AN INVESTIGATION OF THE LIPID FRACTIONS OF PLANT AND BEEF BRAIN TISSUES

by

Robert C. Hiltibran
B.S., Denison University, 1948
M.S., University of Kansas, 1951

Submitted to the Department of Biochemistry and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

November, 1953

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to Dr. Harold J. Nicholas under whose supervision and unfailing interest this investigation was undertaken.

He is also greatly indebted to Drs. Russell C. Mills, Dwight J. Mulford, and Harold J. Barrett, and to Mr. Charles L. Wadkins for their helpful suggestions and assistance in one way or another.

He is also greatly indebted to his wife for her assistance in the preparation of this manuscript.

The writer appreciates the financial support of the General Appropriations Fund of the University of Kansas during this investigation.

BIOGRAPHICAL SKETCH

The candidate was born September 24, 1920, in Urbana, Ohio. He graduated, with Class Honors, from the Urbana Local High School in May 1938. In January 1942, he enlisted in the U. S. Army Air Force, and was honorably discharged from service with the rank of Sergeant, September 3, 1945. Twenty-seven months were spent in overseas service. He enrolled in Denison University, Granville, Ohio in February 1946 and completed the requirements for the Bachelor of Science degree September, 1948. At this time he entered the Graduate School of the University of Kansas, where he remained until June, 1950. From June, 1950 until February, 1951, he was employed as a Research Assistant in the Department of Oncology, University of Kansas Medical Center, Kansas City, Kansas. He returned to the Graduate School, University of Kansas in February, 1951. He was awarded the Master of Science degree from the University of Kansas in June, 1951.

TABLE OF CONTENTS

TABLE OF CONTENTS, CONTINUED

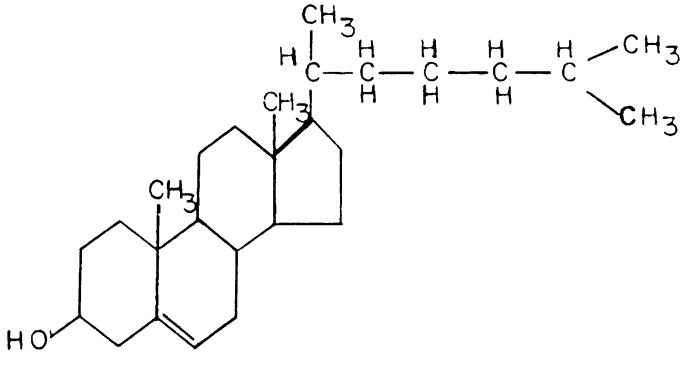
TABLE OF CONTENTS, CONTINUED

INTRODUCTION

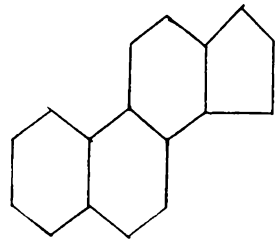
The investigation, the results of which are reported in this thesis, was undertaken to examine the neutral and weakly acidic fractions of the lipids of various plant tissues, and of brain tissue for their content of constituents which might be considered as possible intermediates in the biological synthesis of sterols.

The investigation as carried out was one part of a program designed to study the biological synthesis of sterols, and called the 'Study of the biogenesis of sterols'.

Cholesterol, the most abundant sterol in the animal kingdom has the structure as shown below, which was proposed by Windaus (1), Wieland and Dane (2), and Rosenheim and King (3). The basic sterol nucleus, the cyclopentanoperhydrophenanthrene nucleus, is also shown.



Cholesterol



Cyclopentanoperhydrophenanthrene

There are many substances in nature which contain the sterol nucleus, such as the plant sterols, plant saponins and sapogenins, sex hormones, adrenal cortical steroid hormones, cardiac glycosides, and the polycyclic terpenes. These substances are believed to be synthesized in the tissues in which they are found, however, the mechanisms by which the sterol nucleus is synthesized are not known. As the discussion in the Historical section will show, the sterols can be synthesized from low molecular weight compounds, however, the biosynthetic route from the low molecular weight carbon compounds to the polycyclic compounds remains obscure. It is assumed to be through a series of stepwise conversions, and there is very little known concerning these suspected intermediates. This investigation was undertaken in an attempt to isolate and characterize substances which might have some relation to the biosynthesis of sterols.

The investigation of the lipid fractions of whole mature plants of the plant Plantago rugelii revealed the presence of hentriacontane, ursolic acid, oleanolic acid, and the sitosterol mixture. The distribution of the ursolic acid, oleanolic acid, and the sitosterol mixture in the whole mature plant and in young plants was studied. Whole mature plants of additional species were examined for their ursolic and oleanolic acid content.

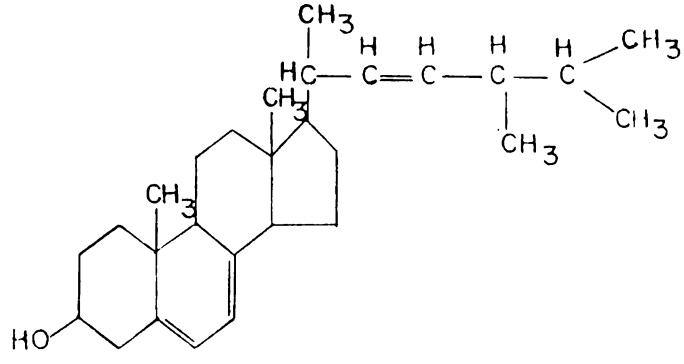
The investigation of the neutral and weakly acidic fractions of beef brain tissue was undertaken. The examination of the weakly acidic fraction did not reveal any identifiable constituents. From the neutral fraction there were isolated and partially characterized three hydrocarbon substances, a low molecular weight oil which contained two oxygen functional groups, and a high molecular weight low-melting compound.

Experiments to indicate the possible relation of these substances to biosynthesis of sterols could not be undertaken in the time alloted.

HISTORICAL

Sterol Formation in Lower Plants

Most of the studies of sterol biosynthesis in lower plants center around the formation of ergosterol, which is the sterol present in largest quantity in most fungi. The structure of ergosterol as shown below was proposed by Windaus (4) in 1932 and has subsequently been found to be correct.



Ergosterol

It is interesting to note that data concerning the formation of this sterol were accumulating long before the structure of the compound was known. Gerard (5) appears to have been the first to record that ergosterol could be synthesized by lower plants when in 1892 he obtained ergosterol from Pencillium glaucum, which had

been cultivated on a medium containing sucrose and tartaric acid as the only carbon-containing compounds. Later (6) he reported the extraction of a sterol, presumably ergosterol, from Mucor mucedo grown on a medium which contained lactose as the sole carbon compound. Pruess, Peterson, Steenbock, and Fred (7) extracted the sterol from a number of fungi cultivated on a medium in which the carbon source was glucose. Ergosterol was isolated from Pencillium puberulum, by Birkinshaw, Callow, and Fishman (8), and from Aspergillus fischeri by Pruess, Peterson, and Fred (9) when these fungi were grown on a medium containing glucose as the only carbon source. The first systematic attack on the problem of sterol synthesis in fungi seems to be that of Bills, Massengale, and Prickett (10), who in 1930 cultivated twenty-nine different yeasts in the same medium and found that the organisms exhibited decidedly different capacities for the elaboration of ergosterol. Exploring the sterol synthesis in yeasts further, Massengale, Bills, and Prickett (11) found that the yeast Saccharomyces cerevisiae produced different amounts of ergosterol when grown on media containing several different sources of carbohydrates. The following carbohydrates were used: xylose, mannitol, alpha-methylglucoside, lactose, melibiose, melezitose, galactose, mannose, fructose, glucose, sucrose, maltose, and raffinose. The authors found that the er-

gosterol content was higher with di- and tri-hexoses than with the mono-hexoses. They did not find any relation between the nitrogen content, non-sterol lipoid, state of nourishment of the yeast, and the amount of sterol formed. They concluded that the synthesis of the sterol nucleus was primarily a product of carbohydrate metabolism.

Sumi (12) in 1933 found that sterol formation in lower plants could occur at any stage of the life cycle, when he observed that in the mushroom Cortinellus shiitake the per cent of ergosterol rose steadily from the button stage to the over-ripe stage.

One of the earliest contributions which indicated a type of intermediate involved in the biological synthesis of sterols was the observation of MacLean and Hoffert (13) in 1926. These authors found that the presence of sulfite in the reaction mixture on which yeast was incubated caused a decrease in sterol production. They did not, however, find a decrease in the elaboration of fatty acids on the same medium containing sulfite. The sulfite was believed to have removed an aldehyde intermediate which was not common to both sterol and fatty acid biosynthesis.

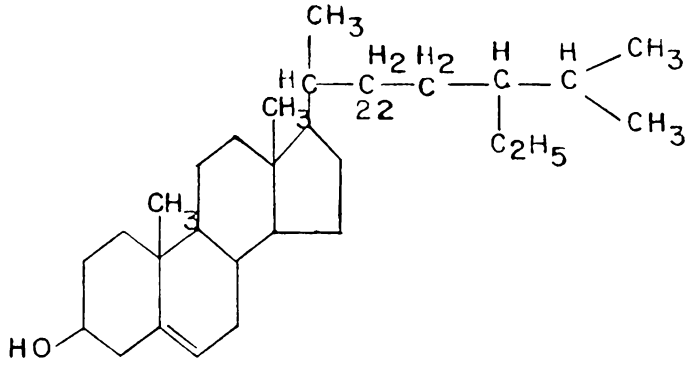
The work of Terroine, Bonnet, Kopp, and Vechot (14) indicated that the mold Sterigmatocystis nigra is capable of utilizing either peptone, sugar, or fat as the source of carbon. With various fats as the source

of carbon sterol was elaborated actually in greater quantity than with either peptone or glucose, but there was no parallelism between the amounts of sterol and fatty acid synthesized in the several cultures.

There will be included, later, additional work on the biological synthesis of ergosterol in fungi.

Sterol Formation in Higher Plants

Early workers in the field of plant sterols did not recognize that plant sterol mixtures were not identical with cholesterol, and considered the sterols from plants and animals to be isomeric. The term phytosterol came into use when the plant sterols were found to be not identical with cholesterol (15). Later the phytosterol was shown to be a mixture of sitosterol and stigmasterol by Windaus and Hauth (16). The structures of sitosterol and stigmasterol are given below (17).



Sitosterol

Stigmasterol = Δ^{22}

The erroneous idea that plant sterols and cholesterol were isomeric, or that they were identical, probably retarded work on the biological synthesis of sterols in higher plants. Lindenmeyer (18) in 1863 appears to have been the first to investigate the biological synthesis of higher plant sterols, when he observed that the seeds of the pea accumulated sterols as they ripen. The ability of plants to synthesize sterols appears early in the young plants. Beneke (19), Schulze and Barbieri (20), Schulze (21), and Terroine, Bonnet, Kopp, and Vechot (14), demonstrated that the sterol content of seeds usually increases during sprouting, even in the dark. Minovici (22) in 1927 made the suggestion that these sterols were derived from agrosterol, a soil constituent which Schreiner and Shorey (23) (1909) regarded as a product of decaying vegetation or mold action.

Marker's (24) work on the biogenesis of the sapogenins is interesting and extensive but deals primarily with transformations occurring about the functional groups on the molecules, however, does not contribute any pertinent information relative to the formation of the sterol nucleus.

It is evident from the above review that practically nothing is known about the process by which the plant sterol nucleus is synthesized in tissues.

Sterol Formation in Higher Animals

Early work on cholesterol in the animal body indicated that cholesterol was associated with pathological conditions (25) and was not a normal tissue constituent. One school of thought formerly held that the sterols of animals were mainly exogenous in origin. This hypothesis was based on: (a) the extremely wide distribution of sterols in plants, (b) the fact that animals are generally incapable of synthesizing heterocyclic compounds, and (c) the belief that cholesterol and the phytosterols were isomeric. These early views led to the conclusion that animals were incapable of synthesizing the steroid nucleus. Bills (26) states, that while there was ample evidence that cholesterol was absorbed by animals, the absorption of phytosterol had not been demonstrated.

With the observation in 1834 by Couerbe (27) that cholesterol is a major constituent of the brain, the idea that plant sterols were the source of all sterols began to change. Couerbe at that time suggested that the brain was a site of cholesterol synthesis. The fact that brain can synthesize cholesterol has only been shown recently. Flint (28) in 1862 regarded cholesterol as an excrementary product originating in the brain. Beneke (29) in 1880 indicated that Flint's view of the excrementitious significance of cholesterol is untenable.

The biosynthesis of cholesterol, or the ability of the animal body to synthesize the sterol nucleus, was not amply demonstrated until about 1920, although Dezani (30) in 1914 had reported that the total cholesterol content of white mice increased, when fed sterol-free diets. Gamble and Blackfan (31) in 1920 fed infants a milk diet and concluded that they had synthesized cholesterol during the course of the experiment. They based their conclusions on the fact that the infants had gained weight during the experiment, indirectly indicating an increase in total cholesterol content. Unfortunately, no cholesterol determinations were made. Gardner and Fox (32) found that adults on a mixed diet excreted 2.5 times as much sterol as was contained in the diet, which likewise indicated that the adults had synthesized cholesterol.

Work on the program began in earnest when Channon (33) fed rats from weaning time until they had reached 100 grams in weight on a cholesterol-free diet, and found that the total cholesterol content increased from 100 to 200 mg. per rat. Later Randles and Knudson (34) found that the cholesterol content of the adult rat, born and raised on a cholesterol-free diet, was many times greater than the cholesterol content at birth, and concluded that cholesterol must have been synthesized from molecules other than sterol in the diet.

Schoenheimer (35) showed that the laying hen synthesizes the cholesterol contained in her eggs, even when phytosterol was available in the feed. Schoenheimer and Breusch (36) in 1933 found that mice on a diet of bread alone synthesized as much cholesterol in a month as they contained in their bodies at the start of the experiment, and also found that the mice could destroy large amounts of cholesterol when administered in the diet.

The problem of plant sterol absorption by animals had not been proved nor disproved conclusively up to 1933. Schoenheimer (37) in a review of some of his work showed that sterols other than cholesterol were not absorbed by animals to any appreciable extent. He and his asscciates (38) fed rabbits for over a year on a diet of sitosterol and found that the total cholesterol content of the rabbits did not increase, while a rabbit fed cholesterol under the same conditions did show a large increase in total cholesterol content. He and his associates (39) extended this work by feeding rabbits a diet of hay and beets, from which a well characterized sterol mixture was separated. The same sterol mixture was recovered from the feces. This indicated the plant sterol mixture had passed through the alimentary tract without being altered qualitatively or quantitatively. To prove the point even more

conclusively he and his associates (40) determined the cholesterol and other sterol content in the thoracic duct after mixtures of cholesterol and plant sterols had been administered to the experimental animals. They found that the plant sterols were not absorbed in quantities which could be detected by the experimental procedures. The conclusions based on the above experimental data were only as valid as the experimental procedures used, which unfortunately were colorimetric determinations, and subject to considerable error. The use of a more critical tool, such as labeled plant sterols, should be used to determine if the plant sterols are absorbed at all.

To add additional evidence on the absorption of sterols, Schoenheimer and his associates (41) administered mixtures of cholesterol and non-irradiated ergosterol to rats, and determined the amount of each in the thoracic duct. The ergosterol content could be determined in much smaller quantities than cholesterol by using quantitative techniques based on the ultraviolet absorption of the ergosterol. The amount of ergosterol found in the thoracic duct of the experimental animal was less than the amount of ultraviolet radiation absorbing component usually present in cholesterol preparations. These results were not in accord with the results of Page and Menschick (42) who found that the ergosterol content of the experimental animals was increased by administra-

tion of ergosterol in the diet. This variance in the results was resolved somewhat when it was found that irradiated-ergosterol was absorbed to a slight extent (43).

Once the conclusions had been reached that animals could synthesize all the sterols necessary for their normal metabolism, the ground work was laid for more detailed developments. Most of those developments are indicated in the subsequent pages of this review.

Theoretical Hypotheses of Cholesterol Biosynthesis

This period was filled with considerable speculation as the following pages will show. Some of the ideas of sterol precursors, based principally on suggestive structural relationships and a common occurrence with sterols in specific tissues, have not been substantiated. One of them (squalene) has recently been shown to be a cholesterol precursor.

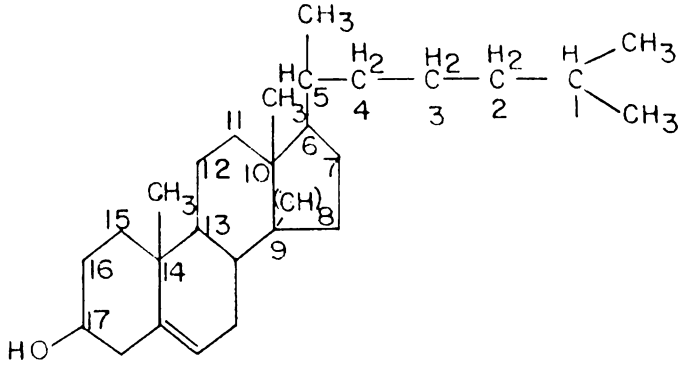
Tsujimoto (44) reported the isolation and characterization from liver oil of members of Squalidae family a highly unsaturated hydrocarbon, which he called squalene. Chapman (45) found another highly unsaturated compound from the liver oil of Centrophorus granulosus and Scymnus lichia, members of the natural family of the Spinacidae and called his compound spinacene. In 1917 (46) he called attention to the possible rela-

tionship of spinacene to the terpenes, and later work showed this relationship to be correct (47). Later, he (48) also called attention to the possible relationship of spinacene to cholesterol, which at that time was considered to be isoprenic in origin. Through the work of Heilbron and his associates (49, 50) squalene and spinacene were shown to be identical, and these authors also called attention to the possible relationship between squalene, on one hand, and stigmasterol and cholesterol on the other. These hypotheses were even more interesting in view of the fact that at the time of their original proposal neither the structure of squalene nor of cholesterol was known.

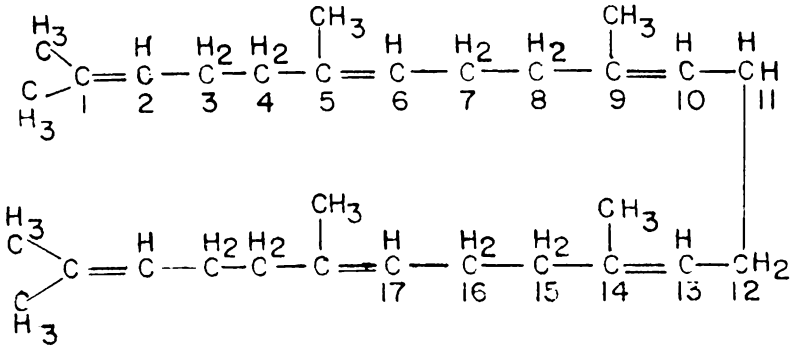
Channon (51) in 1925 administered squalene to rats and found that the unsaturated hydrocarbon was absorbed by the rat. He also found that the cholesterol content of the liver of the rat was increased by 100 per cent, while the content of the unsaponifiable fraction was increased 2.6 times, thus obtaining direct evidence for the possible relationship between the two compounds. Channon's work was the only experimental evidence presented to support the possible relationship between squalene and cholesterol. During the period between 1932 and 1934 the structure of cholesterol was proposed by Windaus and others (1, 2, 3). This structure has been generally accepted as being correct. With the

structure of cholesterol known, several hypotheses for the biosynthesis of cholesterol appeared, with a variety of starting compounds.

Vanghelovici (52) proposed the following general scheme for the biosynthesis of cholesterol by the animal organism: carbohydrate ⟶ saturated fatty acids ⟶ unsaturated fatty acids ⟶ polyisoprenic compounds - carotenoid compounds ⟶ cholesterol. He also proposed the following scheme of cholesterol biosynthesis from squalene based on the comparisons of the cholesterol and squalene molecules. Special attention is called to the numbering of the cholesterol molecule.

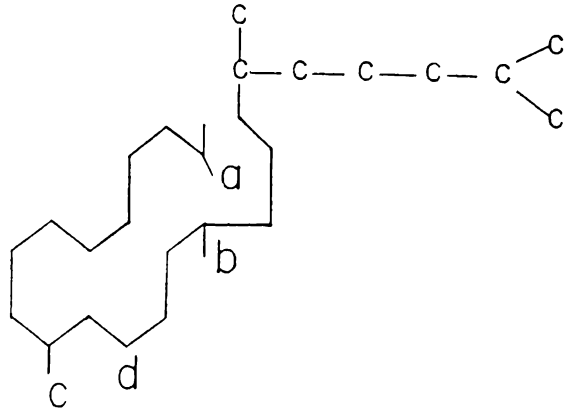


Cholesterol



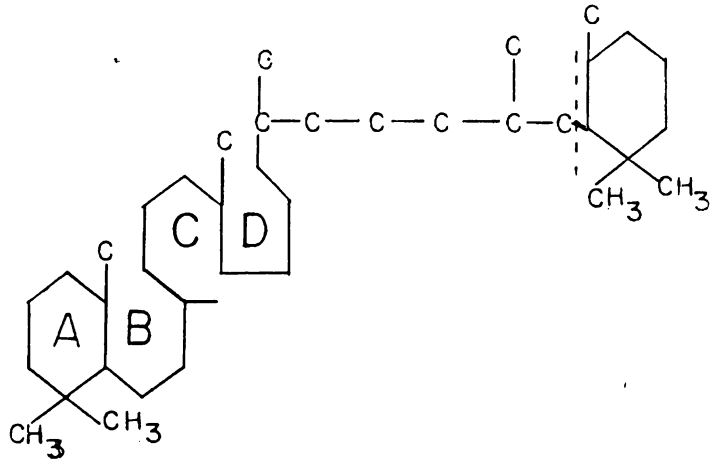
Squalene

Vanghelovici stated that for biogenetic reasons the methyl group should be placed on the carbon-9 rather than on carbon-14. This comment prompted Robinson (53) to remark, "The placing of the methyl group at C-9 is permitting the biogenetic tail to wag the chemical dog." The bulk of the chemical evidence (according to Robinson) indicated that the methyl group was carbon-14 rather than carbon-9. Robinson (54) had proposed a scheme based on similar reasons, but later withdrew his remarks on the basis of the chemical data. He (53) found, however, that by arranging the squalene, or a di-farnesyl type molecule in slightly different manner than had Vanghelovici, the sterol carbon skeleton could be accommodated intact and without previous rearrangement. Robinson's scheme as shown below required the removal of the carbon atoms indicated at points a, b, and c, while point d indicated the point of doubling of the farnesene chains.

