	12.16
"β"-sitosterol	46	137-38	-35.0	83.98	83.38	12.15	12.12
fucosterol	48	124-25	-36.7	84.38	84.18	11.73	11.56
stigmasterol	49	168-69	-43.5	84.38	84.25	11.73	11.48

The experimental work, B, C, and D, for chaulmoogra, cocoanut, and macadamia oils followed the same pattern as that for A, avocado oil for which all experimental procedures were presented in much detail. Repetition of such detail was considered unnecessary in the following three sections although care has been taken to avoid omission of essential and pertinent material and all results and data have been presented completely.

B. Chaulmoogra Oil

The chaulmoogra oil had been obtained from P. K. Sen and Sons of India. The partly solid oil was heated until liquified. Aliquot portions of the thoroughly stirred oil were saponified with 20% alcoholic potassium hydroxide solution. The unsaponifiable fraction was obtained as a yellow solid. The yield of the unsaponifiable fraction obtained from several runs of 400 g. averaged 0.2%.

The unsaponifiable fraction was purified by crystallization from low boiling petroleum ether followed by recrystallization from anhydrous ethyl alcohol. Crystalline white solids separated and were filtered off.

The white solids, subsequently referred to as the sterol mixture, were obtained as needles upon recrystallization from anhydrous ethyl alcohol with a melting point of 132-33°C and a $[\alpha]_D^{25} = -21.5$.

Solubility tests demonstrated that the sterol mixture was soluble in such organic solvents as ether, chloroform, acetone, ethyl acetate, petroleum ether, benzene, pyridine, and hot ethyl alcohol.

The sterol mixture gave positive Liebermann-Burchard and Whitby color reactions showing the presence of sterols. The Tortelli-Jaffe and the Rosenheim

reactions were negative indicating the lack of a diter-tiary double bond between "bridge heads" and a conjugated system in the sterol mixture respectively.

The sterol mixture was subjected to certain standard organic reactions that follow:

Acetylation.

Fifty milligrams of the sterol mixture was treated with an excess of acetic anhydride. After recrystallization from ethyl alcohol 50 mg. of crystals was obtained with a melting point of 124-25°C and a $[\alpha]_D^{25} = -27.9$.

Forty milligrams of the sterol acetate mixture was hydrolyzed with 5% alcoholic alkali. The recrystallized (alcohol) free sterol mixture when re-crystallized weighed 30 mg. with a melting point of 132-33°C and with a $[\alpha]_D^{25} = -21.6$.

Benzoylation.

Forty milligrams of the sterol mixture dissolved in dry pyridine was treated with benzoyl chlor-ide. The tan solids were recrystallized from a small vol-ume of dry acetone. 45 mg. of plates was obtained with a melting point of 143-44°C and a $[\alpha]_D^{25} = -10.1$.

Fifty milligrams of the sterol benzoate mixture was hydrolyzed with 5% alcoholic alkali. The sterols when recrystallized from alcohol weighed 30 mg.

with a melting point of 132-33°C and a $[\alpha]_D^{25} = -21.0$.

Test for Unsaturation.

A few milligrams of the sterol acetate mixture dissolved in acetone was able to discharge the color of a dilute solution of potassium permanganate. The discharge of the purple color demonstrated the presence of unsaturation in the sterol nucleus or side chain.

A few milligrams of sterol acetate dissolved in ether decolorized a bromine-glacial acetic acid solution. This was confirmatory proof for the existence of unsaturation in the sterol mixture.

Formation and Cleavage of the Digitonide.

A 1% alcoholic solution of digitonin was added in excess to 0.2 g. of unsaponifiable matter. The precipitate of digitonide was collected, washed with alcohol and ether, and dried at 80°C. The weight of digitonide amounted to 240 mg. From the weight of the digitonide, the percent of sterol in the unsaponifiable fraction was calculated to be 30. The formation of a digitonide demonstrated the configuration of the 3-hydroxyl group to be β.

Two hundred milligrams of the sterol digitonides was cleaved. The sterol mixture which weighed 35 mg. was recrystallized from absolute ethyl alcohol. The melting point was 133-34°C and the $[\alpha]_D^{25} = -21.7$.

Bromination.

Four grams of the sterol mixture was treated with bromine and digitonin according to the method of Schoenheimer in order to effect a separation of the saturated from the unsaturated sterols. The digitonide which was obtained was decomposed with sodium acetate and alcohol. The yield of saturated sterol was 50 mg. which corresponded to 1.25% of the sterol mixture. Following recrystallization from ethyl alcohol the melting point was 136-37°C and the $[\alpha]_D^{25} = +23.4$. The data are compiled below and the properties compared with stigmastanol (72).

Analysis: Calcd. for $C_{29}H_{52}O$: C, 83.57; H, 12.57.
Found : C, 83.64; H, 12.28

Substance	M.P. °C	$[\alpha]_D$
Sterol	136-37	+23.4
Stigmastanol	140-41	+23.5

Diels-Alder Reaction.

The sterol mixture was treated with maleic anhydride in xylene by the procedure that has been described for the sterol mixture of avocado. No adduct was isolated from the reaction mixture. The solids which were isolated were identified as the original sterol mixture (m.p. 131-133°C) and maleic acid (m.p. 129-30°C). Consequently the conjugated system was not present in the

sterol mixture.

Catalytic Hydrogenation.

Fifty milligrams of the sterol mixture was dissolved in 50 cc. pure ethyl acetate. After the addition of 25 mg. platinum oxide catalyst, the mixture was reduced at 30 pounds pressure. The product obtained by the evaporation of the filtered solution was recrystallized from alcohol. The crystals weighed 37 mg. and melted at 137-38°C. The $[\alpha]_D^{25}$ = +23.1.

Analysis: Calcd. for $C_{29}H_{52}O$: C, 83.57; H, 12.57.

Found : C, 83.51; H, 12.37.

Seventy five milligrams of the sterol mixture was dissolved in 50 cc. pure ethyl acetate and was reduced with hydrogen under the catalytic influence of 35 mg. platinum oxide. When recrystallized from absolute alcohol, the product weighed 43 mg. and had a melting point of 133-34°C. The $[\alpha]_D^{25}$ = +14.0. Data are compiled and compared with the properties of stigmastanol (72) in the following tabulation.

Substance	M.P. °C	$[\alpha]_D^{25}$
Sterol mixture	137-38	+23.1
Acetate of sterol mixture	133-34	+14.0
Stigmastanol	140-41	+23.5
Stigmastanol acetate	135-36	+15.3

The two types of procedure, the Windaus bromination and chromatography, used for the separation of the individual sterols which comprised the sterol mixture of avocado oil were followed for the separation of the sterols in chaulmoogra.