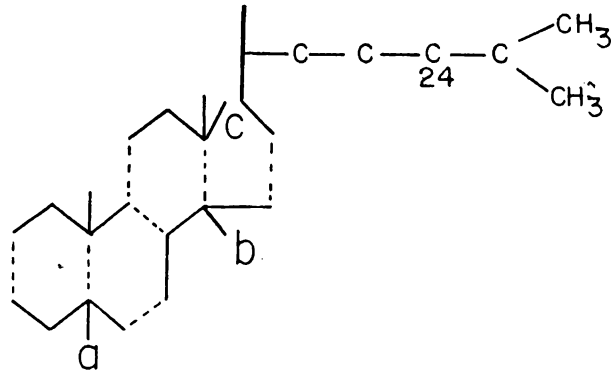


Bryant (55) criticized the hypothesis of Robinson on three points (1) cyclization would evidently begin

near the middle of the squalene chain to avoid the assumption of intermediate large rings, (2) the carbon atom at position 2 of the squalene chain and common to rings C and D was involved in two ring closures, (3) there was no structural peculiarity of the carbon chain forming ring D which would make for the greater probability of a five-membered ring (D) while a six-membered ring would seem more probable. He proposed a scheme of synthesis as shown below in which ring closure was thought of as beginning at the right of ring A already present in the carotene structure. The formation of a five- instead of a six-membered ring at D was attributed to the presence of the four unbranched carbon atoms formed by the symmetrical union of two partially cyclized phytyl chains in carotene, and the aliphatic side chain of cholesterol could be formed by cutting off the last two isoprene units of the symmetrical carotene structure. The fact that this bond was olefinic aids in such a supposition. His scheme is shown below:

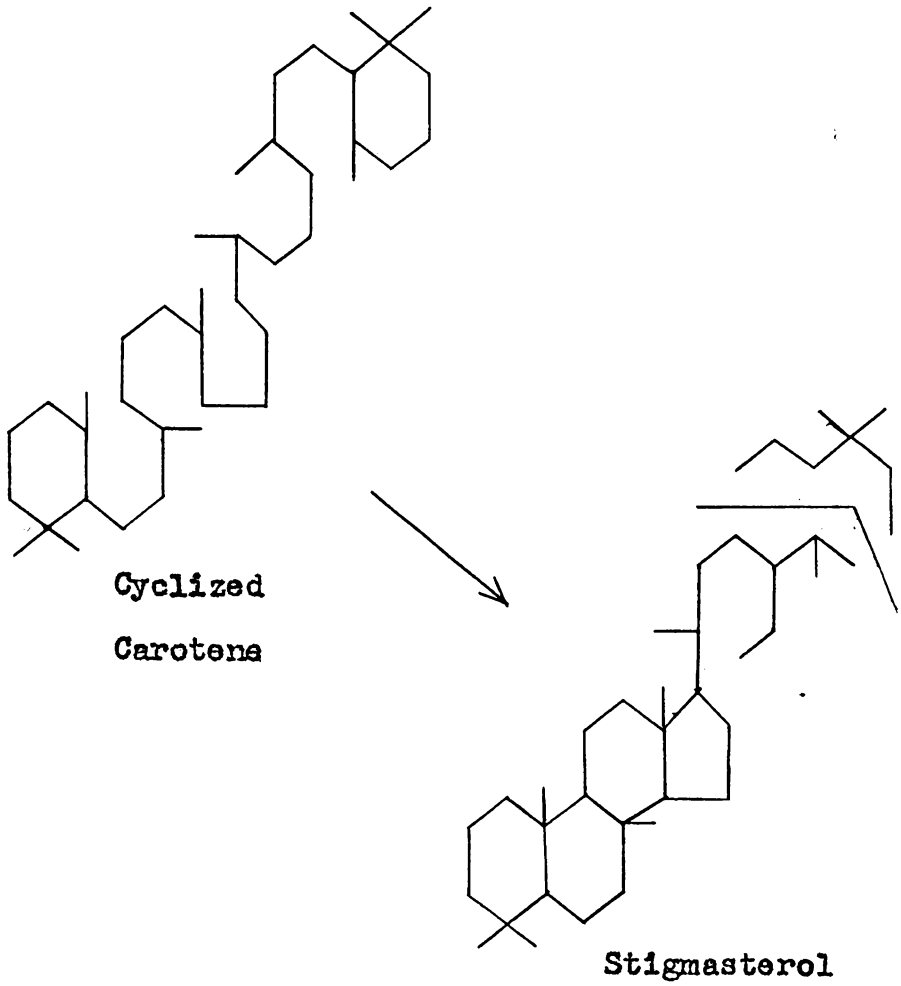


Spring (56) criticized the above two hypotheses on the basis that they were limited only to the cholesterol molecule and did not take into consideration other naturally occurring sterols such as stigmasterol and ergosterol, which differ from cholesterol in possessing a modified side chain. The general isoprene nature of cholesterol is shown in the scheme below. By condensation, cyclization and accompanying disposal of the carbon atoms a, b, and c, the typical cholestane and coprostane structure is obtained. The structure of ergosterol and stigmasterol could be accommodated by the juxtaposition of a seventh isoprene unit adjacent to C-24 with the use of one or two carbon atoms of the unit respectively.



Bryant (57) proposed that the modified side chain could be attributed to local synthetic action following the more fundamental change from the carotenoid to the cholestane structure, or might be produced by ring open-

ing of a more highly cyclized carotenoid derivative as shown below:



Several workers suggested a relation between the sterols and fatty acids. Andre and Canal (58) proposed a conversion of clupanodonic acid, a higher fatty acid, to cholesterol and squalene. Windaus (59) suggested a scheme whereby oleic acid, might be cyclized to civet-

one, which then might give a sterol ring system through hexahydrocivetone and dimethylhexahydrocivetone.

Other workers suggested relationships between carbohydrate and sterols. Reichstein (60) pointed out that the skeleton of the C_{21}-hormones, C_{24}-bile acids, and C_{27}-sterols was theoretically divisible into C_3- or C_5- and C_6- units; he suggested that the synthesis involves a C_3-intermediate of carbohydrate metabolism such as glyceraldehyde or dihydroxyacetone. DuFeu, McQuillin, and Robinson (61) suggested that the ring systems of the sterols could be elaborated from acetone and formaldehyde or their biological equivalents. These authors also suggested that when the hypothesis of sterol formation was made, it would be along the line suggested by them, rather than a scheme based on the condensation of isopentane units. Marker (62) suggested in an extensive report concerning the biogenesis of saponins and sapogenins in plants that the sterols in plants may have their origin in the carbohydrate present in the plant. Miescher and Wieland (63) proposed a scheme of biosynthesis of sterols based on the formation of ethyl-Δ^2-3-methylhexenone-4-carboxylate from two moles of acetoacetic acid and formaldehyde. Ruzicka (64) also proposed that sterols might be biologically synthesized from carbohydrate. Needham (65) cited the schemes proposed by Hall (66) which suggested also that sterols in

plants might be synthesized from the carbohydrate present in the plant.

The above schemes, although of theoretical and historical interest, were not based on direct experimental evidence. Only very recently has the relationship between compounds such as squalene and cholesterol been supported by experimental results. These relationships will be discussed later.

In 1937, Channon and Tristam (67) reinvestigated the administration of squalene to experimental animals and stated; "While no conclusion is possible, the evidence on the whole suggests that squalene is not converted into cholesterol." Kimizuka (68) administered squalene to dogs and found that the quantity of unrecognized non-saponifiable substances of the livers increased, but the quantity of cholesterol did not change. This also indicated that squalene was not converted into cholesterol.

Low Molecular Weight Precursors of Cholesterol

The first experimental evidence for a small molecule playing an integral role in the formation of the steroid nucleus in tissues came, strangely enough, from studies on microorganisms. This finding has played a major role in subsequent developments in our knowledge of cholesterol biosynthesis in animal tissues. As dis-

cussed in the introduction, this development came in a period accompanying the introduction of isotopes as analytical tools. Progress became more rapid when heavy hydrogen and radioactive carbon (C^{14}) were available.

This period was opened by Sonderhoff and Thomas (69) in 1937 when they reported that the unsaponifiable fraction obtained from yeast, which had been grown on a medium containing acetate labeled with deuterium as the only carbon source, contained a higher concentration of deuterium than any other fraction isolated. This indicated a direct employment of acetic acid for the synthesis of yeast sterols. The presence of and the quantity of the heavy isotope could be determined quite easily, and with a high degree of accuracy.

The application of the new technique, the use of a heavy isotope as a label in the study of the biological synthesis of cholesterol in animals, was first used by Block and Rittenberg (70). They administered deuterium labeled acetic acid to adult mice and growing rats, and found that the deuterium concentration of the sterol fraction was approximately three times that of the body fluids at the completion of the experiments.

The concentration of deuterium found in the fatty acids and cholesterol indicated that the higher fatty acids were not intermediates in the biosynthesis of sterols. The same authors (71) found that both carbon

atoms of acetate were used for sterol synthesis. They (72) had reported earlier that at least one-half of the carbon atoms of the cholesterol were derived from acetate, and later Ponticorvo, Rittenberg, and Block (73) reported that forty-five per cent of the carbon atoms of the cholesterol came from the acetate.

Little and Block (74) used acetic acid with both carbon atoms labeled with C^{13} or C^{14} and found the methyl group of acetic acid formed carbon atoms 18, 19, 26, 27, and 17, while the carboxyl group contributed carbon atom 25 and probably carbon atom 10. By assuming that acetate was the only source of carbon, they determined that 15 of the carbon atoms of the cholesterol were derived from methyl groups and 12 carbon atoms from the carboxyl group of acetic acid. From the results which have been mentioned there seemed to be no doubt but that the cholesterol nucleus could be synthesized by animal tissues from small carbon units.

Many other compounds were tested (75) as sources of carbon for cholesterol formation: ethanol, butyric acid, alanine, n-valeric, myristic, propionic, and 10-11 dideuteric-undecylic acids. Of these compounds all gave rise to labeled cholesterol with the exception of propionic acid and undecylic acid, and the compounds which gave rise to labeled cholesterol also produced labeled acetyl groups. The compounds which did not supply car-

bon for the synthesis of cholesterol did not give rise to acetyl groups. Succinic acid (76) was not a precursor of cholesterol, neither was isobutyric acid nor valine, while leucine and isovaleric acid were precursors of cholesterol (77). Leucine and isovaleric acid also were precursors of labeled acetyl groups. N-butyric acid (78) labeled in the α, β and the β, γ positions were only slightly active as cholesterol precursors. Butyric acid (78a) labeled in positions 1 or 3 yielded fragments of different reactivity as found by differences in the amount of C^{14} which was incorporated into cholesterol. The conclusions from this work were that the compounds which served as precursors of cholesterol likewise serve as precursors of labeled acetyl groups, and indicated that the effectiveness as a carbon source for cholesterol synthesis involved the ability of the compounds to supply acetate groups. The effect of compounds with a branched chain was reinvestigated by Zabin and Block (79) with isovaleric acid labeled with C^{13} in the methyl group and C^{14} in the carboxyl group. These workers found that the isopropyl group formed acetyl groups and that the isovaleric acid was a more efficient precursor of cholesterol than was acetate. Later (80), degrading cholesterol synthesized from labeled isovalerate 4, 4' C^{13}, 1 C^{14} and 4, 4' C^{13}, 3 C^{14} as well as from labeled acetate, these authors found the distribution of carbon from the two sources to be the same. The carboxyl carbon was not effectively utilized.

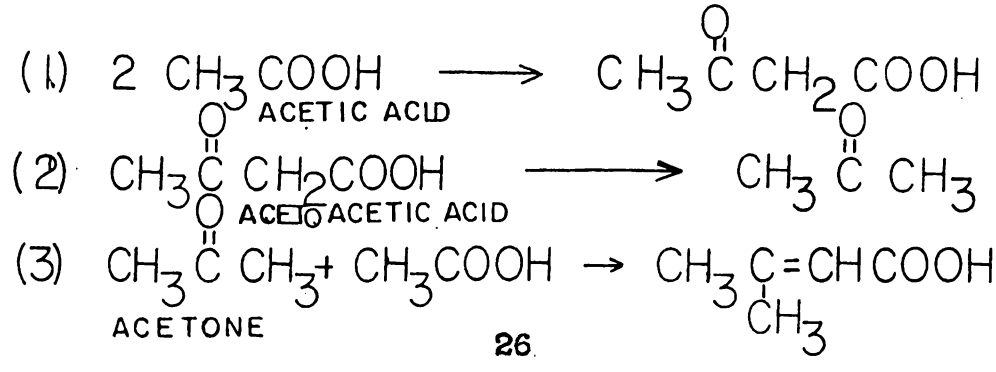
Work turned to the problem of cholesterol synthesis by surviving liver tissue when Block, Borek, and Rittenberg (81) reported the synthesis of labeled cholesterol when the tissue was incubated with labeled acetate or heavy water. Synthesis did not take place under anaerobic conditions nor in slices of kidney, testes, spleen, or gastrointestinal tract. Brady and Gurin (82) found that beta-labeled pyruvic, carboxyl-labeled butyric, hexanoic, and octanoic acids contributed to the formation of labeled cholesterol as well as did carbonyl-C^{14}-acetone, methyl-C^{14}, isovalerate, doubly labeled acetaldehyde and acetoacetate labeled in the carboxyl, carbonyl, and methyl-methylene positions (83). The data indicated that acetoacetate was not degraded to a two carbon unit before utilization, and acetaldehyde was converted more readily into cholesterol and long chain fatty acids than was acetate. Carbonyl labeled acetone (84) was transformed to acetate and cholesterol and it appeared that the acetone was degraded to a metabolically active 2-carbon fragment which was not acetate. Curran (85) incubated 3-C^{14} acetoacetate with surviving rat liver slices and found that the acetoacetate when incorporated into cholesterol did not appear to involve either acetic acid or acetone as intermediates.

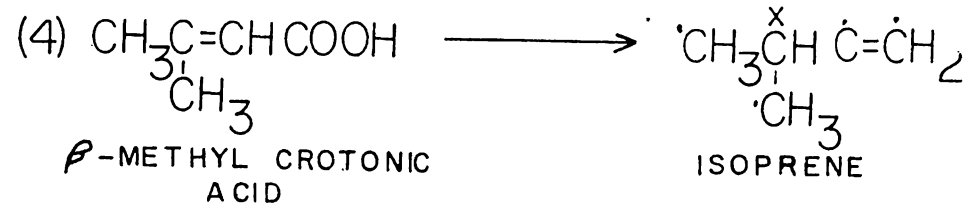
The role of pyruvate for the synthesis of cholesterol was studied by Anker (86) and by Block (87).

Anker's work indicated that pyruvic acid contributed very little carbon for cholesterol synthesis. Block found that pyruvate could provide two carbon units which could be employed for fatty acid synthesis, but this same unit did not appear to be interchangeable with acetate in cholesterol synthesis.

Brady, Rabinowitz, Baalen, and Gurin (88) studied the incorporation of other four-carbon compounds by surviving liver tissue. The following labeled compounds were studied: vinyl-acetic acid, crotonaldehyde, aldol, acetoin, and orsillenic acid. None was found to be important precursors of cholesterol. They found also that labeled formate and formaldehyde were not significantly converted by liver slices to cholesterol. Also, MacKenzie et al. (89) found the labeled methyl group of methionine was not utilized in cholesterol biosynthesis.

Bonner and Arreguin (90) recently obtained evidence for the utilization of acetate in the synthesis of natural rubber by isolated guayule stems. They suggested the accompanying mechanism for isoprene formation from acetic acid:





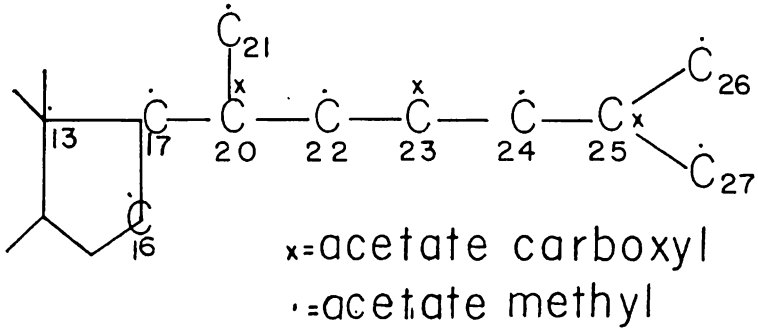
X = ACETATE CARBOXYL CARBON

• = ACETATE METHYL CARBON

The results with rubber, a polyterpenoid substance, gave the first good proof that some particular five-carbon unit was a low molecular weight precursor, although it had been suspected for some time that isoprene or a similar unit was the product which on repeated polymerization or condensation ultimately formed the rubber molecule. Since the sterols had been suspected of being isoprenoid in nature also, the evidence cited here for rubber raised implications for the origin of the steroid molecule too. It was probably the primary reason why several investigators re-investigated the proposals originally made for squalene as a precursor of cholesterol. It is surprising that to this date no report has yet been made on the testing of β-methyl crotonic acid as a precursor of cholesterol. The fact that this five-carbon unit has not yet been reported in animal tissues does not preclude its being absent in such tissues.

Distribution of Acetate Carbon Atoms in Cholesterol

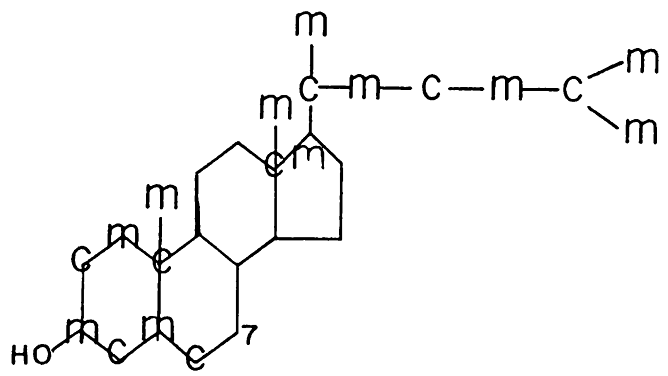
Little and Block (91) have shown that five acetate-methyl and three acetate-carboxyl groups were used in the synthesis of the isooctyl side chain of cholesterol. Wuersch, Huang, and Block (92) undertook a complete degradation of the side chain to determine the sequence of acetate carbon in the isooctyl side chain and found that carbons 21, 22, 24, 26, and 27 were derived from the acetate methyl group and carbon atoms 20, 23, and 25 from the acetate carboxyl group. The sequence is shown below:



The similarities in the structural features between the steroids and various natural products composed of isoprene units raised the possibility that the acetate was converted to cholesterol by way of five-carbon units which might at least be formally related to isoprene. The observed distribution of the methyl and carboxyl carbons of acetate in the cholesterol side chain was entirely consistent with the concept that five-carbon units, formed in the manner suggested by Bonner and and Arreguin (93), could take part and were intermedi-

ates in the biosynthetic process. Carbons 27 to 23 would correspond to the terminal five-carbon unit, and carbons 22, 20, 21, 17, and either carbon 13 or 16 would represent the adjoining five-carbon unit. From evidence obtained earlier it had been tentatively concluded that carbon 17 was derived from a methyl group of acetic acid. Thus the labeling of this ring (D) carbon was in accord with the postulated mechanism.

While the side chain of cholesterol has been completely degraded and the origin of the carbon atoms has been deduced, the work with the steroid nucleus has lagged behind. Cornforth, Hunter, and Popjak (94) published results on the degradation of the sterol nucleus which was carried out to determine the origin of the carbon atoms. The C^{14} cholesterol synthesized by rat liver slices from either carboxyl or methyl labeled acetate was partially degraded by opening ring B and splitting off ring A, by pyrolysis, as 2-methylcyclohexanone. On degradation of the derivative they concluded that carbon atoms 2, 4, 6, and 10 were derived from the carboxyl carbon of the acetate, while carbon atoms 1, 3, 5, and 19 were derived from the methyl carbon of the acetate. The results of Block and his associates (92) coupled with the data given above gave the following distribution of carbon atoms in the cholesterol molecule: (c represents carboxyl carbons, and m represents the methyl carbons).



Cornforth et al. (94) pointed out that the crucial atoms in the scheme appeared to be carbon number 7, and so far all attempts to determine the origin of carbon 7 have been unsuccessful.

Utilization of Acetate for Ergosterol Biosynthesis

The role of acetate in ergosterol biosynthesis was studied by Ottke, Tatum, Zabin, and Block (95), using a mutant strain of Neurospora crassa which was unable to metabolize glucose to the acetate stage, and therefore required acetate for growth. When the mutant was grown on a medium containing sucrose and isotopic acetate labeled by C^{13} in the carboxyl group and by C^{14} in the methyl group, over 90 per cent of the carbon atoms of the ergosterol were found to be derived from the labeled acetate. The ratio of the acetate methyl-carboxyl car-

bons indicated the incorporation of fifteen methyl and thirteen carboxyl groups or fourteen methyl and fourteen carboxyl groups. Isovaleric acid, labeled in the methyl group, was found to participate to a small extent in the ergosterol biosynthesis.

In a later section the relation of Coenzyme A to ergosterol biosynthesis will be discussed.

Hanahan and Wakil (96) synthesized C^{14}-ergosterol from acetic acid labeled in the carboxyl group by using Coenzyme A-enriched yeast. The side chain was partially degraded and the isotope distribution determined. Carbon atoms 23 and 25 were found to have been derived from the carboxyl group of acetic acid. Their data agreed quite well with the data of Wuersch, Huang, and Block (92) on the origin of the terminal six carbon atoms of the side chain.