The unsaturated sterols not affected by digitonin were obtained from the filtrate by debromination with zinc and glacial acetic acid after the saturated sterol digitonide had been removed by filtration (page 54). The weight of unsaturated sterols obtained was 1.8 g. The unsaturated sterols were converted to the acetates with acetic anhydride which were brominated according to the method of Windaus.

One gram of the sterol acetate mixture was dissolved in 10 cc. anhydrous diethyl ether. The cooled solution was brominated with 15.77 cc. of a brominating mixture made by dissolving 5 g. of bromine in 100 cc. glacial acetic acid. The tetrabromide which separated was treated with zinc dust and glacial acetic acid. The product when recrystallized from alcohol weighed 40 mg. The melting point was 138-38.5°C and the $[\alpha]_D^{25} = -54.6$.

Forty milligrams of the doubly unsaturated sterol was hydrolyzed with 5% alcoholic potassium hydroxide. The recrystallized sterol weighed 32 mg. and had a melting point of 167-68°C and a $[\alpha]_D^{25} = -43.5$. The data are compiled and compared with the properties of

stigmasterol (74) in the following tabulation.

Analysis: Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73.
Found : C, 84.52; H, 11.84.

Substance	M.P. °C	$[\alpha]_D$
Sterol	167-68	-43.5
Sterol acetate	138-38.5	-54.6
Stigmasterol	170	-44.7
Stigmasterol acetate	140-41	-55.6

The filtrate from the Windaus bromination procedure described above was treated with one gram of zinc dust and 40 cc. glacial acetic acid. The debrominated sterol acetate was recrystallized from ethyl alcohol. The white crystals weighed 350 mg. and melted at 124.5-25.5°C. The $[\alpha]_D^{25} = -39.9$.

One hundred milligrams of the acetate was hydrolyzed with an excess of 5% alcoholic potassium hydroxide. The product weighed 70 mg.. The melting point was 135.5-136.5°C and the $[\alpha]_D^{25} = -35.6$.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15
Found : C, 84.08; H, 11.79

Fifty milligrams of the product of hydrolysis was dissolved in 4 cc. dry pyridine. The solution was treated with an excess of benzoyl chloride at 100°C for one hour. The reaction mixture was added to water and

the brown solid collected. This was recrystallized from a small volume of dry acetone. The yield amounted to 55 mg. and had a melting point of 146-46.5°C and an $[\alpha]_D^{25} = -13.1$. Data are compiled and compared with the properties of β-sitosterol (26) below.

Substance	M.P. °C	$[\alpha]_D$
Sterol	135.5-36.5	-35.6
Sterol acetate	124.5-25.5	-39.9
Sterol benzoate	146-46.5	-13.1
β-sitosterol	136-37	-36.6
β-sitosterol acetate	125-26	-41.0
β-sitosterol benzoate	146-47	-13.8

The column employed in the chromatography was similar to the one used in the separation of the sterols of avocado oil.

Eight hundred and fifty milligrams of the crude sterol mixture was dissolved in 100 cc. dry benzene and the solution allowed to percolate through the activated alumina. Seventeen separate eluates were collected, eluants removed by evaporation, and residues, designated as sterol fractions 1-17, were obtained. The results of the process are compiled in the following tabulation.

	Eluant	Mg. obtained	M.P. °C	$[\alpha]_D^{25}$
1.	100 cc. benzene	yellow oil	----	
2.	100 cc. benzene	yellow oil	----	
3.	100 cc. benzene	23	139.5-140	+22.8
4.	97 cc. benzene-3 cc. Et_2O	----	----	
5.	90 cc. benzene-10 cc. Et_2O	300	136-137	-35.0
6.	90 cc. benzene-10 cc. Et_2O	150	136-136.5	-36.5
7.	90 cc. benzene-10 cc. Et_2O	30	136.5-137.5	-35.1
8.	50 cc. benzene-50 cc. Et_2O	oil	----	
9.	50 cc. benzene-50 cc. Et_2O	traces	----	
10.	25 cc. benzene-75 cc. Et_2O	35	167-168	-44.0
11.	25 cc. benzene-75 cc. Et_2O	33	166-167	-43.9
12.	25 cc. benzene-75 cc. Et_2O	10	166.5-167.5	
13.	100 cc. Et_2O	oil	----	
14.	97 cc. Et_2O-3 cc. MeOH	traces	----	
15.	50 cc. Et_2O-50 cc. MeOH	traces	----	
16.	25 cc. Et_2O-75 cc. MeOH	130	non sterol	
17.	100 cc. MeOH	70	non sterol	
		581 mg. (sterol)		

Fraction 3 reacted positively toward the Whitby color test and negatively to the Liebermann-Burchard color test. When recrystallized from alcohol 20 mg. of white crystals were obtained. The melting point was 139.5-140°C and the $[\alpha]_D^{25}$ = +22.8. These constants are compared with the comparable constants for stigmastanol (72).

Substance	M.P. °C	$[\alpha]_D$
Sterol	139.5-40	+22.8
Stigmastanol	140-41	+23.5

Analysis: Calcd. for $C_{29}H_{52}O$: C, 83.51; H, 12.57.
Found : C, 83.77; H, 12.27.

Fractions 5, 6 and 7 had the melting points 136-37°C, 136-36.5°C, and 136.5-37°C and the $[\alpha]_D^{25}$ of -35.0, -36.5, amd -35.1, respectively. It seemed obvious that these three fractions were identical. Consequently they were combined.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15.
Found : C, 83.52; H, 11.83.

Seventy five milligrams of the combined fractions was acetylated with an excess of acetic anhydride. The product when recrystallized from alcohol weighed 80 mg. and melted at 126-27°C and $[\alpha]_D^{25}$ = -40.7.

Sixty milligrams of the acetate obtained above was hydrolyzed by 5% alcoholic alkali. The product was recrystallized from alcohol and weighed 40 mg. and melted at 137-38°C and $[\alpha]_D^{25}$ = -36.2.

Seventy five milligrams of the combined fractions was dissolved in 4 cc. dry pyridine and treated with benzoyl chloride. The tan solids obtained by pouring the reaction mixture into water was recrystallized from a small volume of anhydrous acetone. 90 mg. was obtained. The melting point was 146-146.5°C and the $[\alpha]_D^{25} = -13.5$.

A comparison of the constants of Fractions 5, 6 and 7 with the comparable constants for β-sitosterol (26) follows.