Conversion of Squalene to Cholesterol

In 1951 Srere (97) demonstrated that liver tissue from rats which had been previously fed a 1 per cent squalene diet for 30 days did not rapidly incorporate C^{14}-labeled acetate into carbon dioxide. Langdon and Block (98) administered C^{14}-acetate and squalene to rats, and found that the rats utilized the labeled acetate for the synthesis of cholesterol. They found that squalene was a normal constituent of rat liver, 25 gamma were present per gram of rat liver. The incorporation of

acetate into squalene was a very rapid process as maximum isotope concentration was reached within 30 minutes. They (99) administered the biosynthetically labeled squalene to mice and found that the C^{14} of the squalene was incorporated into cholesterol. It was found that C^{14}-squalene prepared from geronylacetone and deuterium-labeled squalene was not converted to cholesterol. These data confirmed the results of Tomkins, Chaikoff, Dauben, and Bradlow (100), who found that synthetically prepared squalene was not incorporated into cholesterol in the rat, and by the rat liver slices. Tomkins, Dauben, Sheppard, and Chaikoff (101) found that feeding natural squalene for 9 days resulted in a marked reduction in the ability of the surviving tissues to convert C^{14}-labeled acetate to cholesterol, but the reduction was not observed in the cholesterol-C^{14} recovery in the experiments in which the rats had been previously fed regenerated squalene. This suggested that natural squalene was a cholesterol precursor, while the regenerated squalene could not be incorporated into cholesterol.

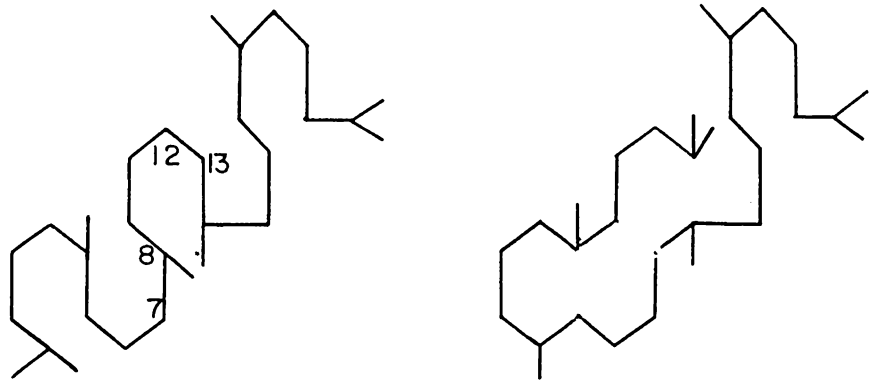
Dauben, Bradlow, Freeman, Kritchevsky, and Kirk (102) from a study of infrared spectra of natural and regenerated squalene, found that natural squalene was of a homogenous trialkylethylene bond structure of the following type: R R' C = CHR'', while regenerated squalene had both the trialkylethylene structure and the unsymmetrical dialkylethylene type RR'C = CH_2. These authors es-

timated between 20-40 per cent of the latter type bond structure was present in the regenerated squalene. Langdon and Block (99) found the infrared spectra of the synthetic and regenerated squalene were identical, but were not identical with the spectrum of natural squalene. Dauben et al. (101) also found the same to be true of their squalenes. If squalene was converted directly to cholesterol, this conferred on the synthesis of cholesterol a very high degree of sterospecificity, which would also be suggested in the ring closure scheme of cholesterol biosynthesis as proposed by Robinson (53).

Langdon and Block (99) concluded that while squalene may or may not be a specific precursor for cholesterol synthesis, the data indicated that squalene was one of the intermediates on the route of incorporation of acetate or some two-carbon intermediate into cholesterol. Dauben et al. (101) concluded that the reason for the inability of regenerated squalene to serve as a precursor of cholesterol was that regenerated squalene did not contain any molecules of the appropriate structural configuration which could serve as a precursor. The work of Langdon and Block (98) indicated that from 5 to 10 per cent of the C^{14} from the labeled squalene could be recovered in cholesterol. From the work of Heilbron et al. (49) it has been shown that natural

squalene is a mixture of at least three isomers, as three hexahydrochloride derivatives have been isolated from the hexahydrochloride derivative prepared from natural squalene. These facts suggested that the natural squalene contained only a small amount of one of the structural isomers which could be used in the biosynthesis of cholesterol. A similar example of such specific requirement for structural isomers was found by Wald (103) in the utilization of vitamin A for the synthesis of visual purple.

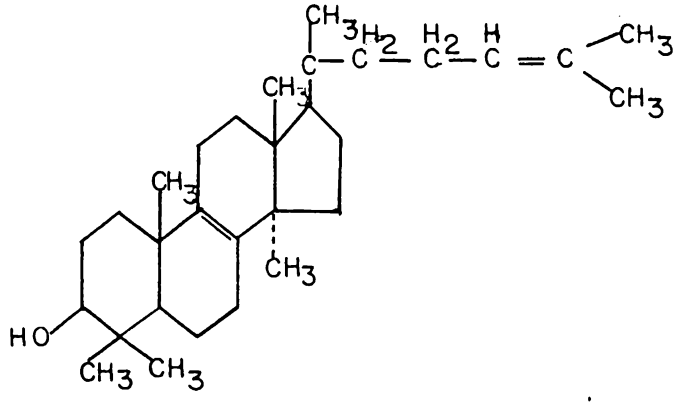
Block and Woodward (104) have presented an alternate scheme for the cyclization of molecules such as squalene in the biosynthesis of cholesterol. Their scheme (A) along with the scheme as proposed by Robinson (B) is presented below:



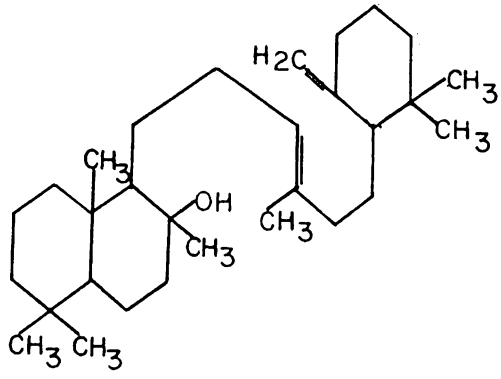
A B

Scheme A altered the arrangement of acetate carbons in cholesterol only at four following positions: C-7, C-8,

C-12, and C-13; to support their theory they have presented evidence for the origin of one of the crucial carbon atoms. Epiandrosterone, obtained by the degradation of labeled cholesterol, was degraded by procedures used for the C-methyl determinations and carbons 10 and 18, and carbons 18 and 19 were assayed for their C^{14} content. The data indicated that either carbon 10 or 13, in addition to the angular methyl groups, contained C^{14}. Cornforth et al. (94) presented evidence to show that carbon 10 was derived from an acetate carboxyl, hence carbon 13 was derived from a methyl group as acetate. Block and Woodward concluded from this evidence that scheme B was untenable, while scheme A was consistent with the new data. Independently, Dauben, Abrahm, Hotta, Chaikoff, Bradlow, and Soloway (105) had proposed such a ring closure as suggested by Block and Woodward. It is rather interesting that scheme A produces a structure with methyl groups on Ring A and at several other positions corresponding to those on lanosterol and the partially cyclic ambrien.



Lanosterol (106)



Ambrein (107)

Relation of Vitamins to Cholesterol Synthesis

Klein (108) reported that Coenzyme A-poor yeast synthesized little or no ergosterol, and the data suggested that the conversion of acetate to steroid, and to lipids in general, occurred through Coenzyme A-catalyzed reactions. Hanahan and Wakil (109) found using either methyl or carboxyl labeled acetate that in the presence of Coenzyme A-deficient yeast very small amounts were incorporated into ergosterol. Coenzyme A-enriched yeast produced ergosterol of very high specific activity from both types of acetate. Coenzyme A-enriched yeast derived from 74 to 84 per cent of the ergosterol carbon atoms from the exogenous acetate, while normal cells contained ergosterol of low specific activity and apparently derived only 8 to 20 per cent of the carbon atoms from the doubly labeled acetate. Klein

and Lipman (110) reported the results of experiments relating the Coenzyme A-level of the yeast Saccharomyces cerevisiae to ergosterol and fatty acid synthesis. These authors found no net increase in lipids. However, there could be demonstrated under the conditions of their experiments a significant difference in the ability of Coenzyme A-poor and Coenzyme A-rich yeast cells to incorporate acetate into fatty acids and steroids. In a later paper these same authors(111) reported experiments with rat liver from Coenzyme A-deficient rats, which showed relative inability to synthesize lipids. They also found a relationship between the Coenzyme A-levels, the synthesis of cholesterol and total lipids.

Guehring, Hurley, and Morgan (112) presented results of cholesterol metabolism in pantothenic acid-, riboflavin-, and pyridoxine-deficient rats which were fed 1 per cent cholesterol diets. The pantothenic acid-deficient rats did not show the increase of cholesterol which the normal, and the riboflavin- and pyridoxine-deficient rats showed.

The work of Becker, Burch, Salomon, Venkitasubramanian, and King (113) indicated that ascorbic acid, while not incorporated directly into cholesterol, did exert a marked effect upon the conversion of 1-C^{14} acetate to cholesterol and other sterols in the adrenal gland of guinea pigs. They found that severely scor-

butic guinea pigs as compared with normal animals incorporated six times as much C^{14} from the labeled acetate into cholesterol.

Some relation between Vitamin E and lipids and cholesterol was indicated by the work of Heinrich and Mattill (114). The work of Collazo, Torres, and Rodriquez (115) indicated there might be some relation of Vitamin A to the synthesis of cholesterol in young rats.

Search for Cholesterol Precursors and Intermediates

Taylor and Gould (116) found that the acetate conversion in surviving liver slices was greatly impaired by the previous feeding of cholesterol. Srere (97) had found that squalene was likewise effective in these conditions. Langdon and Block (117) suggested this might be a good procedure to screen products which might be precursors of cholesterol. These authors incubated labeled acetate with surviving liver slices from rats which previously had been fed the following compounds: squalene, cholesterol, Δ7-cholestenol, 7-dehydrocholesterol, lanosterol, coprosterol, ergosterol, regenerated squalene, and farnesol. They found that only squalene, cholesterol, Δ7-cholestenol, and 7-dehydrocholesterol were active in decreasing the uptake of labeled acetate carbon by the liver slices. This reduction in rate was

accompanied in each case by a significant increase in the total quantity of liver cholesterol.

Schwenk, Werthessen, and Rosenkrantz (118) isolated radioactive 3β, 5α-dihydroxy-6-ketocholestane from C^{14}-labeled cholesterol synthesized by perfusion of surviving liver with acetic acid labeled with C^{14} in the carboxyl group, and from the radioactivity measurements concluded that this compound was not a precursor of cholesterol.

Tomkins, Sheppard, and Chaikoff (119) studied the influence of cholesterol feeding upon hepatic cholesterogenesis and from their results concluded that the cholesterol synthesis in the liver was under homeostatic regulation by dietary cholesterol. These authors found that one feeding of cholesterol to rats decreased the synthesis of cholesterol by the surviving liver slices, when the latter were incubated with labeled acetate. After feeding a 5 per cent cholesterol diet for eight days, cholesterol synthesis in the liver was practically stopped, while smaller amounts of cholesterol depressed the cholesterol synthesis markedly. These same authors (120) studied the cholesterol synthesis by liver when various steroids were administered, by determining the ability of the liver slices to incorporate labeled acetate to cholesterol. They found the following compounds depressed the incorporation of labeled

acetate: Δ^4 -cholestene-3-one (cholestenone), Δ^5 -androsterone-3β-ol-17-one (dehydroisoandrosterone), $\Delta^{5,7}$-3 cholestadiene-3β-ol (7-dehydrocholesterol), and Δ^7-cholestene-3β-ol (cholestenol). The following compounds were inactive: mixed soy bean sterols, ergosterol, Δ^5-pregnene-3-β-ol-20-one (pregnenolone), pregnenolone acetate, cholestane-3β-ol (dihydrocholesterol), diosgenin, Δ^5-cholestene-3β-ol-7-one acetate (7-ketocholesterol acetate).

Previously Anker and Block (121) had shown that cholestenone was not an intermediate in the synthesis of cholesterol.

Langdon and Block (122) reported that the Δ^7-cholestenol accumulated after the feeding of squalene, indicating that Δ^7-cholestenol might be a possible intermediate in the biosynthesis of cholesterol.

Sites of Cholesterol Synthesis

From the above discussion it will be noted that the liver has a marked ability to synthesize cholesterol, and the synthesis of cholesterol in liver homogenates has been reported (123). Popjak and Beeckmans (124) showed by using C^{14} and deuterium labeled acetate that synthesis of cholesterol occurred in the small intestine, stomach, and ovaries. Block, Borek, and Rittenberg (81) found that slices of kidney, testes, spleen and

gastrointestinal tract did not synthesize cholesterol, as indicated by the lack of incorporation of labeled carbon and hydrogen into the cholesterol molecule. The work of Becker et al. (113) indicated that the adrenals of the guinea pig synthesize cholesterol, and confirmed the earlier work of Srere, Chaikoff, and Dauben (125), who found that the surviving adrenal gland could incorporate the labeled acetate into cholesterol. Block and Rittenberg (126) have estimated that the rate of cholesterol synthesis by the rat liver was sufficient to fulfil the total cholesterol requirement of the animal.

Srere, Chaikoff, Treitman, and Burstein (127) studied the extrahepatic synthesis of cholesterol in normal rats, and rats deprived of various organs. The rats tested were eviscerated; eviscerated and ovariectomized, and these rats were deprived of their gastrointestinal tracts, spleens, and pancreases, and their livers were deprived of circulation. Also studied were: eviscerated, ovariectomized; eviscerated, ovariectomized and adrenalectomized; ovariectomized and adrenalectomized animals. These animals incorporated labeled acetate into cholesterol and indicated other sites of cholesterol synthesis were present. They also studied the ability of the various surviving tissues to synthesize cholesterol, and the tissues studied were: liver, gut, testis, kidney, adult skin, baby skin, adult brain,

and baby brain. They found that all the tissues with the exception of adult brain synthesized cholesterol. These results were at variance with the work mentioned previously. However, in these experiments C^{14} was used as an indicator.

Siperstein, Chaikoff, Chernick (128) demonstrated that the aorta and arteries of rabbits and chickens have the ability to synthesize cholesterol. Popjak and Beeckmans (124) found that in rabbits the biosynthesis of cholesterol took place more rapidly in the intestinal tissue than in the liver.

With the ubiquitous distribution of cholesterol, and on the basis of the above results, it would appear that probably all tissues have the ability to synthesize cholesterol, with the possible exception of adult brain tissue.

The brain is one of the most thoroughly investigated organs of the body. As evidence of this one only needs to review the vast amount of literature which appears each year regarding brain. An extensive account of the chemical investigation of brain will not be undertaken here. For a review of the early literature on the brain the reader is referred to the works of Thudichum (129) and Page (130).

As mentioned previously, the work of Couerbe (27) in 1834 indicated that cholesterol was present in brain.

He predicted that the brain had the ability to synthesize cholesterol and the evidence already discussed has indicated that cholesterol can be synthesized by the young brain, at least. Most of the experimental evidence in the literature has indicated that cholesterol and fatty acids of the brain are metabolically inactive. Cholesterol was shown to be relatively inert by Block, Berg, and Rittenberg (131), who injected labeled cholesterol in a dog, and found that the labeled cholesterol was encountered in every organ with the exception of the brain and spinal cord. This work was confirmed and extended by Waelsch, Sperry, and Stoyanoff (132), who found that the deuterium content of the unsaponifiable fraction of rat brain, containing mostly cholesterol, contained very little deuterium after the body fluids had been enriched by the administration of heavy water for periods up to eight days. However, the deuterium level in the brain fatty acid fraction was comparable with the deuterium level of the fatty acid fraction of the depot fatty acids. This indicated that the rate of turnover of fatty acids in the adult rat brain, though slow, was by no means negligible. Later work by Waelsch et al. (133), in which heavy water was administered to experimental rats, indicated that the unsaponifiable lipids and fatty acids were deposited at a rapid rate from the fifteenth to nineteenth day of extrauterine

life. The deposition decreased with increased age of the rats. Their data also indicated that the lipids deposited between the fifteenth and nineteenth days of life were synthesized in the brain. Later these same authors (134), in experiments to determine if the process of myelination or the process of growth was more important in regard to the deposition of lipids, found that the deuterium levels of the unsaponifiable lipids and fatty acid fractions were considerably greater before the process of myelination had occurred. They concluded that as the age of the rat increases the metabolism of cholesterol decreases, as indicated by the decrease in the deuterium level of the unsaponifiable fraction with increasing age.

Using acetate labeled with C^{14} in the methyl group, Srere, Chaikoff, Treitman, and Burstein (127) found that the most active tissues in converting acetate to cholesterol in vitro were: skin and liver of the adult rats, and skin and brain of day old rats. The baby rat brain synthesized cholesterol three times as fast as any other tissue except baby rat skin. These conclusions were based on the percentage of the labeled carbon added to the incubation medium which was converted to cholesterol. They found also that the capacity to synthesize cholesterol was, however, lost with advancing age. Surviving adult brain tissue failed completely to

synthesize cholesterol from the labeled acetate. In conclusion it appears that only very young brain tissue has the capacity to synthesize cholesterol.

EXPERIMENTAL

A. General Procedure for the Isolation of Sterols and Triterpenes from Plants

Each plant and plant part was worked up in the manner described by Marker et al. (24) for the isolation of plant sterols and sapogenins. The plant material was extracted with 95 per cent ethanol, the ethanol removed under reduced pressure, and the residue refluxed with 2N alcoholic hydrochloric acid for two hours. The hydrolyzed mixture was extracted with ethyl ether, the ether solution washed with water, and ethyl ether removed. The residue was saponified with 5 per cent alcoholic potassium hydroxide, cooled, acidified, and extracted with aqueous 5 per cent sodium bicarbonate solution and aqueous 5 per cent potassium hydroxide solution. The ether solution was then washed with dilute acid, the ether washed with water, and ether removed. Only the neutral and weakly acidic (phenolic) fractions were examined. The extraction and fractionation is summarized as follows:

Plant Material

Extracted with 95% ethanol extract concentrated to small volume

Plant residue (discarded)

Ethanol extract

Concentrated to small volume, hydrolyzed with acid, extracted with ether

Aqueous extract (discarded)

Ether extract

Extracted with 5% sodium bicarbonate solution

Strong acid fraction

Ether extract

Acidified and extracted with ether

Extracted with 5% potassium hydroxide

Weak acid fraction (phenolic)

Ether extract

1. Ursolic and oleanolic acids removed as potassium salts

2. Acidified with acid, extracted with ether, washed with water, ether removed by distillation

Washed with dilute acid, washed to neutrality with water, ether removed by distillation

Neutral fraction

Isolation and Identification of Sitosterol from Plantago rugelii

The neutral fractions, after several unsuccessful preliminary attempts by fractional crystallization, were found to be best purified by chromatography on a silica gel column (ratio of one to five of crude product to

silica gel) prepared by the method of Gordon, Martin, and Synge (135). The crude product was placed on the column with the help of a small amount of benzene, and elution of the column with low boiling petroleum ether yielded in the first fractions an orange pigment and a waxy hydrocarbon. Recrystallization of the waxy hydrocarbon from methanol yielded a white product, m. p. 72-75°[1], which appeared to be hentriacontane a $C_{31}H_{64}$ compound (136). Analysis. Calculated for $C_{31}H_{64}$: C, 85.23; H, 14.76. Found: C, 85.13; H, 14.75.

Continued elution with petroleum ether slowly removed the sitosterol, but more rapid removal could be effected by elution with 2 per cent ethanol in petroleum ether. Several recrystallizations from methanol gave a white crystalline product with a melting point of 138-140°, which corresponded to the melting point of sitosterol mixture as given by Fieser and Fieser (137). The melting point was not depressed by mixing with an authentic sample of sitosterol mixture from soy beans. The sitosterol mixture gave a characteristic color with the Liebermann-Burchard color reagent, and the identity of the compound was confirmed by the preparation of an acetate from a composite sample from all the plant parts. This was accomplished by refluxing the composite sample with acetic anhydride in pyridine,

[1]Melting points determined on a Fischer-Johns Bar and are uncorrected.

and working up the reaction products in the usual manner. After several recrystallizations from methanol, a product melting at 127° was obtained; not depressed by admixture with an authentic sample of an acetate, prepared in the same manner from soy bean sitosterol mixture. The melting point also corresponded to the value as recorded by Shriner and Fuson (138).

Separation of Oleanolic and Ursolic Acids

The potassium hydroxide residue was a greenish amorphous mass. This fraction was likewise purified by chromatography on a silica gel column (ratio of one to five of crude product to silica gel). The fractions were placed on the column by using a small amount of warm benzene. Elution with low-boiling petroleum ether was continued until a waxy solid was no longer removed from the column. The waxy solid was discarded. Elution of the column with 2 per cent ethanol in petroleum ether slowly removed the oleanolic and ursolic acids as a mixture. The isomers were found to be readily separated in crude form at this point by boiling in methanol and filtering or decanting the partially cooled methanol solution. The supernatant or filtrate contained the oleanolic acid, as ursolic acid was only slightly soluble in hot methanol. An alternate purification of the crude fraction, occasionally used was that of King et al. (139). This did not require chromatography.

Purification of Oleanolic Acid as the Acetate

The oleanolic acid was isolated in pure from by converting the acid to the acetate derivative, and the acetate isolated from the reaction mixture in the usual manner. Samples from each part of the plant melted sharply between 258-261°, after several crystallizations from methanol. A composite sample, from all the plant parts containing oleanolic acid, consisted of needles which melted between 259-261°, after two crystallizations from methanol. Winterstein and Stein (140) recorded 259-264° as the melting point of oleanolic acid acetate. Analysis:[1] Calculated for $C_{32}H_{50}O_4$: C, 77.06; H, 10.58. Found: C, 76.80; H, 9.88.

Oleanolic acid was obtained in pure form by saponifying samples of the acetate with 5 per cent alcoholic potassium hydroxide, and the free compound obtained by working up the reaction mixture in the usual manner. The free acid so obtained, after several crystallizations from methanol, consisted of needles and melted sharply between 295-302°. Winterstein and Stein (140) gave 305-308° as the melting point of free oleanolic acid. None of the samples from the various plant parts showed a melting point depression when mixed with an authentic sample of free oleanolic acid.[2] Likewise a composite sample of all of the free acid fractions,

---

[1]Carbon and hydrogen analyses were made by the Micro-Tech Laboratories, Skokie, Illinois.

[2]Obtained from the collection of Dr. C. E. Sando, U. S. Dept. of Agriculture, Washington, D. C.

after two crystallizations from acetone, consisted of needles. They gave the characteristic purple color with the Liebermann-Burchard reagent, and melted between 302-305°. Analysis. Calculated for $C_{30}H_{48}O_3$: C, 78.89; H, 10.58. Found: C, 78.66; H, 10.38.

Oleanolic Acid Benzoate

A composite sample of the free acid was boiled with benzoyl chloride in pyridine, and the derivative isolated from the reaction products in the usual manner. A benzoate, m. p. 259-261°, was obtained after several recrystallizations from methanol. Obata (141) recorded the melting point of oleanolic acid benzoate as 260-261°.

Purification of Ursolic Acid as the Acetate

The crude ursolic acid obtained as described above consisted of an amorphous, green powder, which even after many attempts to obtain a pure compound by crystallization from various solvents, remained a green amorphous powder. It was found that the best method of achieving purification was to prepare the acetate in the usual manner. The crystalline derivative was isolated from the reaction products. After recrystallization from acetone or methanol, it melted at 284-289°. A composite sample from all the plant parts consisted of needles after two crystallizations from acetone, and melted at

286-287°. This value agreed very well with 288-289°, as recorded by Rowe et al. (142), and the value of 289-290° as recorded by Sando (143). Analysis. Calculated for $C_{32}H_{50}O_4$: C, 77.06; H, 10.10. Found: C, 77.04; H, 10.12.

Free Ursolic Acid

Saponification of ursolic acid acetate from each part of the plant parts with 5 per cent alcoholic potassium hydroxide gave an amorphous white powder, m. p. 260-285°, and numerous attempts to obtain the free acid in crystalline form failed. The ursolic acid was obtained in crystalline form by treatment of the saponified product according to the procedure of King, Chatterjee, and Parks (139). The resulting precipitate was recrystallized from methanol and yielded small needle-like crystals, which melted at 283-285°. The melting point was not depressed when mixed with an authentic sample of ursolic acid.[1]

A composite sample from all the plant parts, after two recrystallizations from ethanol, consisted of needles, m. p. 283-285°, and agreed very well with the value of 282-284° as given by Rowe et al. (142) and the 284-285° as reported by Sando (143) and Jacobs (144)

[1]Kindly supplied from the collection of Dr. C. E. Sando at U. S. Dept. of Agriculture, Washington, D. C.

for free ursolic acid. The melting point of the composite sample was not depressed when mixed with an authentic sample of ursolic acid. The free compound gave the characteristic color of a triterpene hydroxy acid with the Liebermann-Burchard color reagent. Analysis. Calculated for $C_{30}H_{48}O_3$: C, 78.89; H, 10.58. Found: C, 78.62; H, 10.80.

Preparation of Ursolic Acid Acetate Acid Chloride

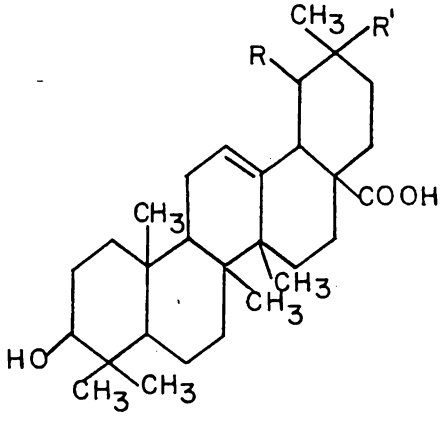
The procedure used for the preparation of this derivative was given by Rowe, Orr, Uhl, and Parks (142). A small sample of ursolic acid acetate was used. Recrystallization from high boiling petroleum ether gave white needle crystals, m. p. 221°. Rowe et al. (142) recorded the melting point of the acid chloride as 222° (corrected) and Goodson (145) reported a melting point of 224-226°.

Preparation of Ursolic Acid Monoacetyl Methyl Ester

A portion of the acid chloride, prepared as above, was refluxed four to six hours with methanol. The reaction mixture was mixed with water and precipitate removed with ethyl ether. Several recrystallizations of the product from methanol gave a white crystalline solid which melted at 247°. The melting point of 246-247° was recorded by Sando (143).

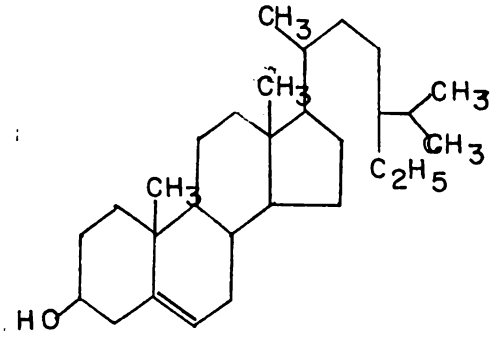
Results

A detailed search of the lipid fractions of the whole mature plants of Plantago rugelii revealed only the presence of hentriacontane, ursolic acid, oleanolic acid and the sitosterol mixture. The structures of ursolic acid (146), oleanolic acid (147), and sitosterol (148) are given below.



URSOLIC ACID: R=CH_3
R' = H

OLEANOLIC ACID: R = H
R = CH_3



SITOSTEROL

Some quantitative relationships between the sterol, sitosterol, and the triterpenes, ursolic and oleanolic acids, are given in Table I. The figures representing the quantities of triterpenes and sitosterol should be accepted as only roughly quantitative because of the difficulties associated with the isolation in pure form.

TABLE I

DISTRIBUTION OF TRITERPENES AND STEROL IN PLANTAGO RUGELII

Plant Part	Weight in Gms.	Yield - Gms. Ursolic Acid	Oleanolic Acid	Sitosterol
Young Plant				
Roots	565	0.9		0.8
Leaves	3819	2.3		3.8
Mature Plant				
Roots	1004	0.9		1.2
Leaves	2625	5.0	2.0	2.9
Seed stalks	3363	0.5	1.0	0.9
Flower parts	4016	6.0	1.5	4.3

Sitosterol and ursolic acid were found to occur in all parts of the plants studied, however, oleanolic acid was found only in the aereal parts of the mature plants. The leaves were found to contain the largest amounts of oleanolic and ursolic acids.

Investigation of Additional Plants

A number of whole mature plants were examined for the presence of ursolic and oleanolic acids. Both of these carboxylated triterpenes form insoluble sodium and potassium salts. Hence on shaking an ether solution of the free acids with 5 per cent potassium hydroxide or

sodium hydroxide, the potassium or sodium salts (respectively) readily precipitate out as a heavy deposit. If present in good quantity, they can, therefore, be detected in plant extract hydrolysates by this method. The formation of a precipitate by this crude procedure does not insure absolutely the presence of the two acids. However, the absence of a precipitate, is sufficient to insure the absence of the two acids in appreciable quantity. Of the plants investigated by this procedure (Table II), only Lamb's Quarters yielded oleanolic acid. The examination of the neutral fractions has not been completed.

TABLE II

ADDITIONAL WHOLE MATURE PLANTS EXAMINED FOR URSOLIC AND OLEANOLIC ACIDS

Technical Name Common Name	Family[1]	Quantity of Plant (Gms.)
Verbena hastata Blue vervain	Verbenaceae	1,000
Xanthium orientale Cocklebur	Compositae	3,838
Asclepias syriaca Common Milkweed	Asclepiadaceae	4,420
Ambrosia artemisiifolia Common Ragweed	Compositae	3,322
Erigeron annuus Daisy Fleabane	Compositae	1,200
Vernonia noveboracensis Ironweed	Sunflower	5,448
Chenopodium album Lamb's Quarters	Chenopodiaceae	3,973 (mature plant)
Polygonum pennsylvanicum Smartweed	Polygonaceae	3,759

[1] J. M. Fogg, Jr., "Weeds of Lawn and Garden," Univ. of Penn. Press, Philadelphia, Pa. (1945).

B. Exploratory Investigation of Beef Brain Tissue

Brain tissue consists principally of four distinct types of chemical substances:

(1) water
(2) phospholipids, cerebrosides
(3) cholesterol
(4) protein.

To obtain all of the neutral and weak acid material of the brain tissue lipid extracts, a thorough extraction of brain tissue was performed. However, this method had the disadvantage of removing water, phospholipids and cholesterol from the brain, they were impurities as far as this work was concerned. The operations described here were purely exploratory, and many of the steps were designed to effect some preliminary fractionation to make subsequent isolation and identification of the constituents easier. During the work the interest became centered on a fragrant, oil mixture in the neutral fraction.

General Procedures for Extraction and Fractionation

The sixty-five beef brains were obtained as soon as possible after their removal from the animals, minced by hand and placed in five gallon glass jugs containing 95 per cent ethanol. The liquor was removed from the brain tissue by filtration and the ethanol was distilled off under reduced pressure. The residual brain tissue was ground in a meat grinder, placed in a large metal

continuous extractor, and thoroughly extracted with ethanol, acetone, and chloroform in succession. The residues remaining after the removal of the solvents were combined and contained considerable amount of water, originally present in the brain.

The water was removed by distillation on a steam bath under reduced pressure. When the extract became concentrated, excessive foaming occurred. This was controlled by adding antifoaming agent. The final traces of water were removed by co-distillation with benzene-ethanol and the excess benzene-ethanol removed by evaporation.

The residue so obtained was extracted thoroughly with acetone. This effected a crude separation of cholesterol from phospholipids. The acetone extracts were treated with 5 ml. of a saturated alcoholic solution of magnesium chloride per 1000 ml. of acetone solution and allowed to cool. This removed any phospholipid material which might have been present in the acetone solution. The precipitate which formed was removed by filtering and discarded. The acetone was then removed by distillation, leaving a whitish crystalline residue. The cholesterol was removed from this residue by repeated fractional crystallizations from hot acetone.

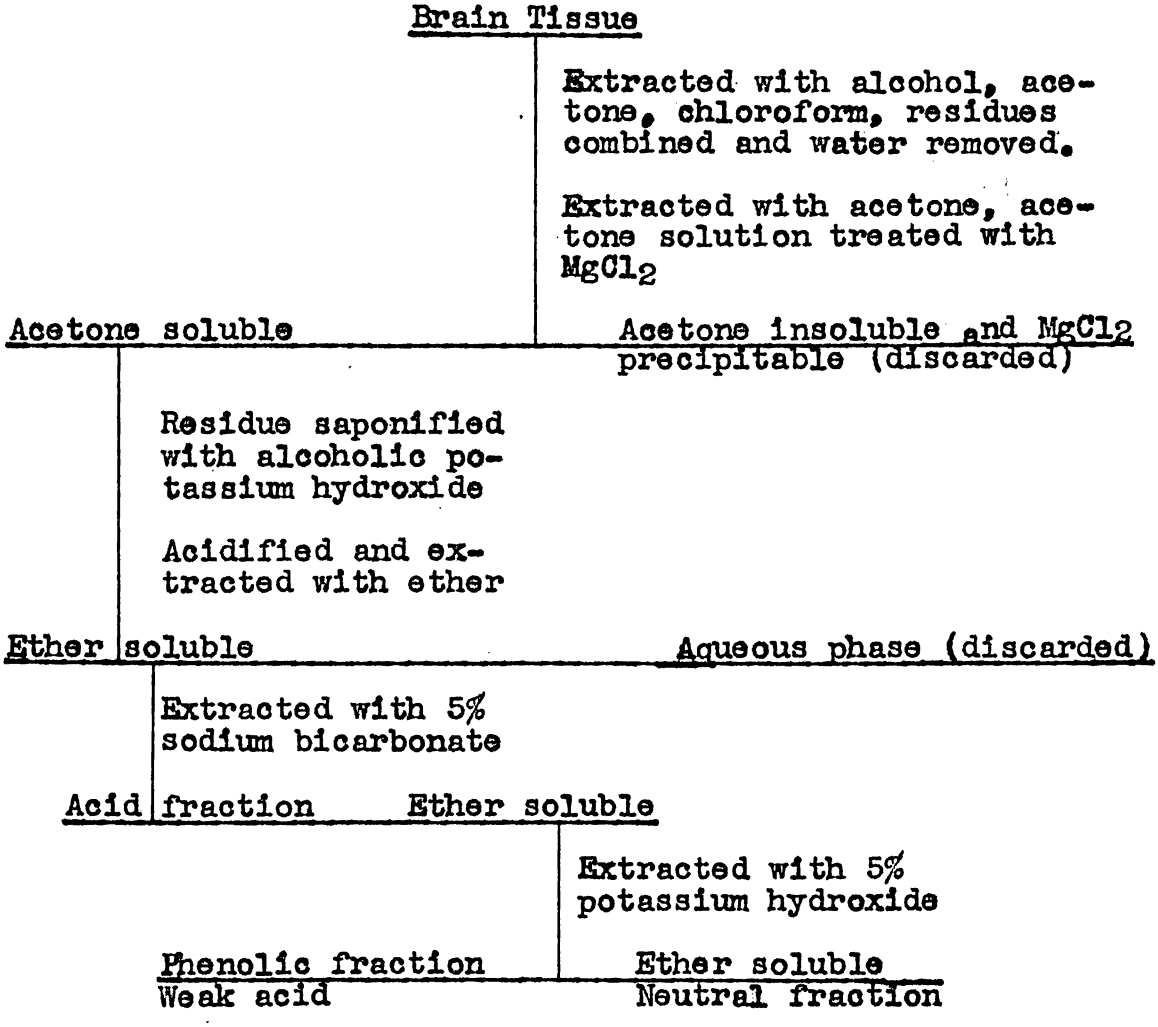
The acetone-soluble material was saponified in 4 liters of alcoholic potassium hydroxide (the alcohol

solution contained 800 grams of potassium hydroxide) for two hours under an atmosphere of nitrogen. The saponified mixture was cooled, acidified, and extracted with ethyl ether. The ether solution was washed with water. Emulsification difficulties were encountered during this operation, and the wash water was discarded. The ether solution was extracted with aqueous 5 per cent sodium bicarbonate solution, and extraction continued until the acidification of the last extract did not form any precipitate. The acidified extract was extracted with ethyl ether, the ether solution washed with water, and the ether removed by distillation. The residue constituted the strong acid fraction. Subsequent work up of this fraction, which will not be described, indicated that the fraction did not contain any compounds of interest at present.

The ether solution was next extracted with aqueous 5 per cent potassium hydroxide until the last extract did not form any precipitate when acidified. Extraction of the acidified extract with ethyl ether, and work up in usual manner, yielded the weak acid or phenolic fraction.

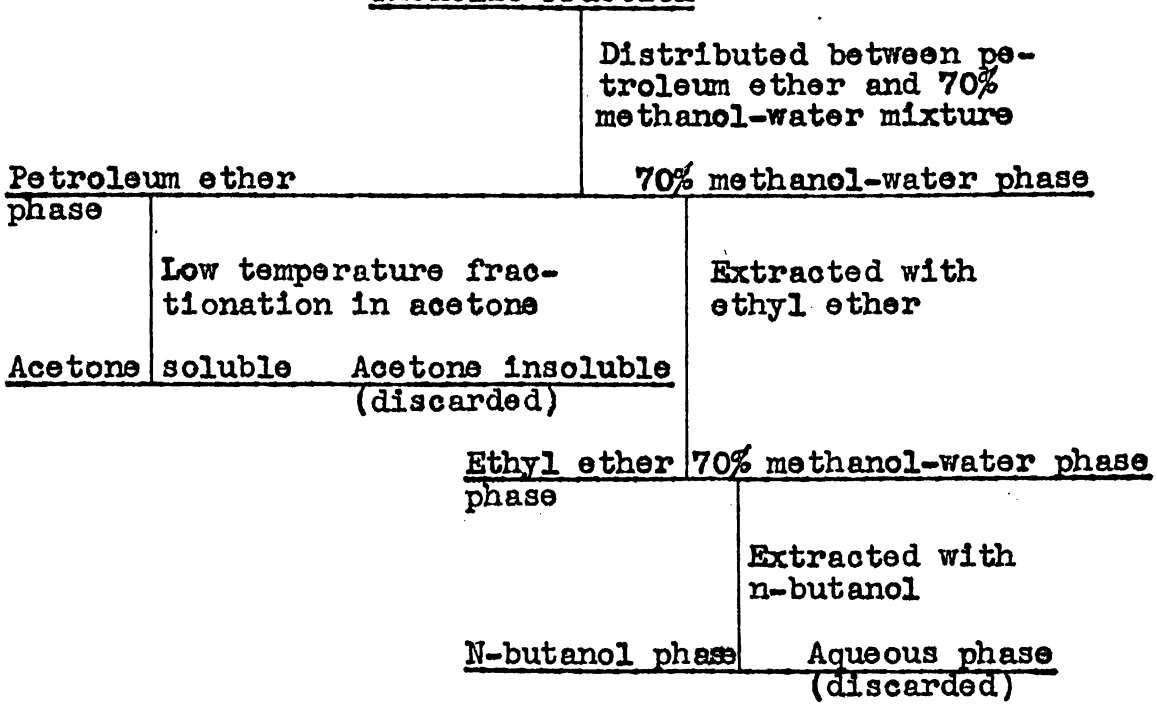
The remaining ether solution was washed with dilute hydrochloric acid (50 ml. of acid diluted to 500 ml. with water), washed with water and ether removed. The residue constituted the neutral or non-saponifiable fraction.

The extraction of the brain tissue and fractionation of the extract is summarized in the following flow sheet:



Work up of the Phenolic Fraction

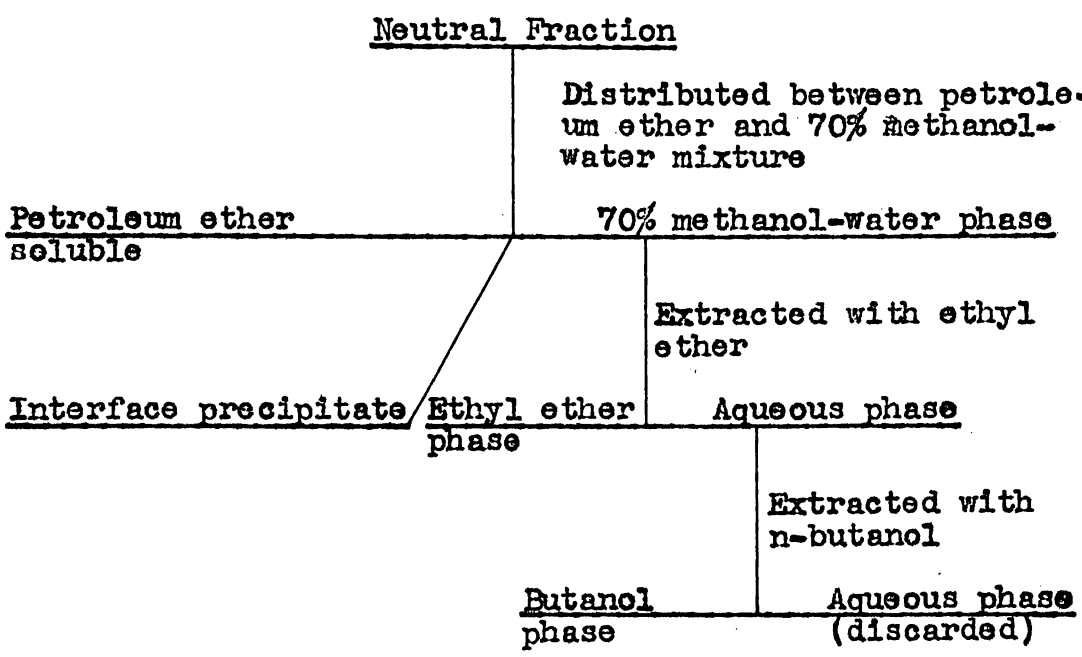
The phenolic fraction was treated as indicated in the following flow sheet.



Further examination of the various fractions shown on the above flow sheet revealed no substances present of interest for further investigation. Therefore, a detailed description of the work on the phenolic fraction will not be given.

Work up of the Neutral Fraction

The neutral fraction was treated as indicated in the flow sheet. Examination of butanol, ethyl ether phases and interface precipitate fractions did not yield any identifiable material, and will not be discussed further.



Chromatographic Fractionation of the Petroleum Ether Soluble Fraction

This fraction had been stored under petroleum ether in the refrigerator until it could be examined. Cholesterol was removed by repeated recrystallization from hot acetone. Eighty-two grams of yellowish-brown solid remained after the removal of the solvent from the mother liquor of the above crystallizations. The fraction was suspended in petroleum ether and introduced in equal quantities into two-100 gram chromatograph columns containing a mixture of 50 grams of magnesium silicate and 50 grams of Celite. The following fractions were collected:

Fraction 1. Elution of each column with one liter of low boiling petroleum ether yielded a combined total of 62 grams of brownish crystalline solid, which liquified readily on a steam bath. Continued elution with petroleum ether yielded 29 grams of cholesterol.

Fraction 2. Elution with 2 per cent ethanol-petroleum ether mixture yielded 3.403 grams of yellowish-brown gum which readily liquified on a steam bath, but was not very soluble in benzene or acetone at room temperature. Further elution of the column was not undertaken.

Examination of Fraction 2

Recrystallization of the fraction from acetone-methanol mixture yielded yellowish-orange crystalline clumps, m. p. 66-80°. Recrystallization from acetone-methanol mixture, after removing some insoluble material, yielded orange microspheres, m. p. 60-75°.

Five hundred milligrams of fraction 2 were refluxed for 30 minutes with 20 ml. of 5 per cent alcoholic potassium hydroxide solution under nitrogen. When the solution was extracted with ether without previous acidification, difficulty was encountered. The mixture was acidified, and extracted with ether. The extraction of the ether solution with aqueous 5 per cent potassium hydroxide solution did not remove any material from the ether. This indicated that the fraction consisted of neutral material. The ether was removed leaving a yellowish-brown gum.

Recrystallization of the residue from methanol-acetone, after decanting from some insoluble material, yielded orange crystalline clumps, m. p. 60-67°. The melting point was not depressed when mixed with an original sample of material, m. p. 80-100°.

An acetate was prepared, using 688 mg., and after several recrystallizations from acetone, yellowish clumps were obtained, m. p. 101-103°. One-hundred mg. of the acetate were saponified by refluxing with 5 per cent alcoholic potassium hydroxide for 30 minutes under an atmosphere of nitrogen. Work up of the saponified mixture in the usual manner yielded 94 mg. of a waxy solid. Recrystallization from acetone, yielded white crystalline clumps, m. p. 90-91°. The substance was not examined further at this point.