Substance	M.P. °C	$[\alpha]_D$
Sterol (5, 6, 7)	137-38	-36.2
Sterol acetate	126-27	-40.7
Sterol benzoate	145-45.5	-13.3
β-sitosterol	136-37	-36.6
β-sitosterol acetate	125-26	-41.0
β-sitosterol benzoate	146-47	-13.8

Fractions 10, 11, and 12 reacted positively to the Whitby color test. The fractions were recrystallized from ethyl alcohol and specific rotations determined. Fractions 10, 11, and 12 melted at 167-68°C, 166-67°C, and 166.5-67.5°C and had an $[\alpha]_D^{25} = -44.0$, -43.9, and ----, respectively. It seemed obvious that the three fractions were identical and they were combined.

Analysis: Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73.

Found : C, 84.44; H, 11.83.

Fifty milligrams of the combined fractions was acetylated with acetic anhydride in the usual way. The product was recrystallized from ethyl alcohol. The white crystals weighed 40 mg. The melting point was 138-39°C and the $[\alpha]_D^{25} = -55.0$. Data are compiled and compared with the properties of stigmasterol (74) as follows.

Substance	M.P. °C	$[\alpha]_D$
Sterol (10,11,12)	167-68	-44.0
Sterol acetate	138-39	-55.0
Stigmasterol	170	-44.7
Stigmasterol acetate	140-41	-55.6

Fractions 16 and 17 whose total weight was 100 mg. were treated separately by the Whitby color test. Both tests were negative. These solids were waxy substances and were eluted last with a relatively polar mixture of ether and methanol. No further study was made.

The sterol mixture from chaulmoogra oil was resolved into three individual sterols.

In Table 2 are compiled data for carbon-hydrogen values, melting points, and specific rotations for the individual sterols which were isolated from chaulmoogra oil.

Table 2

Chaulmoogra Oil - Individual Sterols

Sterols	Isolated on page	M.P. °C	$[\alpha]_D^{25}$	Carbon		Hydrogen	
				calc.	found	calc.	found
stigmastanol	56	136-37	+23.4	83.57	83.64	12.57	12.28
stigmastanol	62	137-38	+23.1	83.57	83.51	12.57	12.37
"β"-sitosterol	59	135.5-36.5	-35.6	83.98	84.08	12.15	11.79
stigmasterol	59	167-68	-43.5	84.38	84.52	11.73	11.84
stigmastanol	62	139.5-40	+22.8	83.57	83.77	12.57	12.27
"β"-sitosterol	62	136-37	-35.0	83.98	83.52	12.15	11.83
stigmasterol	63	167-68	-44.0	84.38	84.44	11.73	11.83

C. Cocoanut Oil.

The cocoanut oil was purchased from Magnus, Maybee, and Reynard, Inc. of New York City. The unsaponifiable fraction was isolated by the method that has been described above. The content of unsaponifiable matter based on several runs averaged 0.4%. This fraction, however, unlike the unsaponifiable matter from chaulmoogra oil and avocado oil was not solid at room temperature. Samples placed in a refrigerator solidified readily but reverted to oils on being exposed to the temperature of the room.

Attempts were made to secure crystalline compounds from the unsaponifiable fractions by recrystallization from low boiling petroleum ether and ethyl alcohol. There was difficulty in securing crystals of good quality by these methods. The melting point of the solids that were finally obtained covered a wide range from 110-120°C and had an $[\alpha]_D^{25} = -15.6$.

Solubility tests demonstrated that the sterol mixture was soluble in such organic solvents as ether, chloroform, acetone, ethyl acetate, petroleum ether, benzene, pyridine, and hot ethyl alcohol.

The sterol mixture was subjected to the four color tests that have been described previously. The Liebermann-Burchard and Whitby color tests were positive showing the presence of sterols. The Rosenheim and Tortelli-Jaffe tests were negative indicating the lack of ditertiary

double bond between "bridge heads" and a conjugated system in the sterol mixture respectively.

Fifty milligrams of the sterol mixture was acetylated with acetic anhydride. The solids were recrystallized from alcohol. 42 mg. were obtained of melting point 125-27°C and an $[\alpha]_D^{25} = -30.5$.

Fifty milligrams of the sterol mixture dissolved in a few cc. of dry pyridine was treated with benzoyl chloride. The reaction mixture was poured into a mixture of ice and water. The resulting yellow oil did not solidify after standing in a refrigerator for a week. The oil was taken up by low boiling petroleum ether. Upon the spontaneous evaporation of the petroleum ether the benzoates were obtained as white crystals with a slight yellowish tinge. The benzoates were recrystallized from a small volume of anhydrous acetone. 50 mg. were obtained with a melting point of 143-44°C and a $[\alpha]_D^{25} = -9.7$.

Forty five milligrams of the benzoates was hydrolyzed with 5% alcoholic alkali. The product when recrystallized from alcohol weighed 32 mg. and melted at 127-28°C. The $[\alpha]_D^{25} = -21.2$.

A few milligrams of the sterol acetate mixture in ether decolorized a solution of bromine in glacial acetic acid. This demonstrated the presence of unsaturation in the nucleus or side chain of the sterol mixture.

A 1% alcoholic solution of digitonin made up as described above was added in excess to 0.2 g. of unsaponifiable matter. The precipitate of digitonide which formed was collected, washed with alcohol and ether and dried. The weight of digitonide amounted to 210 mg. On the basis of the weight of digitonide the percent of sterol in the unsaponifiable fraction was calculated to be 26.3.

200 mg. of the digitonide was cleaved according to the recommendations of Westgate. The free sterols so obtained weighed 37 mg. and melted between 125-27°C. The $[\alpha]_D^{25} = -19.5$.

Two grams of the crude sterol mixture was treated with bromine and digitonin according to the method of Schoenheimer in order to effect a separation of the saturated from the unsaturated sterols. The 200 mg. of digitonide which separated was cleaved immediately with sodium acetate and alcohol. The sterols which were obtained weighed 40 mg. and represented the stanol or saturated fraction of the sterols present. The weight obtained corresponds to 2% of the total sterols isolated. The stanol fraction was recrystallized from alcohol and melted between 130-133°C and had an $[\alpha]_D^{25} = +20.0$. The wide melting point range indicated a mixture of saturated forms.

The sterol mixtures dissolved in xylene was treated with maleic anhydride by the procedure that has been described above. No adduct could be detected but the

original sterol mixture (m.p. 127-28°C) and maleic acid (m.p. 128-29°C) were isolated. Therefore, the conjugated system was not present in the sterol mixture.

One hundred milligrams of the sterol mixture was dissolved in 50 cc. pure ethyl acetate. 50 mg. of platinum oxide was added to the solution and the reaction mixture was reduced at 30 pounds pressure. The product obtained by the evaporation of the filtered reaction mixture was recrystallized from alcohol. The weight of solids obtained was 63 mg. and melted between 127-31°C and had an $[\alpha]_D^{25} = +19.6$. Recrystallization failed to reduce the rather wide melting point range which indicated a mixture.