Rechromatography of Fraction 1

A preliminary run was made with 5 grams of this fraction on a fifty-fifty part silica gel-Celite column. Elution with petroleum ether indicated that further fractionation was affected. The remaining 57 grams were chromatographed on a 200 gram silica gel-Celite column of similar composition. The following fractions were collected:

Fraction 3. Elution with low boiling petroleum ether yielded 8.06 grams of yellow oil.

Fraction 4. Further elution with petroleum ether yielded more cholesterol.

Fraction 5. Continued elution with petroleum ether yielded 1.19 grams of a white solid.

Fraction 6. Elution with 2 per cent ethanol in petroleum ether yielded 21.9 grams of a brownish crystalline solid.

After the elution with the 2 per cent ethanol-petroleum ether mixture the column was discarded.

Work up of Fraction 5

The complete fraction was recrystallized from acetone. Transparent needles and plates were obtained, which melted between 138-143°. Preparation of an acetate was undertaken with 792 mg. of the fraction in the usual manner. Subsequent work-up of the reaction mixture, followed by several recrystallizations of the reaction products from acetone-methanol mixture yielded jagged needles which melted between 113-115°. The melting point of cholesterol acetate was recorded by Anderson (149) as 116°.

Work up of Fraction 6

The brownish crystalline solid was recrystallized twice from acetone, and an orange gummy solid was obtained which melted between 40 and 100°.

The orange gummy solid was warmed with methanol, the solution was allowed to cool slightly, and the supernatant was decanted from some yellow oil. A voluminous precipitate was observed, after the solution had been allowed to stand in the refrigerator overnight. Recrystallization of the precipitate from acetone-methanol solution yielded yellowish clumps, which melted between 90 and 100°. An acetate was prepared in the usual manner from 778 mg. of the fraction. Recrystallization of the reaction product from acetone-methanol mixture, fol-

lowed by decolorization with charcoal in benzene yielded white irregular crystals which melted between 92 and 94°. The melting point of this acetate corresponded very well with the melting point of the acetate prepared from a previous fraction, see page 65.

Isolation and Attempted Characterization of Hydrocarbon Material from Fraction 3

A small amount of Fraction 3, 0.217 grams, was saponified under nitrogen for 30 minutes with 5 per cent alcoholic potassium hydroxide solution and the neutral material was obtained in the usual manner. The extraction with potassium hydroxide was performed to insure that only neutral material was present. As only the original product was recovered, the starting material was considered neutral.

A small amount of Fraction 3 decolorized bromine in carbon tetrachloride without the evolution of gas. It was concluded that the fraction was unsaturated or contained some unsaturated material.

Chromatography on Alumina

The material which constituted Fraction 3 appeared to be an unsaturated hydrocarbon mixture. A portion, 0.217 grams, was chromatographed on an alumina column according to the procedure of Sobel (150) for the puri-

fication of squalene. The material was eluted from the column with a small volume of petroleum ether. As a control, a sample of squalene was chromatographed on a similar column. The behavoir of the two fractions was very similar. From these observations it was concluded that Fraction 3 was either squalene, a similar hydrocarbon, or a mixture.

The infrared absorption curve[1] indicated that Fraction 3 and squalene were not identical, as shown in Infrared Spectra 1 and 2, page 69.

Fraction 3 and squalene did not exhibit any selective absorption in the ultraviolet region[2] from 220 to 310 mμ. The absorption curves of Fraction 3 and squalene are given by Ultraviolet Spectra 1 and 2 respectively, page 70.

Attempted Preparation of a Hydrochloride

From preliminary observations concerning Fraction 3, it appeared that the fraction might be squalene. Squalene is best characterized by the preparation of the hexahydrochloride, formed by passing dry hydrogen chloride gas through an acetone solution of the squalene according to the method of Heilbron et al. (49). A sample

---

[1]Infrared spectrum obtained using NaCl prism in a Model 21 double-beam recording Spectrophotometer manufactured by Perkin Elmer & Corporation. All spectra are recorded in frequency (cm^{-1}).

[2]All ultraviolet spectra made using a Beckman DU Spectrophotomer. One mg. sample was dissolved in 95% redistilled alcohol and diluted to 100 ml.

Fig. 1

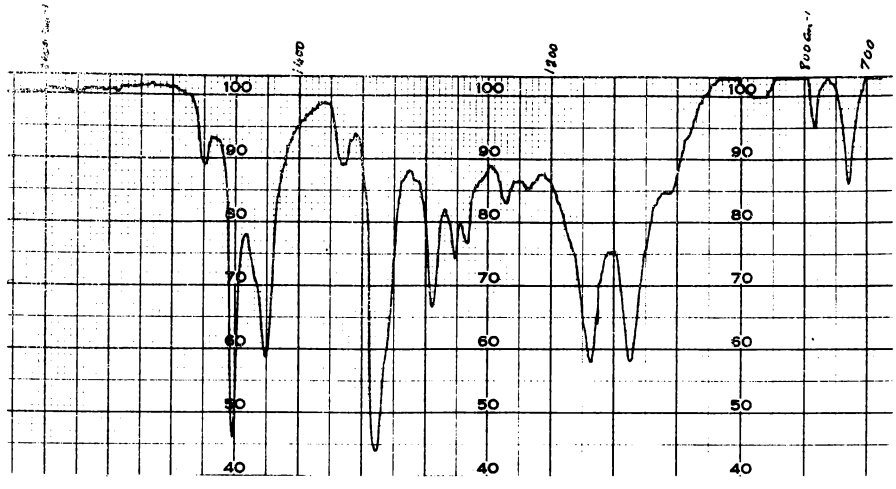


Fig. 2

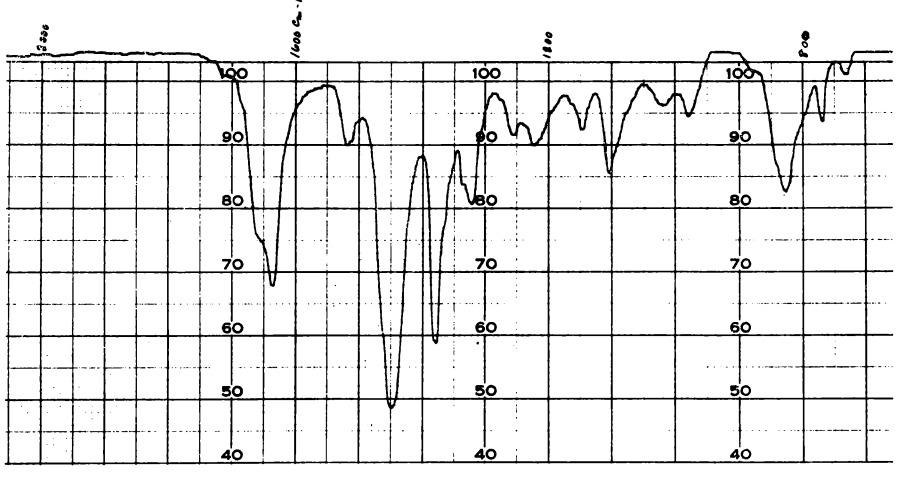


Fig. 3

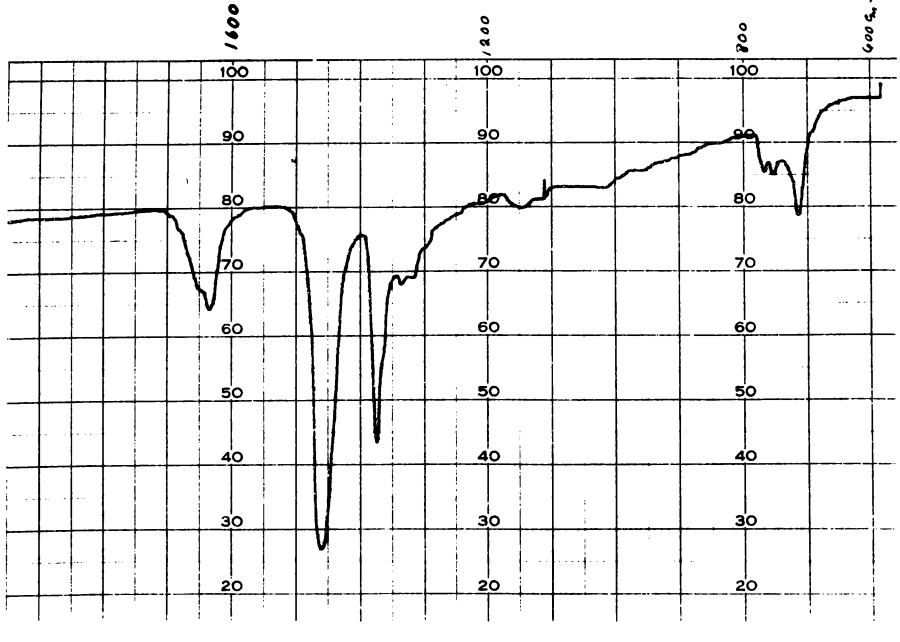


Fig. 1

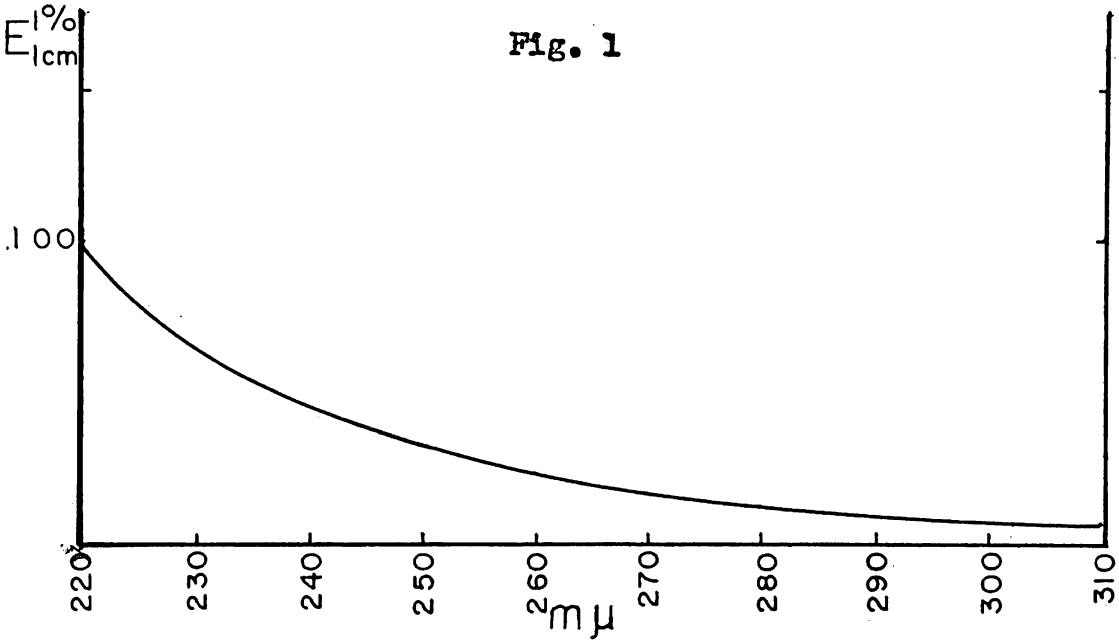


Fig. 2

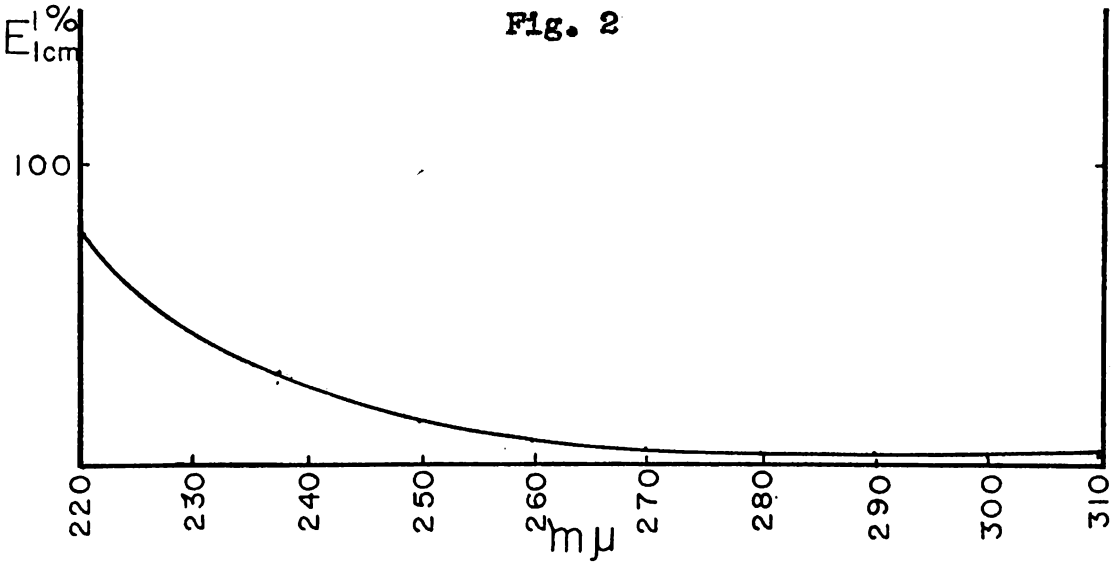
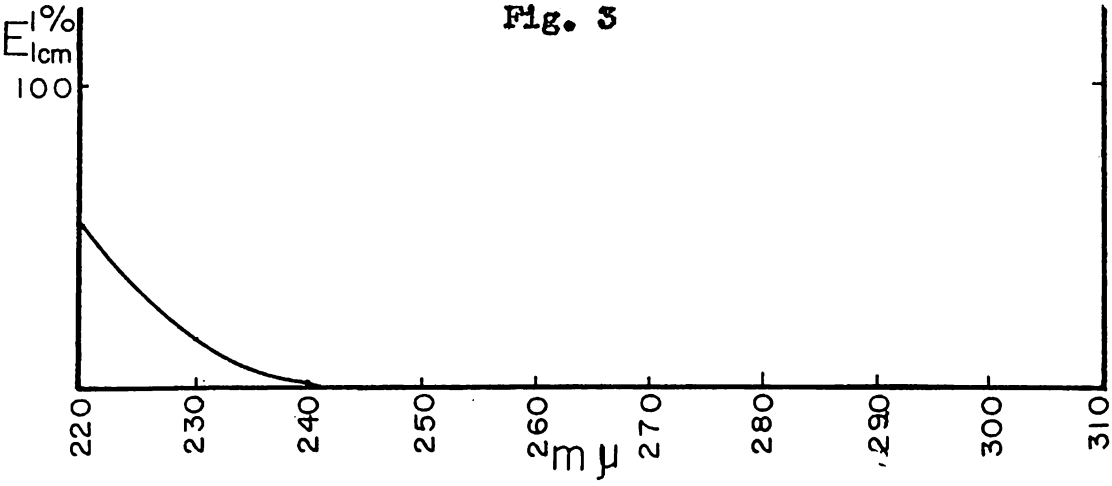


Fig. 3



of squalene was chromatographed and the hydrochloride prepared by passing dry hydrogen chloride gas for about 30 minutes through a cooled acetone solution of the squalene. A white crystalline product formed, which after recrystallization from acetone melted between 111-126°. The melting point agreed with the value of 110-126° recorded by Heilbron et al. A small sample of Fraction 3, 516 mg., was used and when introduced into the acetone solution previously saturated with dry hydrogen chloride gas, the solution turned yellow. After passage of the gas for approximately 20 minutes a white-yellow precipitate formed. Difficulty was encountered in the purification of this mixture. Recrystallization from acetone yielded an oil at room temperature. Leaching the oil with methanol, decanting the methanol, and cooling yielded a quantity of white solid, m. p. 43-51°. Several recrystallizations from acetone or acetone-methanol mixture gave white irregular crystals, m. p. 60-61°. Analysis. Calculated for $C_{30}H_{50}$-6HCl: C, 57.7; H, 8.9; Cl, 33.9. Found: C, 80.54; H, 13.37; Cl, 2.28.

From the above data, it was concluded that the substance was not squalene, and was probably a mixture of unsaturated hydrocarbons.

Further Examination of Fraction 3

Several of the colorless fractions from the silica gel-Celite columns were combined, yielding 345 mg. of Fraction 3. This was subjected to chromatography for purification.

The fraction was taken up in petroleum ether, placed on a 40 gram alumina column and eluted with 500 ml. of low boiling petroleum ether. Then 241 mg. were recovered by distillation of the petroleum ether. It was again introduced onto a 40 gram alumina column and eluted with 500 ml. of low boiling petroleum ether, and 237 mg. of colorless oil were recovered. The oil was dried overnight over P_2O_5 under water-pump aspirator vacuum. Found: C, 86.10, 85.86%; H, 14.00, 13.90%. Molecular weight: 180. These data corresponded to an empirical formula of $C_{13}H_{25}$. Refractive Index (32°) 1.4650.

These data confirmed the original suggestion that Fraction 3 was a hydrocarbon.

Iodine Number

An attempt was made to determine the iodine number by the Yasuda (151) method, as given by Fitelson (152), but it was not satisfactory and a low value was indicated.

Vacuum Distillation of Fraction 3

A portion of Fraction 3, 1.349 grams was distilled at 0.01 mm. The bath temperature was slowly raised to

200°, at which temperature some distillate formed. The distillation temperature slowly rose from 130 to 140° and then dropped. Increasing the bath temperature had no effect, and so the distillation was discontinued. A light yellow oil was obtained, which weighed 0.255 grams. This fraction was chromatographed on alumina and 0.238 grams were recovered. Refractive index (31°) 1.4629, which agreed very well with the value obtained after extensive purification by chromatography on alumina.

C-methyl Determinations

One hundred and thirty-nine milligrams of Fraction 3 were subjected to C-methyl determination as described by Kuhn and Roth (153). There were 65 grams of acetic acid produced per mole of the compound. The purpose of this determination was to find if the substance was branched. The value, while not quantitative, indicated that the fraction contained some material which contained a branched carbon chain. This would also indicate that the fraction might be a mixture.

Preparation of a Bromide

Preparation of a bromide was attempted with 365 mg. of the colorless oil in anhydrous ether. No solid product could be obtained from the mixture.

Summary

From the neutral fraction of the acetone-soluble material from brain tissue, a small amount of fragrant oil was isolated. This substance was not squalene, nor did it contain any squalene. The analyses which were performed on this oil indicated that it was probably a mixture of hydrocarbons. It was felt that the work should be repeated with carefully purified solvents.

There was also isolated from the neutral fraction a low melting substance which appeared to form an acetate.

C. Investigation of the Second Quantity of Beef Brain Tissue

The following fractionation of the lipid fraction of fresh brain tissue was undertaken to verify the presence of the oily hydrocarbons as indicated by the exploratory work. For this work all solvents were distilled twice or otherwise treated to insure the absence of oily contaminants as impurities. The general process is a simplification of that previously described, and was primarily designed to obtain the acetone-soluble material from brain tissue. Only a small amount of hydrocarbon was obtained by acetone extraction, but by extracting of the brain tissue with chloroform an additional quantity of fragrant oil was obtained.

An additional fragrant oil, more polar than the mixture previously found, was isolated during this investigation.

General Procedures for Extraction and Fractionation

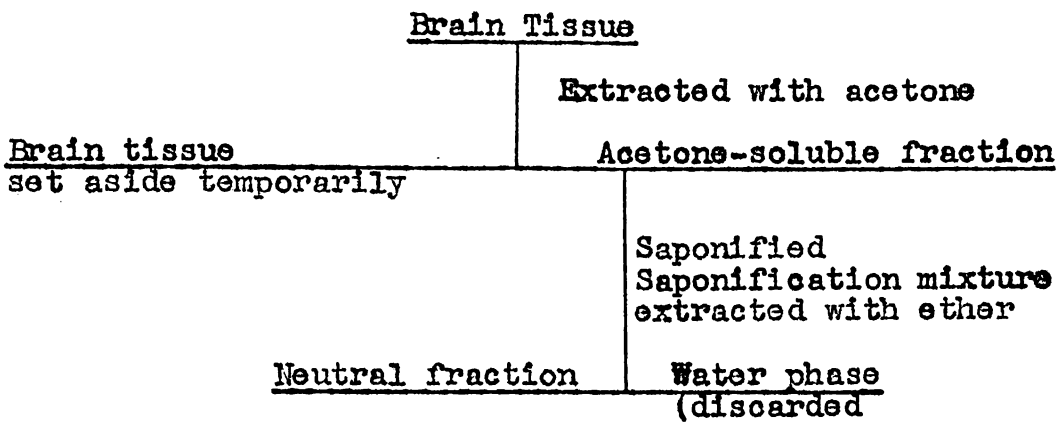
One hundred beef brains were obtained immediately from the animals, ground with a meat grinder and placed in double-distilled acetone in four 5-gallon glass jugs. After several extractions with fresh acetone the acetone was removed, the brain tissue was ground again, and extraction was continued in a metal continuous extractor. The acetone was removed from the extract, leaving a con-

siderable amount of water and solid material. The water was removed by distillation under reduced pressure and the remaining mass was repeatedly extracted with hot acetone. The acetone-soluble material so-obtained consisted of a whitish-yellow crystalline mass.

Cholesterol was removed from this mass by repeated recrystallization from hot acetone. When the removal of the cholesterol had been almost completed, the acetone was removed by distillation from the mother liquor, with the final traces removed under a stream of dry nitrogen on a steam bath. There were 459 grams of a yellow-brown material obtained, which softened readily on a steam bath.

This mass was saponified for 30 minutes under nitrogen with 1500 ml. of 5 per cent alcoholic potassium hydroxide. The saponified mixture was cooled and extracted with ethyl ether in the usual manner. Only the neutral fraction was saved.

The ethyl ether solution (containing the neutral material) was washed four times with distilled water, and the ether was removed. Then 210 grams of yellow crystalline solid material, which possessed a marked fragrant, penetrating lemon-like odor, remained. The whole mass became gummy when heated on a steam bath. The extraction and fractionation procedures are summarized below:



Chromatographic Fractionation of Neutral Material

Since this material still appeared to contain much cholesterol and was too bulky to chromatograph intact, it was treated as follows. The fraction was triturated with two-600 ml. portions of petroleum ether, the reddish-yellow petroleum ether solution decanted, and the petroleum ether removed, leaving 54 grams of an orange-red semi-solid crystalline mass.

The white solid remaining behind after the decantation was set aside as petroleum ether decanted residue.