The two types of procedure used for the separation of the individual sterols in avocado and chaulmoogra oil were followed for the separation of the individual sterols in cocoanut oil.

The unsaturated sterols were obtained from the filtrate of the Schoenheimer procedure[14] by debromination with zinc and glacial acetic acid. After recrystallization the white crystals weighed 1.3 g. and melted at 138-40°C. The $[\alpha]_D^{25} = -39.0$.

One gram of the crude acetates was dissolved in 12 cc. dry ether and the cold solution brominated with a brominating mixture of bromine and glacial acetic acid prepared as described above. No tetra bromide which

would correspond to a doubly unsaturated sterol was precipitated. The solution was treated with zinc dust and glacial acetic acid. The debrominated sterol acetates were recrystallized from ethyl acetate. 600 mg. of crystals was obtained. The crystals melted at 131-32.5°C and had an $[\alpha]_D^{25} = -43.4$.

One hundred milligrams of the sterol acetates was hydrolyzed by treatment with 5% alcoholic alkali. The product when recrystallized from alcohol weighed 76 mg., melted between 139-40.5°C, and had an $[\alpha]_D^{25} = -39.9$. It was not possible to determine whether the product was homogeneous or a mixture of monounsaturated sterols. Consequently the sterol mixture was subjected to chromatographic analysis.

One gram of the crude sterol mixture was dissolved in 100 cc. dry benzene and the solution allowed to percolate through the alumina column. The column was eluted with solvents and solvent mixtures in the order shown. Eighteen separate eluates were collected, eluants removed by evaporation, and residues, designated as sterol fractions 1-18, were obtained. The results are compiled in the following tabulation.

Eluant	Mg. obtained	M.P. °C	$[\alpha]_D^{25}$
1. 100 cc. C_6H_6	----	----	----
2. 100 cc. C_6H_6	----	----	----
3. 100 cc. C_6H_6	35	129-31	+ 21.7
4. 100 cc. C_6H_6	trace	----	----
5. 97 cc. C_6H_6- 3 cc.Et_2O	----	----	----
6. 90 cc. C_6H_6- 10 cc.Et_2O	100	136-37	-36.4
7. 90 cc. C_6H_6- 10 cc.Et_2O	175	136.5-37	-36.1
8. 90 cc. C_6H_6- 10 cc.Et_2O	150	136-37	-36.9
9. 75 cc. C_6H_6- 25 cc.Et_2O	10	136-37	----
10. 75 cc. C_6H_6- 25 cc.Et_2O	----	----	----
11. 50 cc. C_6H_6- 50 cc.Et_2O	40	144-45	-41.7
12. 25 cc. C_6H_6- 75 cc.Et_2O	100	144-45	-42.0
13. 25 cc. C_6H_6- 75 cc.Et_2O	30	143-44	-42.5
14. 100 cc.Et_2O	trace	----	----
15. 97 cc. Et_2O- 3 cc. MeOH	55	51-52	----
16. 90 cc. Et_2O-10 cc. MeOH	30	51-52	----
17. 50 cc. Et_2O-50 cc. MeOH	50	52-53	----
18. 50 cc. Et_2O-50 cc. MeOH	10	51-52	----
	640 mg.(sterol)		

Fraction 3 was recrystallized from ethyl alcohol, and melted between 129-31°C and had a $[\alpha]_D^{25}$= +21.7. This fraction was positive to the Whitby color test and gave a negative Liebermann-Burchard color test but on

standing a faint color did appear. This suggested that the saturated fraction was contaminated by unsaturated forms.

Fractions 6, 7, and 8 were recrystallized separately. The melting points of fractions 6, 7, and 8 were 136-37°C, 136.5-37.5°C, and 136-37°C and $[\alpha]_D^{25}$ were -36.4, -36.1, and -36.9, respectively. Each of these fractions reacted positively to the Whitby color test. On the basis of their physical constants the three fractions were combined.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15 .
Found : C, 83.51; H, 12.10.

Fifty milligrams of the combined fractions was acetylated with an excess of acetic anhydride in the usual way. When recrystallized from alcohol 45 mg. was obtained. The melting point was 124-25°C and the $[\alpha]_D^{25} = -40.6$.

Fifty milligrams of the combined fractions was converted to the benzoate by treatment with benzoyl chloride in a solvent of anhydrous pyridine. Following recrystallization from anhydrous acetone the crystals weighed 50 mg. melted 145-46°C, and had an $[\alpha]_D^{25} = -13.5$. Data are compiled and compared with the properties of β-sitosterol (26) in the following tabulation.

Substance	M.P. °C	$[\alpha]_D$
Sterol (6,7,8)	136-37	-36.4
Sterol acetate	124-25	-40.6
Sterol benzoate	145-46	-43.5
β-sitosterol	136-37	-36.6
β-sitosterol acetate	125-26	-41.0
β-sitosterol benzoate	146-47	-13.8

Fractions 11, 12, and 13 reacted positively to the Whitby color test. Following recrystallization from alcohol, the melting point and specific rotation of each fraction was determined to be 144-45°C, 144-45°C, 143-44°C, and $[\alpha]_D^{25}$ to be -41.7, -42.0, and -42.5 respectively. The fractions were combined.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15.

Found : C, 83.52; H, 12.06.

Seventy five milligrams of the combined fractions was acetylated with an excess of acetic anhydride in the usual way. The product when recrystallized from alcohol weighed 58 mg., melted between 141-42°C, and had an $[\alpha]_D^{25}$ = -45.8.

Fifty milligrams of the combined fractions was treated with benzoyl chloride. The product which was worked up in the usual way and recrystallized from acetone weighed 55 mg. and melted between 148-49°C. The $[\alpha]_D^{25}$ -19.5.

Data are compiled and compared with the properties of γ-sitosterol (23) in the following tabulation.

Substance	M.P. °C	$[\alpha]_D$
Sterol (11,12,13)	144-45	-42.0
Sterol acetate	141-42	-45.8
Sterol benzoate	148-49	-19.5
γ-sitosterol	145-46	-42.4
γ-sitosterol acetate	143-44	-46.1
γ-sitosterol benzoate	152	-20.0

Fractions 15, 16, 17, and 18 were recrystallized from ethyl alcohol. Each of the fractions purified in this way was definitely crystalline in character. The melting points of the four fractions were approximately the same, ranging from 51-53°C. Each fraction did not respond to the Whitby color test. They were not characterized.