The orange mass decanted in the petroleum ether was examined as follows. Two grams were placed on an 80-gram alumina column and eluted with 24-30 ml. portions of petroleum ether. Combination of the residues yielded a small quantity of a colorless oil with a characteristic lemon-like odor. This oil appeared to be very similar to the oil described in the previous section on brain. The results with the alumina column indicated

that the desired fractionation had been effected, and so the remaining 52 grams of the petroleum ether-soluble fraction were divided between two 200-gram alumina columns, and the following fractions collected:

> Fraction 1. Elution of the two columns with 400 ml. of petroleum ether each yielded 0.669 grams of a colorless fragrant oil with a lemon-like odor. Yellow bands were moving down the columns, so the elution was stopped and this fraction taken before any colored material had been eluted.
>
> Fraction 2. This fraction was eluted from the columns by 1900 ml. of petroleum ether in each column, 0.728 grams of a deep-yellow crystalline solid were obtained.
>
> Fraction 3. Elution was continued using petroleum ether until all of the colored bands had been removed. This required about 2,000 ml. of solvent for each column, and 0.674 grams of solid material were obtained. The continued elution of the column yielded only cholesterol; elution was discontinued.

The fractionation of the petroleum ether decanted material is summarized by the diagram below.

Petroleum ether decanted material

	Eluted with 400 ml. of solvent
Fraction 1 (Hydrocarbon)	Fraction on column
	Eluted with 1900 ml. of solvent
Fraction 2	Fraction remaining on column
	Eluted with 2,000 ml. of solvent
Fraction 3	Fraction remaining on column (column discontinued)

The previous work on extracts of brain tissue indicated the presence of hydrocarbon material. Fraction 1 exhibited very similar properties to those observed previously.

Examination of Fraction 1

The entire fraction, 0.699 grams, was introduced onto a 30-gram alumina column and elution with 300 ml. of petroleum ether yielded a colorless, fragrant oil weighing 0.611 grams. The following data were obtained on this substance. The purity of the product could not be assured. However, it was possible to confirm the fact that brain tissue does contain some hydrocarbon substances.

Density (26°) 0.857 (w/v)
Refractive index (28.5°) 1.4662
Iodine number Low (determination not very satisfactory).
Optical rotation None
C-methyl determinations The quantity of acetic acid obtained indicated the presence of material containing a branched chain.
Found: C, 86.10 85.88; H, 13.98 13.84
Molecular weight (Rast) 230
Empirical formula $C_{16}H_{32}$

The infrared absorption spectrum of Fraction 1 is given by Infrared Spectrum number 3, page 69. Comparison with the Infrared Spectrum number 1, the absorption spectrum of hydrocarbon material from the exploratory investigation of brain, indicated a similar, yet not identical fraction.

The ultraviolet absorption spectrum of Fraction 1 is given by Ultraviolet Spectrum number 3, page 70.

Fraction 1 did not exhibit selective absorption over the range studied.

The remaining portion of Fraction 1 was stored in a stoppered flask filled with nitrogen in the refrigerator until the extraction and reworking of the fraction could be completed.

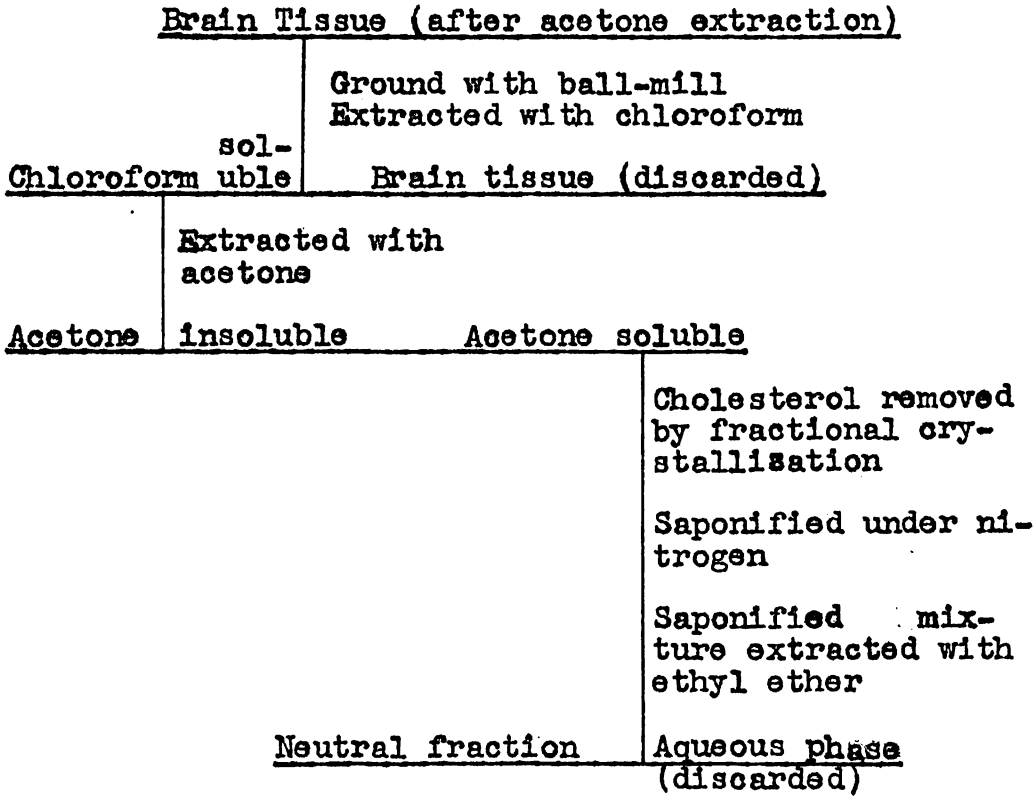
Rechromatography of Fractions

Several fractions mentioned previously were rechromatographed on alumina, and the first material eluted with low-boiling petroleum ether was set aside as hydrocarbon material. The fractions rechromatographed were the residue from the petroleum ether triturations described on page 77 and Fractions 2 and 3 described on page 78. Continued elution yielded in some cases solid material, which was rechromatographed and the small amounts of viscous hydrocarbon obtained were set aside. The detailed description of these procedures will not be given. The hydrocarbon material mentioned was stored under nitrogen in the refrigerator until it was used.

Extraction of the Brain Tissue with Chloroform

The dried brain tissue remaining after the acetone extraction was ground in a ball-mill and thoroughly extracted in the continuous extractor with double-distilled chloroform. The chloroform was removed by distilla-

tion and final traces of chloroform removed by using a water-aspirator pump and steam bath. The large mass of yellowish-brown gum, which consisted mostly of phospholipid, was thoroughly extracted with hot acetone. The following diagram indicates the process in outline form.



The cholesterol was removed from the acetone soluble portion by repeated recrystallization from hot acetone. After removal of the solvent 200 grams of brownish solid, which was a semi-fluid at room temperature and possessed a strong pungent odor, remained.

The residue was saponified with 1500 ml. of 5 per cent alcoholic potassium hydroxide for 30 minutes under

an atmosphere of nitrogen, and the neutral fraction was obtained in the usual manner. Removal of the ethyl ether yielded a fraction weighing 90 grams. The fraction was dissolved in petroleum ether and divided between four 200-gram alumina columns. The following fractions were collected

> Fraction 1. This fraction was obtained by eluting each column with 100 ml. of petroleum ether. The residues, which were combined, consisted of a colorless, fairly mobile oil, and possessed the characteristic lemon odor.
>
> Fraction 2. This fraction was obtained by eluting each column with 3 liters of petroleum ether and consisted of a yellow-orange oil with an intense lemon odor. The residues were combined into one fraction.
>
> Fraction 3. Elution of the columns with 2 per cent ethanol in petroleum ether until all the colored material had been eluted, yielded 43 grams of a yellow-orange solid.
>
> Fraction 4. Elution of the columns with 5 per cent ethanol petroleum mixture yielded 10 grams of a light brown waxy solid.

Fractions 1 and 2 were set aside to be combined into the "hydrocarbon fraction". While Fractions 3 and 4 were treated separately and will be described later.

The work is summarized as follows:

Chloroform Neutral Fraction

	Placed on four 200-gram alumina columns Elution with 100 ml. of petroleum ether
Fraction 1	Portion remaining on column
	Elution with 3 liters of petroleum ether
Fraction 2	Portion remaining on column
	Elution with 2% ethanol-petroleum ether mixture
Fraction 3	Portion remaining on column
	Elution with 5% ethanol-petroleum ether mixture
Fraction 4	Residue (discarded

Hydrocarbon Fraction

The fractions described on page 80 and Fractions 1 and 2 obtained from the chloroform extract of brain tissue described on page 82, when combined consisted of a dark yellow oil and weighed 5.42 grams. The mixture possessed a very strong lemon odor. It did not appear that additional fractionation could be accomplished by chromatographic procedures. Fractional distillation under reduced pressure was, therefore, undertaken. However, before the fraction was subjected to fractional distillation, it was examined as follows:

Density (26°) 0.901 (w/v)
Refractive index (27°) 1.4928

The infrared and ultraviolet absorption spectra are given by Infrared Spectrum number 4, page 84, and Ultra-

Fig. 4

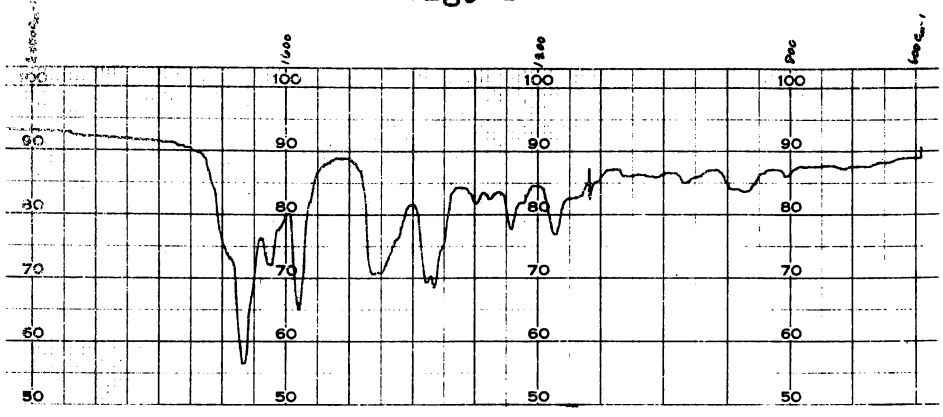


Fig. 5

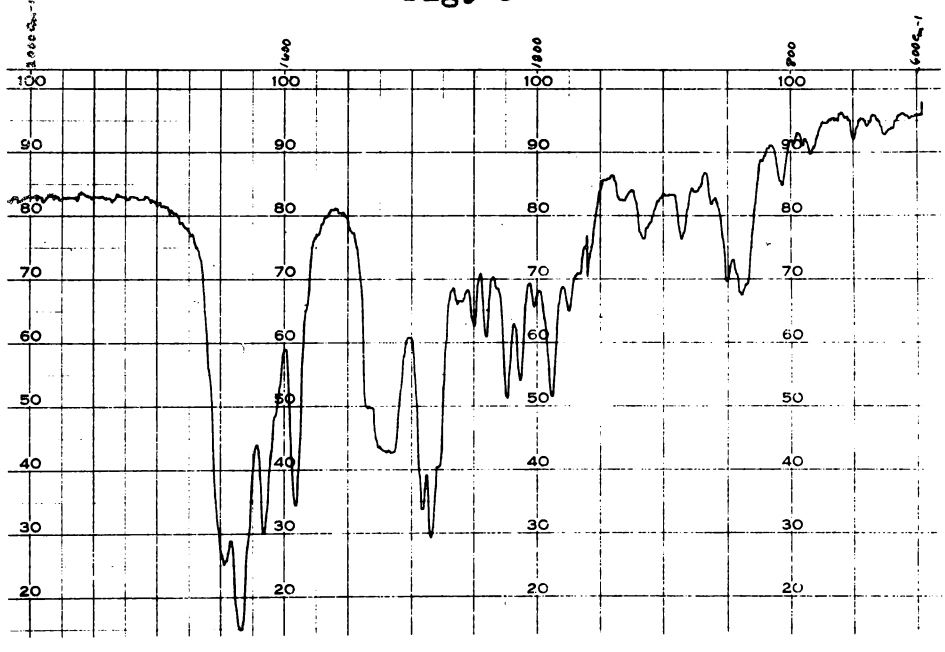


Fig. 6

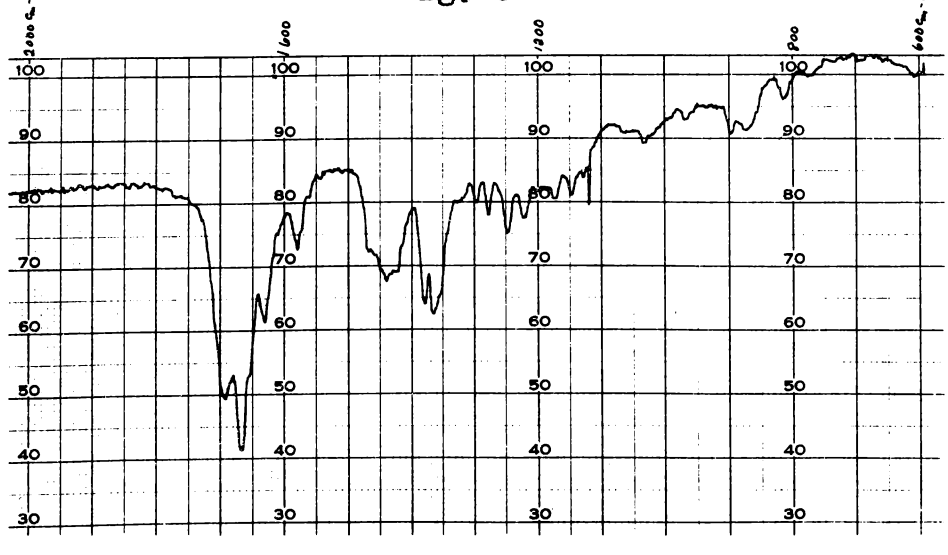


Fig. 4

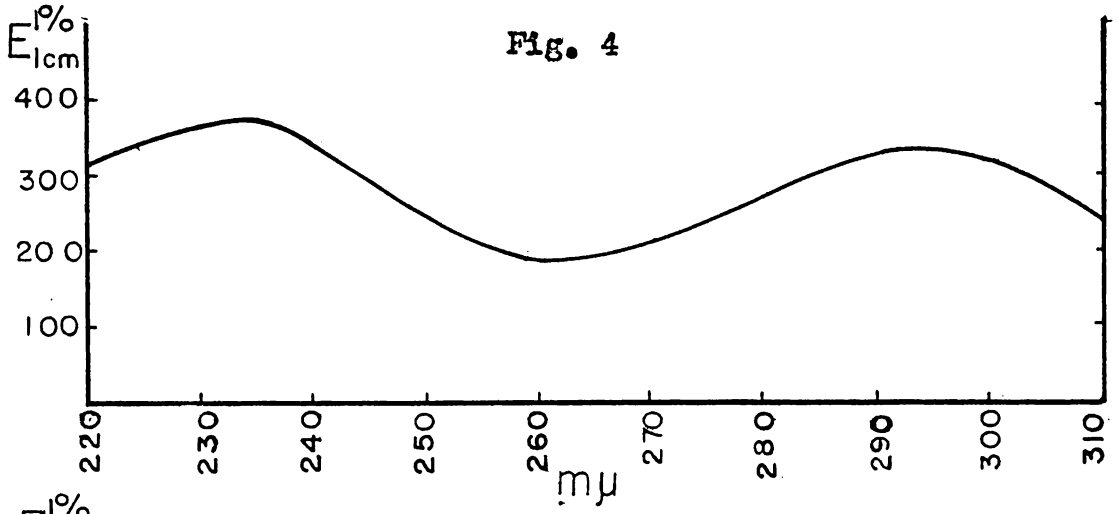


Fig. 5

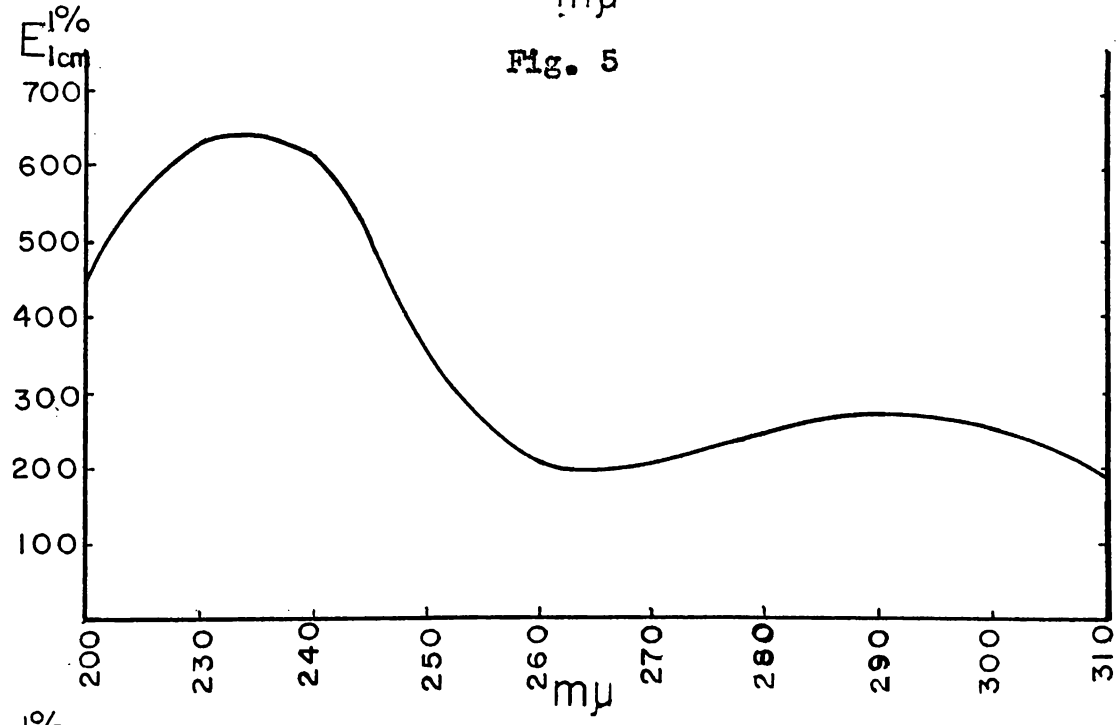
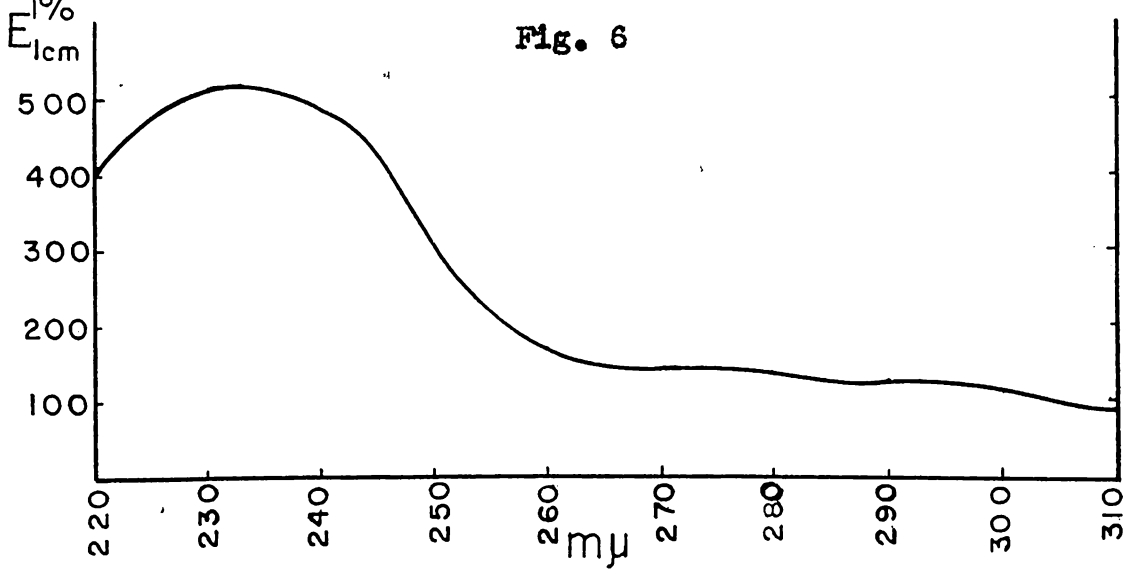


Fig. 6



violet Spectrum number 4, page 85. These spectra were used for reference purposes for following the separation of the fractions.

Fractional Distillation of the Hydrocarbon Fraction.

The 4.907 grams were distilled as indicated below:

Fraction #	Distilling P	Distilling T	Yield
1	5 mm.	94-100°	811 mg.
2	5	110-140	989
3	1	90-100	151
4	0.1	130-140	586
5	residue		2.155 g.
Total recovery			4.292 g.

Fractions 1, 2 and the residue were selected for further study.

Redistillation of Hydrocarbon Fraction 1

Fraction 1 was examined before it was redistilled.

Refractive index (21.5°) 1.4910
Infrared Spectrum 5, page 84
Ultraviolet Spectrum 5, page 85

An attempt was made to distill this fraction at atmospheric pressure (730 mm.). When the silicone bath temperature had been slowly raised to 230° the fraction turned dark. The distillation was discontinued and upon cooling the odor of olefinic gas in the distilling flask was observed. The distillation apparatus had been flushed thoroughly before distillation. Later the fraction was subjected to distillation under reduced pressure as follows:

Fraction #	Distilling P	Distilling T	Yield
6	0.08 mm.	40-52°	138 mg.
7	0.08	52-58	195
8	residue		263
Total recovery			696 mg.

Fraction 7 was selected for further examination and the fraction was analyzed as given below:

Infrared Spectrum 6, page 84
Ultraviolet Spectrum 6, page 85
Iodine number 151, 159
Optical rotation none
Refractive index (25°) 1.4876
Found: C, 76.58%; H, 10.02
Molecular weight 248.

Discussion

The infrared spectrum of the composite fraction (Infrared Spectrum 4) indicated a hydrocarbon. However, the infrared absorption curve of the fractionally distilled fraction indicated the presence of a carbonyl group, as shown by the absorption in the 1700 cm^{-1} region. Since a carbonyl group was indicated, the identity of the material as a hydrocarbon was questionable. Due to the lack of sufficient material, the presence of the carbonyl group could not be verified.

The composite fraction exhibited selective absorption at 234 and 292-296 mμ, (Ultraviolet Spectrum 4). The ultraviolet absorption curve of Fraction 1 (Ultraviolet Spectrum 5) exhibited a very pronounced peak at 234 mμ (E, 1%, 1 cm. - 650). Further purification by distillation removed all of the component which absorbed ultraviolet radiation in the 292-296 mμ region. Thus

it would appear that the composite fraction was a mixture of at least two components, and that separation had been effected by distillation.

The iodine numbers indicated the presence of two double bonds, and the absorption peak at 234 mμ indicated that the double bonds were conjugated.

In just a few days after the fraction had been obtained by distillation, even though stored in a stoppered flask in the refrigerator under an atmosphere of nitrogen, the fraction became a glassy gum. Also, the sample which had been sealed in a glass tube likewise became a glassy gum. The sample had not been sealed under nitrogen. No provisional formula was calculated for this fraction, as the carbon, hydrogen, and molecular weight values should be accepted with some reservation.

Redistillation of Hydrocarbon Fraction 2

The following analyses were performed on this fraction before it was redistilled:

Infrared Spectrum 7, page 89
Ultraviolet Spectrum 7, page 90
Refractive index (21.5°) 1.5069

The entire fraction, 989 mg., was redistilled as follows:

Fraction #	Distilling P	Distilling T	Yield
9	0.05 mm.	46-52°	92 mg.
10	0.05	58-62	390
11	Residue		360
Total recovery			842 mg.

Fig. 7

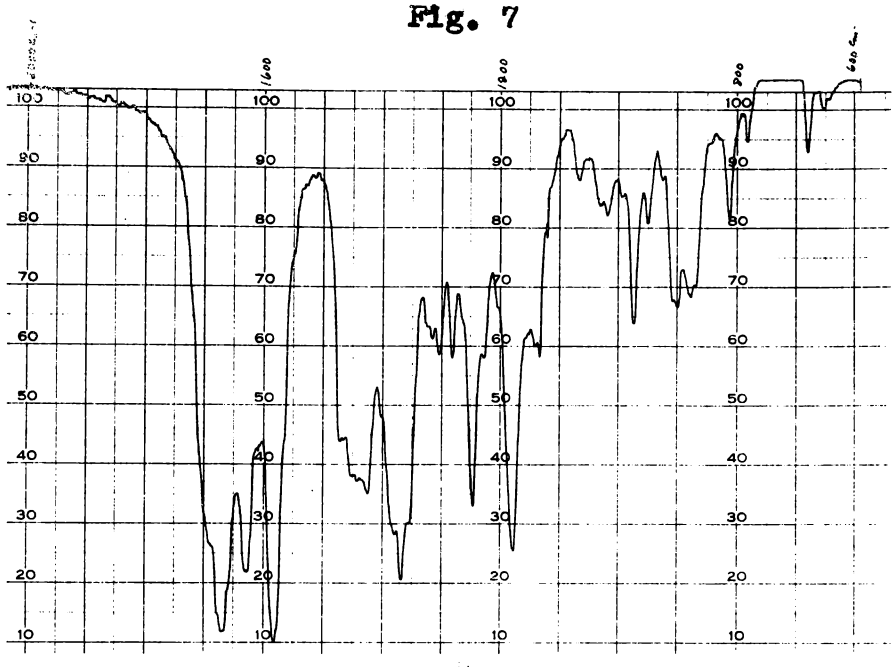


Fig. 8

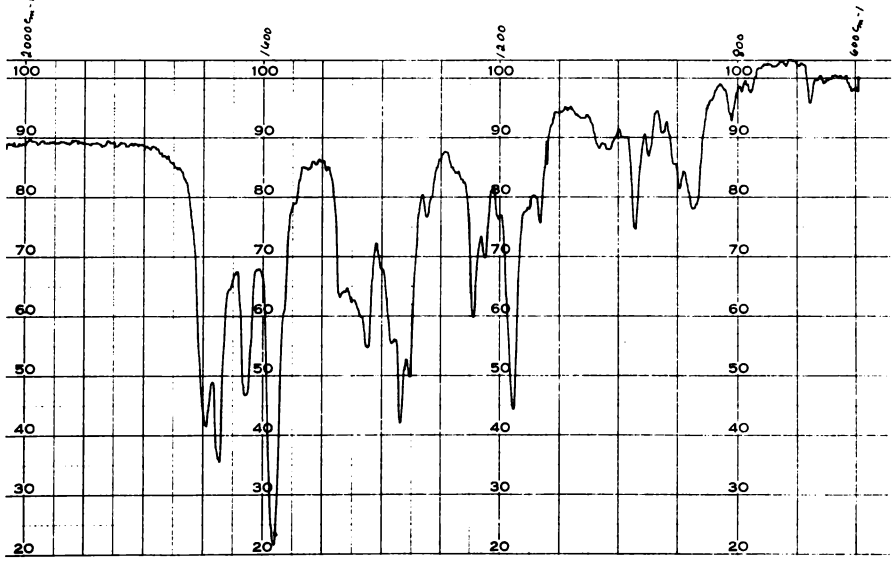


Fig. 9

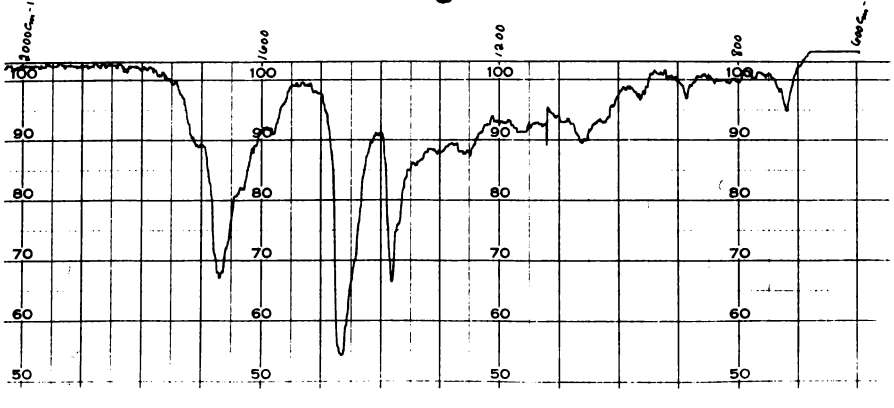


Fig. 7

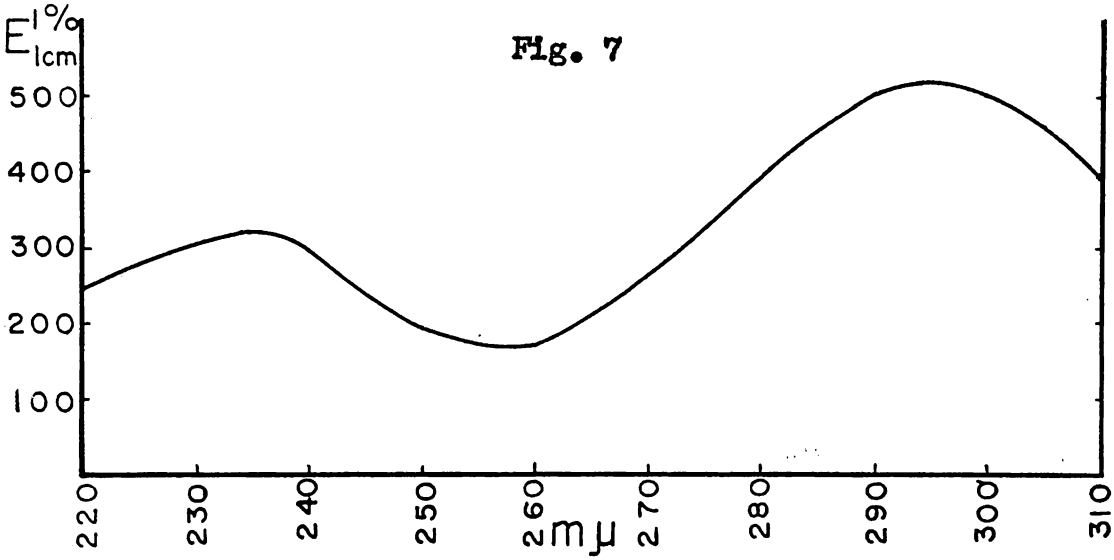


Fig. 8

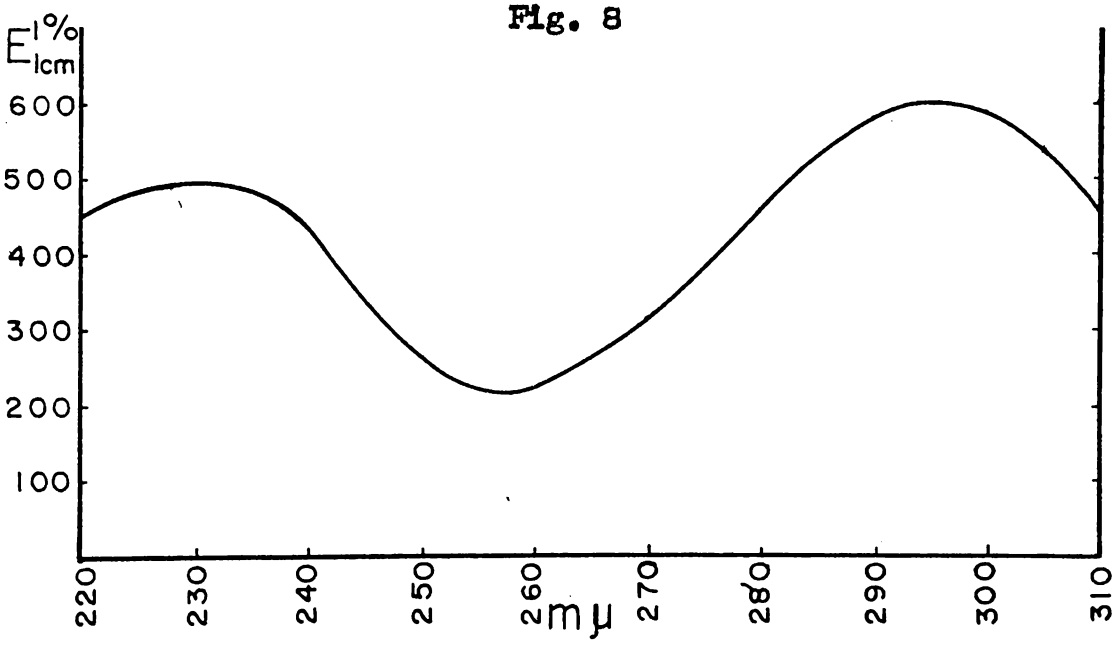
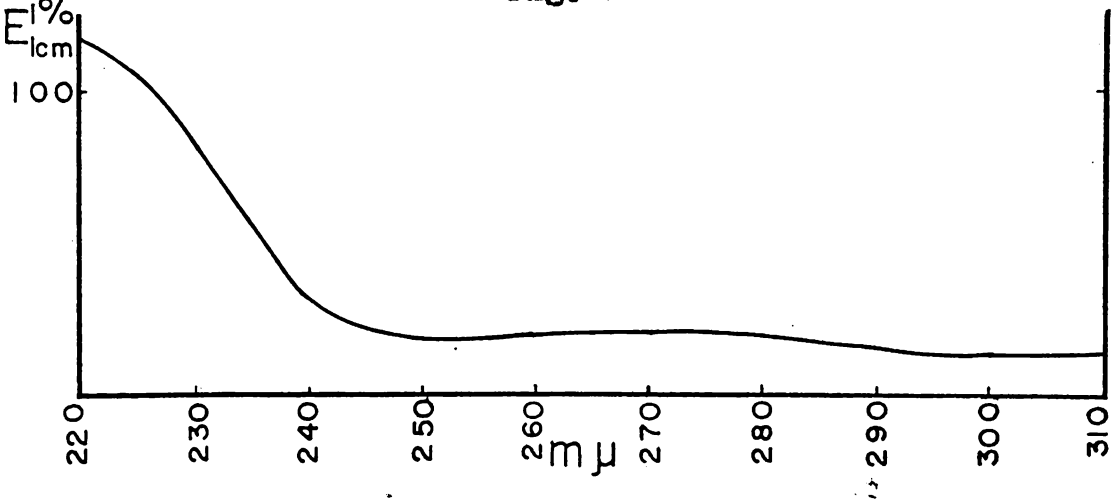


Fig. 9



Fractions 10 and 11 were very hazy after they had been distilled. It appeared that the haziness was due to the contamination of the fractions by the silicone stop-cock lubricant which had been used on the ground glass joints. The two fractions were taken up in a small amount of petroleum ether and introduced onto two 25-gram alumina chromatograph columns, and eluted with 1200 ml. of petroleum ether. One hundred and two, and 182 mg. respectively were recovered from the columns. Fraction 10 possessed the strong lemon odor, and was analyzed as indicated below:

Infrared Spectrum 8, page 89
Ultraviolet Spectrum 8, page 90
Refractive index (25°) 1.5068
Iodine number 200, 250
Optical rotation none
Elemental analysis C, 68.38%; H, 8.87%
Molecular weight 214

Discussion

A comparison of Infrared Spectra 4, 7, and 8 likewise revealed considerable differences, especially Spectra 4 and 8, including the presence of an absorption band in the 1700 cm^{-1} region. This absorption band was not present in Spectrum 4, the spectrum of the parent fraction. Differences were observed in the ultraviolet absorption spectra of these fractions; Spectra 4, 7, and 8. The E, 1%, 1 cm. values in the 234 mμ region were found to decrease in Spectrum 7. However, a considerable increase in E, 1%, 1 cm. value was observed in Spectra 8,

the spectrum of material used for Spectrum 7 after redistillation. There were increases in E, 1%, 1 cm. values in the 292-296 mμ region, as the fraction was purified by fractional distillation.

The iodine number indicated the presence of two double bonds in the fraction, and the ultraviolet absorption indicated that the double bonds were conjugated. Very good agreement was observed in the refractive index values observed for Fractions 2 and 10, 1.5069 and 1.5068 respectively.

Inasmuch as this fraction also polymerized to a glassy gum in a few days even though special precautions had been taken, no empirical formula was prepared.

Examination of the Non-Distillable Residue

An infrared absorption analysis (Spectra 9, page 89) was made on this fraction prior to chromatography on an alumina column. The entire fraction, 2.155 grams, was placed on a 50-gram alumina column with the aid of petroleum ether, and eluted with 500 ml. of petroleum ether. First elutions yielded 973 mg. of a colorless, faintly fragrant oil. Continued elution with 800 ml. of 2 per cent ethanol-petroleum ether mixture yielded 1.147 grams of a dark red oil with rather pleasant odor. No work was undertaken with this red oil

The colorless oil was analyzed as follows:

Infrared Spectrum 10, page 94
Ultraviolet Spectrum 9, page 90
Refractive index (25°) 1.4741
Density (37°) 0.853 (w/v)
Iodine number 56.6, 59.8
Optical rotation none
Found: C, 85.72%, 85.85; H, 13.83%, 13.91
Molecular weight 376
Provisional formula $C_{27}H_{52}$
Carr-Price test (154) pink in 10 minutes
Lieberman-Burchard color test green color

The elemental analysis indicated that this compound was a hydrocarbon containing 27 carbon atoms. This is very interesting in that cholesterol contains the same number. One double bond was indicated from the provisional formula, and confirmed by the iodine number of 56.6 and 59.8.

No optical activity was observed and no selective absorption was observed in the region of 220 to 300 mμ of the ultraviolet region. Comparisons of Infrared Spectra 9 and 10 revealed a very simple spectrum for the parent compound and the purified sample, with only differences noted in the regions 960 and 1070 cm^{-1}. In comparison with Spectrum 4 several differences were observed.

Potassium Permanganate Oxidation

In an attempt to obtain information concerning the location of the double bond, 174 mg. of the sample were subjected to oxidation by potassium permanganate in acetone. No identifiable products were obtained from the reaction mixture.

Fig. 10

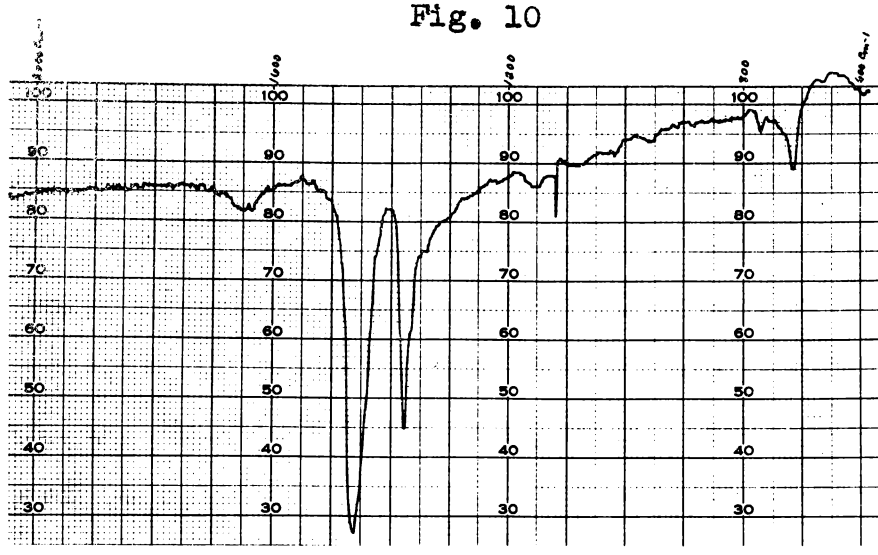


Fig. 11

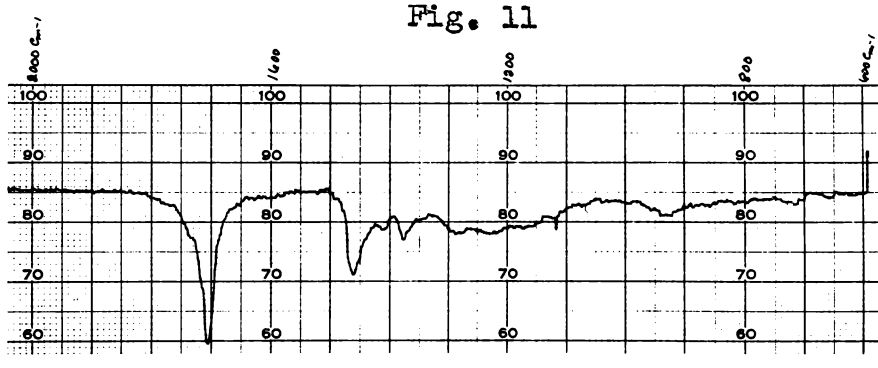
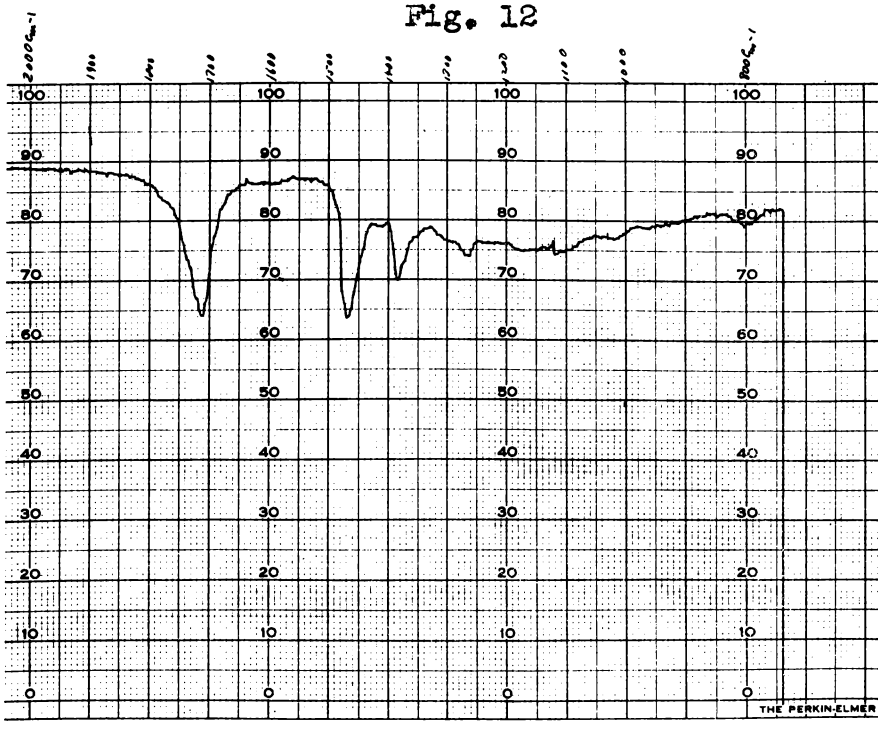


Fig. 12



Preparation of Bromide

The preparation of a bromine derivative was attempted using 163 mg. of the sample in dry ether. One drop of a bromine solution caused the solution to become colored for at least ten minutes. This indicated that the compound reacted very slowly, if at all with bromine. The solvent was removed, the residue taken up in ethyl ether, and bromine solution was added. The mixture was allowed to stand over night in the refrigerator, then distributed between ether and water. The ether was washed and removed. Attempts to recrystallize the residue from methanol or acetone were not successful. It was concluded that the compound did not yield a solid bromide.

Ozonolysis

A sample of the hydrocarbon, 307 mg., was subjected to ozonolysis in an attempt to locate the double bond. The resulting gelatinous ozonide was decomposed by hydrogen peroxide, and the reaction mixture was steam distilled. From the distillate of about 50 ml., 7.0 mg. of a substance with a very musty odor were obtained. Due to the small quantity of material only an infrared absorption study was made (Spectrum 11, page 94). A carbonyl group was indicated by the absorption band at 1700 cm^{-1}, but the substance was not further characterized.

From the non-distillable residue, 207 mg. of a white oil with a "fatty" odor were obtained by ether extraction. The fraction solidified in the refrigerator. An infrared absorption curve (Infrared Spectrum 12, page 94), indicated the presence of a carbonyl group by a slight peak at 1700 cm^{-1}. The fraction was chromatographed on a 30-gram alumina column and eluted with 700 ml. of petroleum ether, yielded 69.0 mg. The infrared absorption curve of this fraction (Spectrum 13, page 97), indicated no carbonyl group. The analysis. Found: C, 85.5; H, 14.4. These data and the molecular weight determination of 256 gave a provisional formula, $C_{18}H_{36}$. This fraction represents a considerable portion of the original molecule C_{18} but unfortunately was not suitable for identification purposes.