The sterol mixture from cocoanut oil was resolved into a saturated sterol fraction and two individual sterols.

In Table 3 are compiled data for carbon-hydrogen values, melting points, and specific rotations for the individual sterols which were isolated from cocoanut oil.

Table 3.

Cocoanut Oil - Individual Sterols

Sterols	Isolated on page	M.P. °C	$[\alpha]_D^{25}$	Carbon		Hydrogen	
				calc.	found	calc.	found
"β" -sitosterol	72	136.5-37.5	-36.1	83.98	83.51	12.15	12.10
"γ" -sitosterol	73	144-45	-42.0	83.98	83.52	12.15	12.06

D. Macadamia Oil

The oil was obtained from Grade 1 macadamia nuts which were purchased from the Hawaiian Macadamia Nut Company of Honolulu. The oil was expressed by means of a Carver press by pressure which was applied at room temperature. Approximately 2500 cc. was obtained from fourteen pounds of nuts. The macadamia nut oil is light brown and opaque. It is completely liquid both at room temperature and at the temperature of refrigeration.

The unsaponifiable fraction was obtained in the usual way by the saponification of 400 g. lots of oil with 20% alcoholic potassium hydroxide followed by extraction of the diluted soap solution with commercial ether. The water white ethereal extracts were washed and dried. The unsaponifiable portion was obtained by the removal of the solvent. The greenish-yellow solids had a slight tendency to liquefy particularly when the weather was warm. On the basis of several runs of 400 g. the average yield of unsaponifiable matter in the oil was 0.3%.

The yellow solids were dissolved in a minimum amount of low boiling petroleum ether and the filtered yellow solution placed in an ice box. The solids which separated were recrystallized from anhydrous alcohol and had a melting point of 111-119°C and an $[\alpha]_D^{25} = -14.3$.

Recrystallization did not reduce the wide melting point range markedly.

Solubility tests were carried out with the standard organic solvents. The results indicated that the solids were soluble in the common organic solvents but the solubility in such solvents as petroleum ether and ethyl alcohol was greater than was the case for the other sterol mixtures examined previously. The sterols were obtained as needles when recrystallized from anhydrous ethyl alcohol.

The sterol mixture gave positive Liebermann-Burchard and Whitby color reactions showing the presence of sterols. The Tortelli-Jaffe and the Rosenheim reactions were negative indicating the lack of ditertiary double bond between "bridge heads" and a conjugated system in the sterol mixture respectively.

Sixty milligrams of the sterol mixture was acetylated with acetic anhydride. The solids resembling plates when recrystallized from ethyl alcohol weighed 50 mg. and melted between 125-28°C. The $[\alpha]_D^{25} = -19.5$.

Fifty milligrams of the sterol mixture dissolved in a few cc. dry pyridine was treated with benzoyl chloride. The reaction mixture was poured into cooled water. The resulting yellow oil did not solidify on long standing in the refrigerator. The oil was taken up with five cubic centimeters of low boiling petroleum

ether and the petroleum ether allowed to evaporate spontaneously. Stoutish plate-like crystals were obtained that weighed 60 mg., melted at 103-104°C, and had an $[\alpha]_D^{25} = -10.5$.

A solution of the sterol acetate mixture in acetone decolorized a dilute solution of potassium permanganate. The discharge of the purple color demonstrated the presence of unsaturation in the sterol nucleus or side chain. A solution of the sterol acetate mixture in ether decolorized a solution of bromine in glacial acetic acid. This confirmed the existence of unsaturation in the sterol mixture.

A 1% alcoholic solution of digitonin was added to 0.2 g. of unsaponifiable matter. The digitonide was collected, washed with ether and dried. The weight of digitonide was 300 mg. The percent of sterols in the unsaponifiable matter based on the weight of the digitonides was calculated to be 37.5. 250 mg. of the digitonide was cleaved in the usual way. The sterol mixture when recrystallized from alcohol weighed 42 mg. and melted between 128-30°C. The specific rotation, $[\alpha]_D^{25} = -22.1$.

Two grams of the sterol mixture was dissolved in 400 cc. of warm ethyl alcohol. The solution was treated with normal alcoholic bromine and digitonin according to Schoenheimer. The digitonides which were formed weighed 350 mg. The digitonides were cleaved

immediately with sodium acetate in alcohol. The stanol fraction so obtained weighed 60 mg. This corresponded to 3% of the sterol mixture. The melting point was 126-30°C and the $[\alpha]_D^{25} = +9.5$. The rather wide melting point range indicated a mixture of forms.

No adduct was obtained when 100 mg. of the sterol mixture was treated with maleic anhydride in xylene. The sterol mixture did not contain the conjugated system. of double bonds.

One hundred milligrams of the sterol mixture dissolved in 50 cc. ethyl acetate was reduced with 100 mg. platinum oxide and hydrogen. The product was recrystallized several times from ethyl alcohol. The crystals weighed 55 mg., melted between 131-34°C, and had an $[\alpha]_D^{25} = +27.5$. Since recrystallizations did not reduce the melting point range, it was concluded that the reduction product consisted of a mixture of saturated forms.

The saturated sterol fraction had been removed as digitonide according to the method of Schoenheimer. The unsaturated sterols were obtained from the resulting filtrate and were separated by the method of Windaus into two mixtures, designated as A and B, the former a mixture of di-unsaturated sterols and the latter a mixture of mono-unsaturated sterols. Both mixtures were subjected to chromatography.

One gram of the crude acetates dissolved in 15 cc. dry ether was brominated with a brominating mixture of bromine and glacial acetic acid after the method of Windaus. The tetra bromides which were formed and which melted over a wide range (m.p. 160-75°C) were treated with zinc and glacial acetic acid. The mixture of sterol acetates containing two double bonds weighed 360 mg., melted between 130-35°C, and had an $[\alpha]_D^{25} = -21.1$.

Three hundred and thirty milligrams of the acetate mixture was hydrolyzed by 5% alcoholic potassium hydroxide. The white crystals when recrystallized from alcohol weighed 230 mg., melted between 150-55°C, and had an $[\alpha]_D^{25} = -23.4$. This portion was designated as A.

The column employed was similar to the one described above with the exception that the volume of eluants employed was 50 cc. instead of 100 cc. The method of analysis in other respects was similar to those described.

Two hundred milligrams of the unsaturated sterol mixture designated as A isolated above was dissolved in 50 cc. pure, dry benzene and the solution allowed to percolate through the alumina. Eleven separate eluates were collected, eluants removed by evaporation, and residues, designated as sterol fractions 1-11, were obtained. The results of the procedure are compiled in the following tabulation.

Analysis of A

	Eluant	Mg. obtained	M.P. °C	$[\alpha]_D^{25}$
1.	50 cc. C_6H_6-(soln.)	----	----	----
2.	50 cc. C_6H_6	----	----	----
3.	50 cc. C_6H_6	----	----	----
4.	48 cc. C_6H_6-2 cc. Et_2O	trace	----	----
5.	45 cc. C_6H_6-5 cc. Et_2O	5	158-62	----
6.	45 cc. C_6H_6-5 cc. Et_2O	90	166-68	-43.7
7.	45 cc. C_6H_6-5 cc. Et_2O	2.5	140-43	----
8.	25 cc. C_6H_6-25 cc. Et_2O	5	146-49	----
9.	20 cc. C_6H_6-30 cc. Et_2O	75	163-64	-2.3
10.	20 cc. C_6H_6-30 cc. Et_2O	trace	----	----
11.	50 cc. Et_2O	----	----	----
		178 mg.		

No optical rotations could be determined for fractions 4, 5, 7, 8, and 10 due to lack of sufficient material.

Fifty milligrams of fraction 6 was acetylated with acetic anhydride and yielded an acetate which weighed 55 mg., melted at 137-38°C, and had an $[\alpha]_D^{25} = -53.7$. Data are compiled and compared with the properties of stigmasterol (74) in the following tabulation.

Analysis: Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73

Found : C, 84.35; H, 11.99

Substance	M.P. °C	$[\alpha]_D$
Sterol fraction 6	166-68	-43.7
Sterol acetate of fraction 6	137-38	-53.7
Stigmasterol	170	-44.7
Stigmasterol acetate	140-41	-55.6

Fraction 9 when recrystallized from alcohol, melted between 163-64°C, and had an $[\alpha]_D^{25} = -2.3$. This fraction was acetylated with acetic anhydride and recrystallized from alcohol. The crystals melted at 135-36°C and had an $[\alpha]_D^{25} = +27.9$. Data are compiled and compared with the properties of α_1-sitosterol below (23).

Analysis: Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73.

Found : C, 83.98; H, 11.77.

Substance	M.P. °C	$[\alpha]_D^{25}$
Sterol fraction 9	163-64	-2.3
Sterol acetate of fraction 9	135-36	+ 27.9
α_1-sitosterol	166	-1.7
α_1-sitosterol acetate	137	+ 29.0

The filtrate from the Windaus bromination which contained the sterol dibromides was debrominated with zinc and glacial acetic acid. The sterol acetates that were isolated weighed 550 mg. after recrystallization from alcohol, melted at 130-34°C, and had an $[\alpha]_D^{25} = -42.5$.

The mono-unsaturated sterol mixture was obtained from the acetates by treatment with 5% alcoholic potassium hydroxide. From 500 mg. of acetates 300 mg. of the sterol mixture was obtained. It melted at 137-40°C, and had an $[\alpha]_D^{25} = -38.5$. This portion was designated as B.

Using the column and procedure described briefly above, 200 mg. of the sterol mixture designated as B was dissolved in 50 cc. dry benzene and allowed to percolate through the column of alumina. Eleven separate eluates were collected, eluants removed by evaporation, and residues, designated as sterol fractions 1-11, were obtained. The results follow.

Analysis of B

Eluant	Mg. obtained	M.P. °C	$[\alpha]_D^{25}$
1. 50 cc. C_6H_6(soln.)	----	----	----
2. 50 cc. C_6H_6	----	----	----
3. 49 cc. C_6H_6- 1 cc. Et_2O	trace	----	----
4. 45 cc. C_6H_6- 5 cc. Et_2O	5	133-34	----
5. 45 cc. C_6H_6- 5 cc. Et_2O	35	136-37	-35.8
6. 45 cc. C_6H_6- 5 cc. Et_2O	50	136-37	-35.5
7. 45 cc. C_6H_6- 5 cc. Et_2O	trace	----	----
8. 25 cc. C_6H_6-25 cc. Et_2O	10	140-42	----
9. 25 cc. C_6H_6-25 cc. Et_2O	75	144-45	-42.1
10. 25 cc. C_6H_6-25 cc. Et_2O	4	143-44	----
11. 50 cc. Et_2O	----	----	----
	180 mg.		

Fractions 4, 8, and 10 could not be characterized due to lack of material.

Fractions 5 and 6 both melted at 136-37°C and had $[\alpha]_D^{25}$ of -35.8 amd -35.5 respectively. Therefore, the two fractions were combined.

Thirty milligrams of the combined fractions yielded 32 mg. of an acetate by acetylation with acetic anhydride. The crystals melted at 125.5-26.5°C and had an $[\alpha]_D^{25}$ = -40.4.

By treatment of 30 mg. of the combined fractions with benzoyl chloride, 35 mg. of the benzoate, which melted at 145-46°C and had an $[\alpha]_D^{25}$ = -13.1, were obtained. Data are compiled and compared with the properties of β-sitosterol (26) below.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15.

Found : C, 83.62; H, 12.17.

Substance	M.P. °C	$[\alpha]_D$
Sterol (5 and 6)	136-37	-35.7
Sterol acetate	125.5-26.5	-40.4
Sterol benzoate	145-46	-13.1
β-sitosterol	136-37	-36.6
β-sitosterol acetate	125-26	-41.0
β-sitosterol benzoate	146-47	-13.8

Thirty five milligrams of fraction 9 was acetylated with acetic anhydride. When recrystallized from ethyl alcohol, 35 mg. was obtained. The melting point

was 139-41°C and the $[\alpha]_D^{25} = -46.1$.

Fraction 9 formed a benzoate with benzoyl chloride which weighed 30 mg., melted at 148-49.5, and had an $[\alpha]_D^{25} = -19.4$. Data are compiled and compared with the properties of γ-sitosterol (23) in the following tabulation.

Analysis: Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.15.

Found : C, 83.59; H, 11.94.

Substance	M.P. °C	$[\alpha]_D$
Sterol fraction 9	144-45	-42.1
Sterol acetate of fraction 9	139.5-41	-46.1
Sterol benzoate of fraction 9	148-49.5	-19.4
γ-sitosterol	145-46	-42.4
γ-sitosterol acetate	143-44	-46.1
γ-sitosterol benzoate	152	-20.0

The sterol mixture from macadamia oil was resolved into a small stanol fraction and four individual sterols.

In Table 4 are compiled data for carbon-hydrogen values, melting points, and specific rotations for the individual sterols which were isolated from macadamia oil.

Table 4.

Macadamia oil - Individual sterols

Sterols	Isolated on page	M.P. °C	$[\alpha]_D^{25}$	Carbon		Hydrogen	
				calc.	found	calc.	found
stigmasterol	81	166-68	-43.7	84.38	84.35	11.73	11.99
"α_1"-sitosterol	82	163-64	- 2.3	84.38	83.98	11.73	11.77
"β"-sitosterol	84	136-37	-35.5	83.98	83.62	12.15	12.17
"γ"-sitosterol	85	144-45	-42.1	83.98	83.59	12.15	11.94

In Table 5 is compiled a summary of data for carbon-hydrogen, melting point, and specific rotation for the individual sterols that have been isolated from the four tropical oils.

Table 5

Oils	Sterols	M.p. °C	$[\alpha]_D^{25}$	Carbon calc.	Carbon found	Hydrogen calc.	Hydrogen found
		136.5-137.5	-35.7				11.92
	β-sitosterol	137-138	-35.6	83.28	83.62	12.15	12.12
AVOCADO	fucosterol	144-145	-36.7	84.38	84.10	11.23	11.56
		167-170	-43.5		84.27		11.51
	stigmasterol	168-169	-43.7	84.38	84.35	11.23	11.48
	stigmasterol	136-137	+33.4		83.34		12.28
		132.5-140	+29.8	83.57	83.27	12.52	12.27
CHERIMOYA		132.5-137.5	-32.3		84.00		11.79
	β-sitosterol	136-137	-33.8	83.28	83.52	12.15	11.83
		167-170	-43.5		84.32		11.54
	stigmasterol	167-168	-41.8	84.38	84.34	11.23	11.53
CUCUMBER	β-sitosterol	136.5-137.5	-36.1	83.28	83.51	12.15	12.10
	γ-sitosterol	144-145	-42.0	83.98	83.52	12.15	12.06
	β-sitosterol	136-137	-35.5	83.98	83.62	12.15	12.17
	α-sitosterol	163-164	- 2.3	84.38	83.98	11.23	11.27
MACADAMIA	stigmasterol	166-168	-43.7	84.38	84.35	11.23	11.29
	γ-sitosterol	144-145	-42.1	83.98	83.52	12.15	11.94

V DISCUSSION AND CONCLUSIONS

The isolation of a sterol mixture from each of the four oils investigated was carried out by approximately the same procedures: preparation of the oil for use, saponification, extraction, and recrystallization. Difficulties and differences for individual oils were discussed in the experimental section in their proper places. Inasmuch as such mixtures were prominent in the earlier work on sterols and were often reported as separate compounds and because in themselves the mixtures possess fairly sharp melting points, they were first studied in this research as mixtures and then were separated into individual sterols which were found to correspond in properties to particular sterols described in literature.

The two color reactions, the Whitby and the Liebermann-Burchard, which are general reactions for sterols, were shown by all sterol mixtures. Two reactions, the Tortelli-Jaffe and Rosenheim, which are specific for particular groupings were negative.

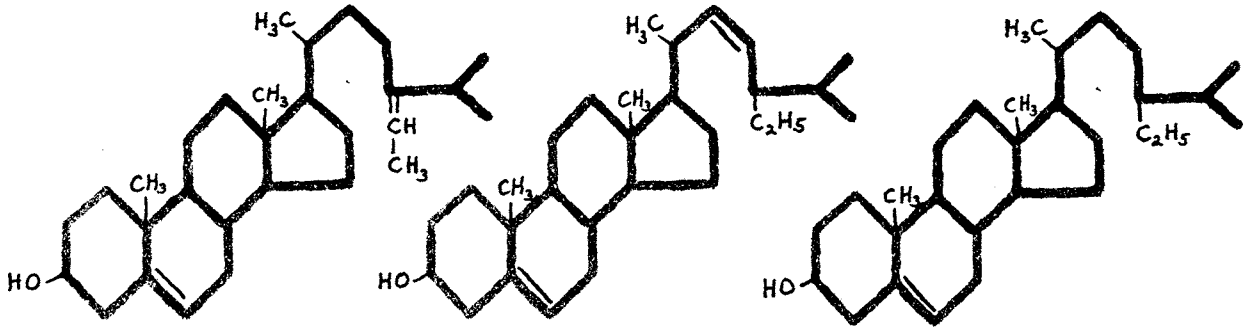
Acetylation was accomplished for all sterol mixtures and for individual sterols showing the presence of hydroxyl groups in all. Benzoylation led to the same conclusion.

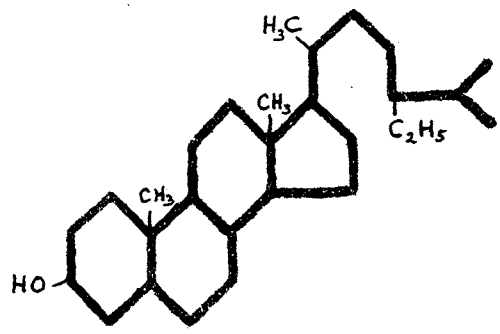
Hydrogenation and bromination showed that all sterol mixtures contained some unsaturated sterols. Hydrogenation of the sterol mixtures from avocado and

chaulmoogra oils resulted in the formation of only one compound found to be stigmastanol. This result was consistent with ultimately finding the separate sterols, β-sitosterol, fucosterol, stigmasterol and stigmastanol, in these two oils. Hydrogenation would convert the first three of these to stigmastanol. See Figure 2. Hydrogenation of the sterol mixtures from cocoanut and macadamia oil did not lead to single products and this result is in accord with the individual sterols ultimately isolated from these two oils.

It is known that sterol acetate tetrabromides are insoluble and have high melting points while the dibromides are soluble and have low melting points. This constitutes a basis for the separation of sterol mixtures if both types of bromides can be prepared. For avocado sterol mixture such a separation was made showing the presence of one-double bond and two-double bond components. This was in accord with the later identification of β-sitosterol, fucosterol, and stigmasterol in avocado oil. Similar results of bromination for the three other sterol mixtures verified the conclusions reached with regard to individual sterol in chaulmoogra, cocoanut, and macadamia oils.

It is established that unsaturated sterols which have been brominated do not form digitonides while saturated (therefore not able to be brominated) sterols can be converted to digitonides. This constitutes a basis for separation of sterols in mixtures. When applied to the





stigmastanol

Figure 2. Conversion of the Sterols of Avocado and Chaulmoogra to Stigmastanol

sterol mixtures from the four oils, no digitonides were formed for avocado oil indicating that only unsaturated sterols were present in avocado sterol mixtures. This was in agreement with sterols later identified. Similarly for the other three oils, the bromination-digitonin treatment proved the presence of both saturated and unsaturated sterols for all three. These results were later verified when individual sterols were identified.

It is known that most natural sterols possess the 3 β-hydroxy spatial configuration, i.e. the hydroxyl is spatially on the same side of the ring as are the two angular methyl groups. All sterols possessing this configuration form digitonides. Crude, unsaponifiable portions obtained from the four oils (before the isolation of purified sterol mixtures) were treated with digitonin. In all four cases digitonides were formed showing that all four oils contained sterols possessing the 3 β-hydroxy stereo configuration. This result was substantiated by the proof that all of the sterols identified in the sterol mixtures possessed this configuration.

Assuming that all natural sterols contain the 3 β-hydroxy configuration by preparing the digitonides from the crude unsaponifiable portions of the oils, it was possible, by weighing the digitonides, to secure a rough approximation of the percentage of sterols in the unsaponifiable portion. For avocado this was found to be 55%, for chaulmoogra 30%, for cocoanut 26%, and for macadamia 37%. These percentages indicate whether or not oils are

good sources of sterols. For the same oils these percentages were in agreement with the values obtained by Westgate.

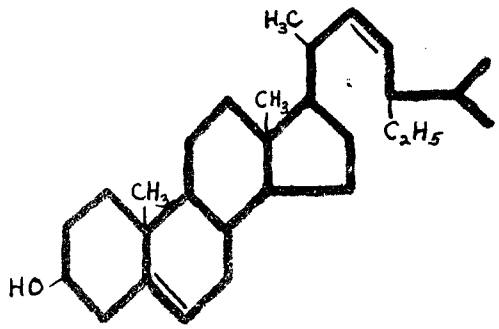
The Diels-Alder reaction was found to be negative for the four sterol mixtures, showing that none of the sterols in the oils studied possessed conjugated systems such as react with maleic anhydride in the Diels-Alder reaction. The result was in accord with the failure on sterol mixtures of the Rosenheim Color test which is designed to detect the conjugated system.

It should be recalled here that historically it has been shown that much of the earlier research on sterols was done upon extremely intimate mixtures of closely related and difficultly separable sterols. These mixtures usually possessed sharp melting points, showed optical rotation, and responded to sterol reactions. In fact, for a long time they were considered to be individual sterols. Such intimate mixtures were isolated from the four oils investigated here and individual sterols obtained from them by chromatography. Westgate (2), working in this laboratory, in 1935-1938, isolated intimate sterol mixtures throughout his studies of avocado, kukui, chinawood, chaulmoogra, and cocoanut oils, but reported that he was unable to separate the mixtures into individual identifiable sterols by various chemical procedures. Chromatography has served in the present work to accomplish these separations.

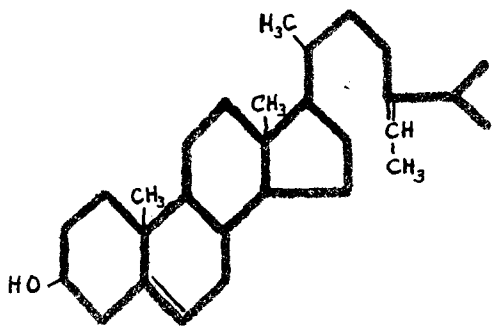
Studies of sterol mixtures and of

individual sterols chromatographically obtained and comparison of their properties, structures, and carbon and hydrogen content with known sterols have established the presence of β-sitosterol, fucosterol, stigmasterol in avocado oil; sigmastanol, β-sitosterol, stigmasterol in chaulmoogra oil; β-sitosterol, γ-sitosterol in cocoanut oil; and stigmasterol, β-sitosterol, γ-sitosterol, α_1-sitosterol in macadamia oil. In all, six sterols were identified. Further work needs to be done on the structure of γ-sitosterol and α_1-sitosterol.

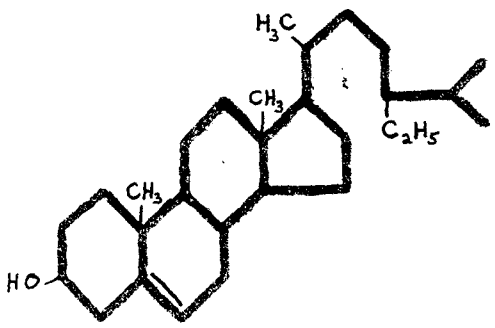
The structures of these, with the exception of α_1-sitosterol, are shown below:



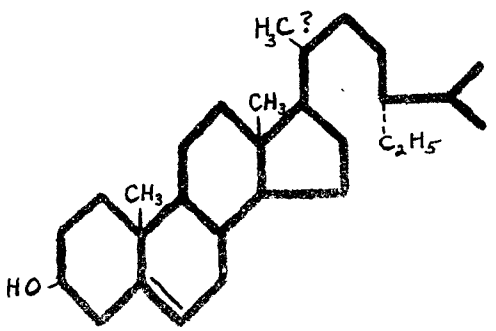
stigmasterol



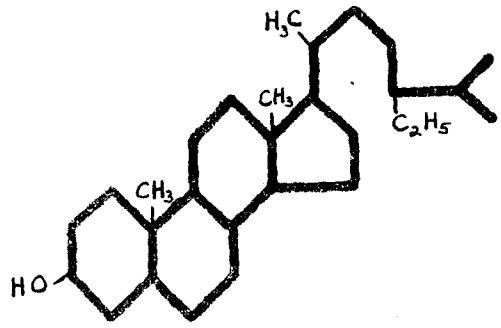
fucosterol



β-sitosterol



γ-sitosterol



stigmastanol

VI SUMMARY

1. Four tropical oils, avocado, chaulmoogra, cocoanut, and macadamia were investigated as sources of sterols.

2. The sterols were isolated from the unsaponifiable fractions which were obtained by the saponification of the oils by alcoholic alkali.

3. Chemical and physical studies showed the sterol portions to be sterol mixtures and confirmed investigations which established the fact that plant sterols consisted of intimately related and difficultly separable mixtures.

4. The sterol mixtures were separated chromatographically and chemical and physical data compiled on the individual sterols.

5. The identities of the individual sterols were determined by correlation of their chemical and physical data with corresponding data for known sterols in the literature.

6. Avocado oil was shown to contain three individual oils: β-sitosterol, stigmasterol, and fucosterol.

7. Chaulmoogra oil was shown to contain three individual oils: stigmastanol, β-sitosterol, and stigmasterol.

8. Cocoanut oil was shown to contain a small stanol fraction and two individual sterols: β-sitosterol and γ-sitosterol.

9. Macadamia oil which was studied for the first time was shown to contain a small stanol fraction and four individual sterols: β-sitosterol, stigmasterol, δ-sitosterol, and α,-sitosterol.

10. Avocado oil was found to be the best source of sterols.

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