Examination of Chloroform Fraction 2

This fraction (obtained as described on page 82) readily became soft and partially fluid on the steam bath, despite continued heating under nitrogen on a steam bath to remove solvent. The fraction was recrystallized from hot methanol and a voluminous precipitate was removed. Recrystallization of the precipitate from acetone several times yielded white irregular plates melting between 150-151° (cholesterol). The removal of the solvent from the mother liquors by the

Fig. 13

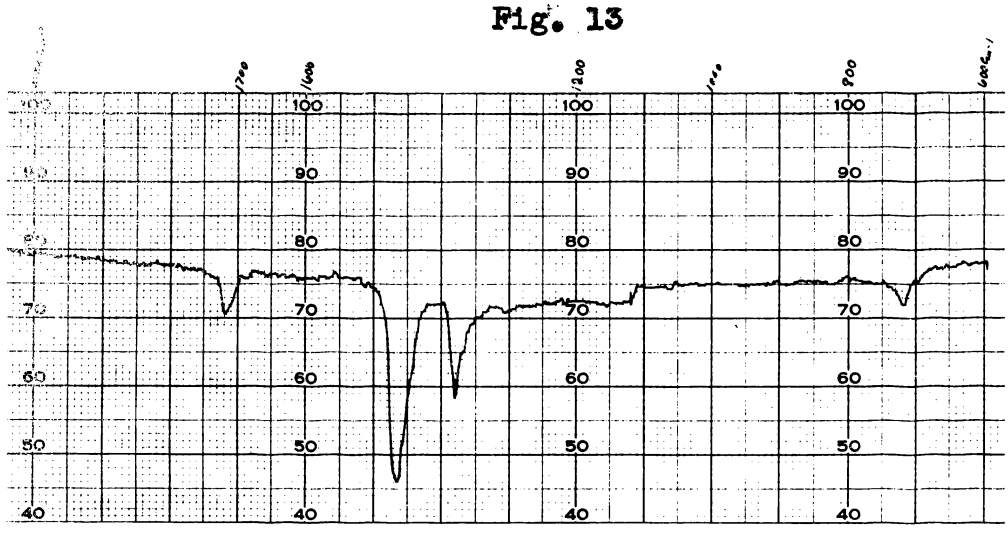


Fig. 17

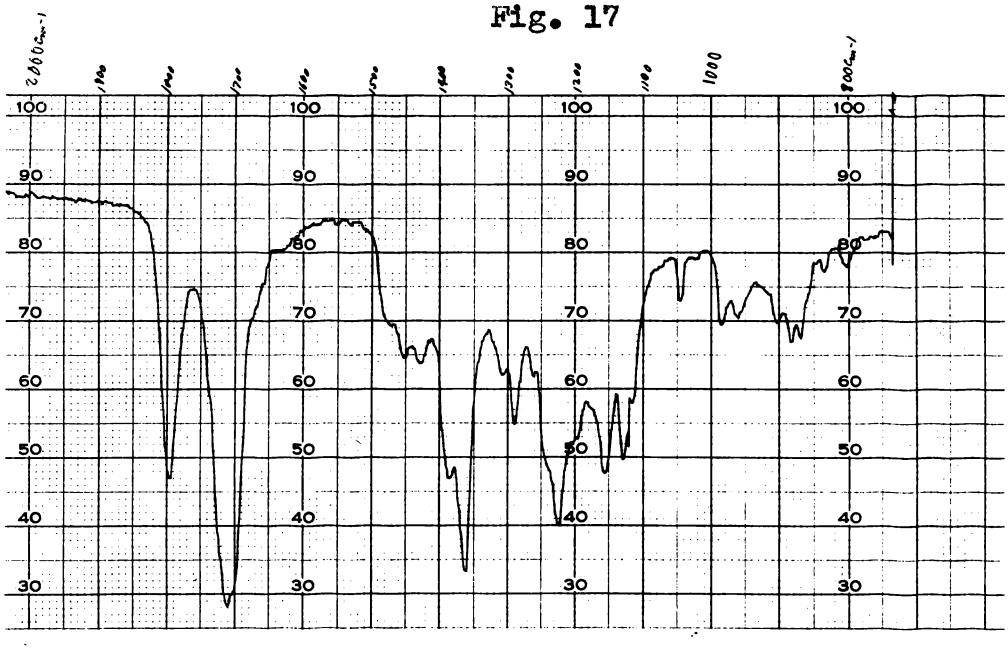
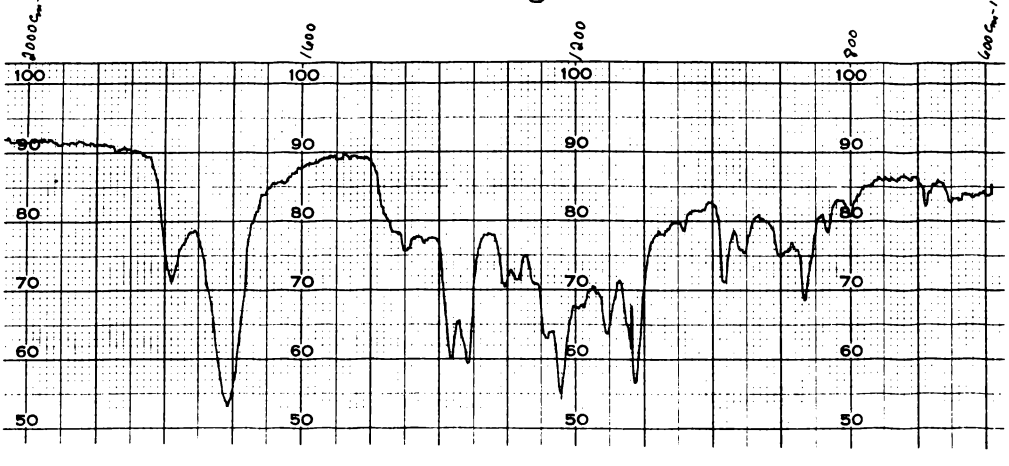


Fig. 18



methanol crystallization yielded a large quantity of deep red oil. A solid material precipitated from the oil when it was placed in the refrigerator.

Fractional Distillation of Deep Red Oil

Original oil:

Refractive index (22°) 1.4989

The oil, 6.680 grams, was fractionally distilled as follows:

Fraction #	Distilling P	Distilling T	Yield
1	0.01 mm.	$50-60^{\circ}$	1.683 g.
2	0.01	60-80	1.322
3	Residue		3.959

Most of Fraction 1 distilled between $54-58^{\circ}$, while the majority of Fraction 2 distilled between $68-78^{\circ}$. These two fractions were analyzed as given below:

Fraction 1

Infrared Spectrum 14, page 99
Ultraviolet Spectrum 10, page 100
Refractive index (21.5°) 1.4832

Fraction 2

Infrared Spectrum 15, page 99
Ultraviolet Spectrum 12, page 100
Refractive index (21.5°) 1.4893

Examination of Fraction 1

Fraction 1 was redistilled as given below:

Fraction #	Distilling P	Distilling T	Yield
4	0.04 mm.	$32-54^{\circ}$	0.214 g.
5	0.04	58-60	0.762
6	Residue		0.530

Fig. 14

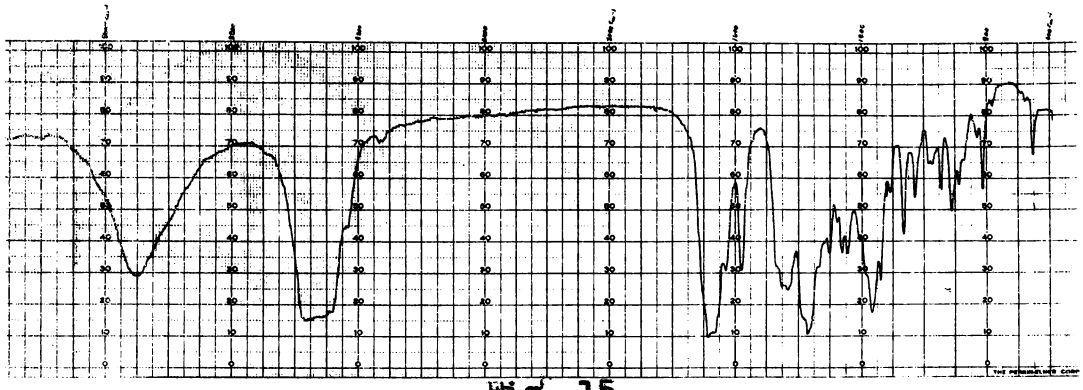


Fig. 15

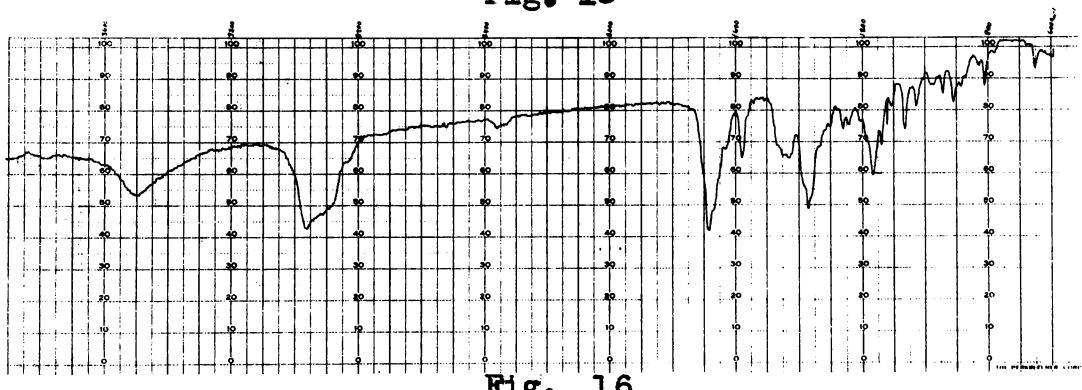


Fig. 16

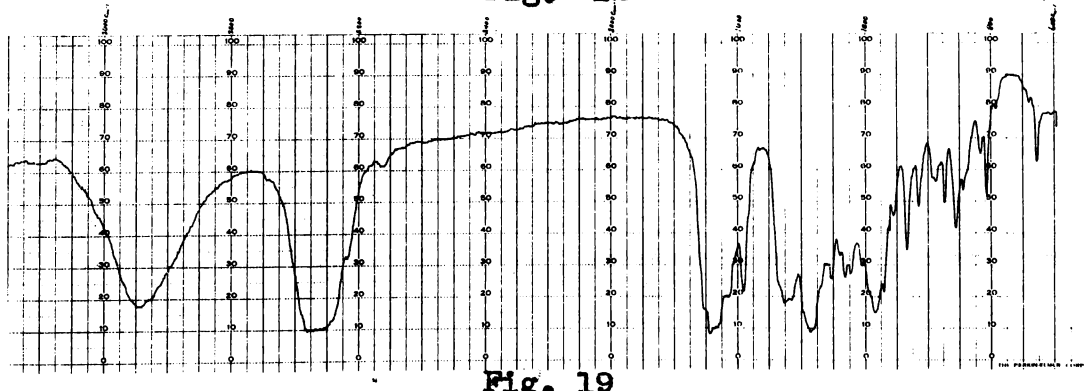


Fig. 19

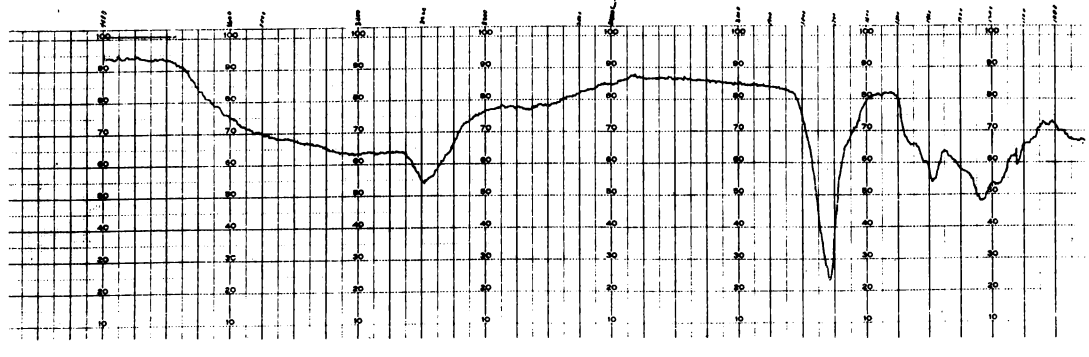


Fig. 10

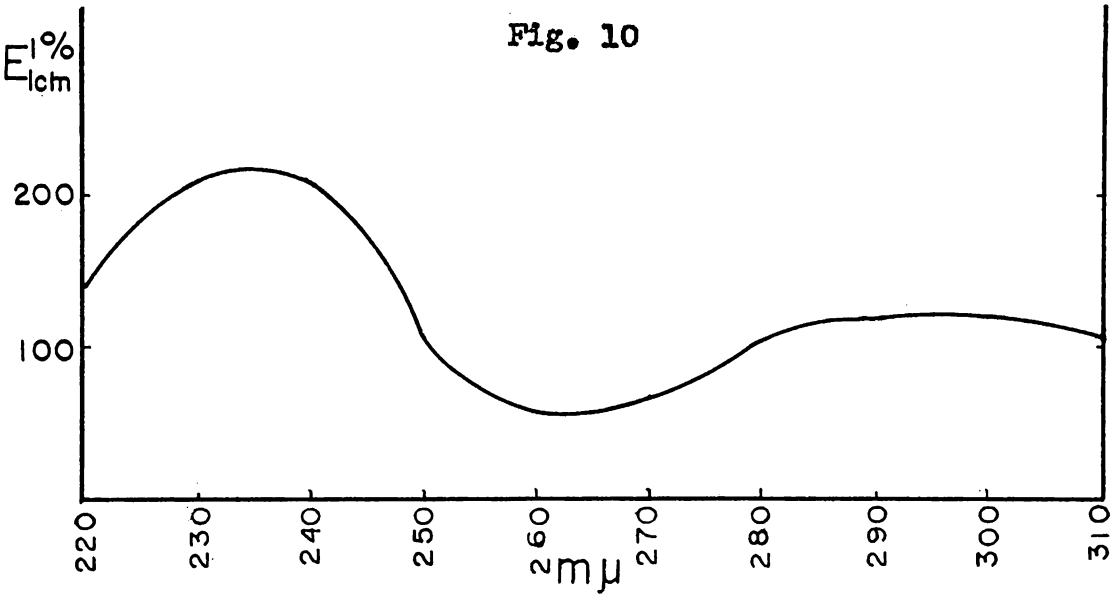


Fig. 11

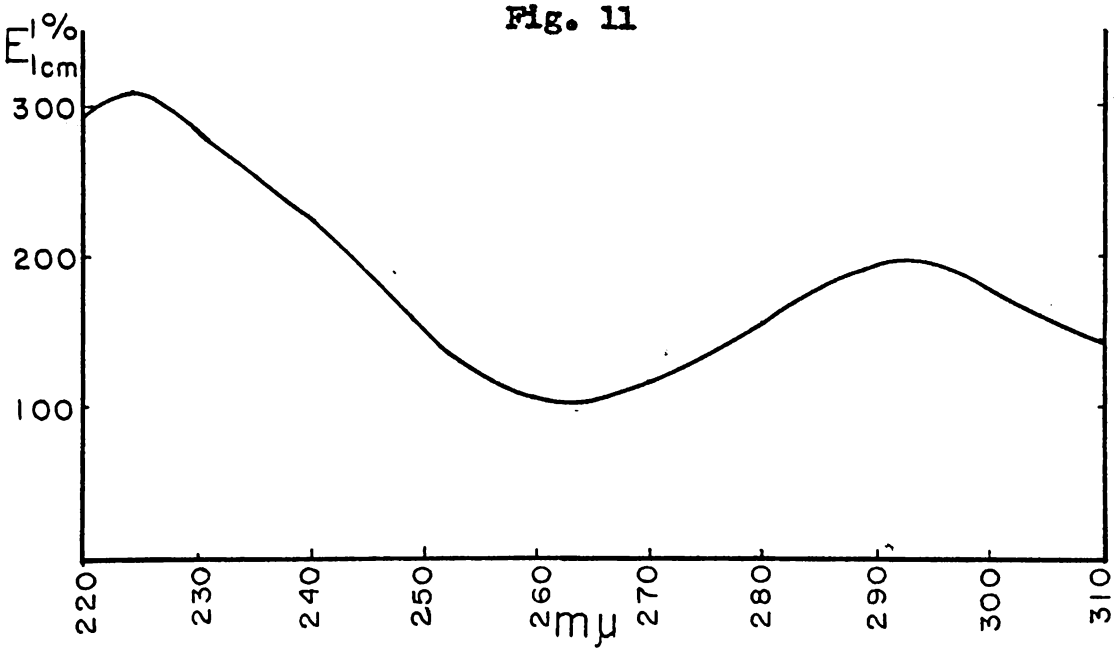
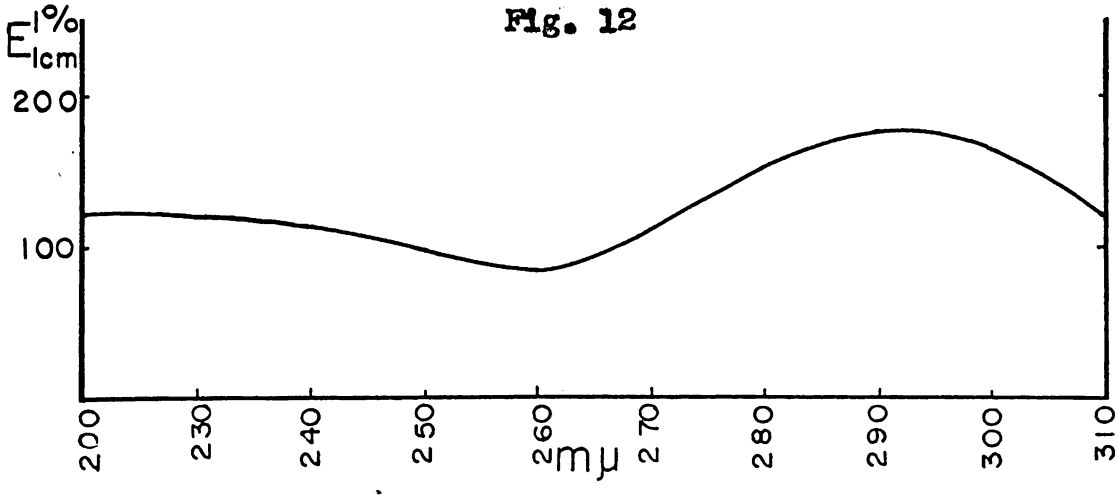


Fig. 12



Fraction 5 was selected for further examination and the data are given below:

Infrared Spectrum 16, page 99
Ultraviolet Spectrum 11, page 100
Refractive index (25°) 1.4826
Density (30°) 0.958 (w/v)
Iodine number 325, 346
Optical rotation none
Analysis: C, 74.16 H, 10.43 O, 15.41
74.40 10.35 15.35
Molecular weight (Rast) 206
Formula $C_{13}H_{21}O_2$
Carr-Price yellow-green in 10 minutes
Lieberman-Burchard negative

Discussion

Fractional distillation of the deep red oil gave two fractions which exhibited very similar infrared spectra (Infrared Spectra 14 and 16), with absorption bands at 3500 cm^{-1}, the hydroxyl group absorption band, and the absorption band at 1700 cm^{-1}. Differences were observed in the ultr aviolet absorption curves. A slight absorption peak was observed in the spectrum of Fraction 1 in the 234-236 mμ region, and only a slight peak in the 292 mμ region in Fraction 2. On the basis of the close relationship to one another, even though the boiling points of the two fractions were much different, Fraction 1 was selected for redistillation, and when redistilled a fraction weighing 762 mg. was obtained.

An inspection of the Infrared Spectra 14 and 15 did not reveal any large differences in the spectrum of the two fractions. However, some slight changes occurred in

the ultraviolet absorption study, in that one absorption peak was observed at 224 rather than 234 mμ and the slight shift in the 294 mμ region. The E, 1%, 1 cm. values are 308 and 187 for the redistilled fraction and 226 and 139 for the parent fraction, indicating that some purification had been effected.

Molecular weight determination and carbon, hydrogen analyses gave the provisional formula of $C_{13}H_{21}O_2$. The iodine number indicated the presence of three double bonds in the fraction, two of which appeared to be conjugated.

Preparation of a Bromide

The first derivative to be attempted was the preparation of a bromide, using 230 mg. An oil was recovered upon recrystallization of the reaction residue from ethyl acetate, and later, recrystallization from methanol. Although a liquid bromide might have been formed, this was not useful for characterization.

Preparation of 2, 4-Dinitrophenylhydrazone

The next derivative attempted was the preparation of a carbonyl derivative using 2, 4-dinitrophenylhydrazine hydrochloride. An oil remained after recrystallization from various solvents had been attempted.

Action of Potassium Permanganate

The fraction, 204 mg., was subjected to the action of potassium permanganate in acetone. The permanganate

solution was decolorized by the fraction, and the addition of permanganate was continued until the permanganate color persisted for several minutes. The reaction mixture was extracted with ethyl ether, but unfortunately the ether was contaminated with peroxides, which exploded during the removal of the ether. This loss was indeed unfortunate in that the sample used for this oxidation consisted of the last redistilled sample.

Examination of Fraction 2

Preparation of a Phenylurethane

Due to the close similiarity of Fractions 1 and 2, work was continued using Fraction 2. So 240 mg. were used to prepare a phenylurethane in an effort to establish the presence of the hydroxyl group as indicated by the infrared absorption analysis. The attempt was unsuccessful. Later the preparation of a phenylurethane was tried again, using 261 mg. and this attempt was also unsuccessful.

Preparation of a Semicarbazone

Two hundred milligrams of the fraction were used in the preparation of a semicarbazone, but this attempt also failed. A gum was obtained which could not be crystallized. Preparation of derivatives was discontinued at this point.

Ozonolysis

Four hundred and seventy-four mg. of Fraction 2 in carbon tetrachloride were subjected to ozonolysis and the mixture (after decomposition of the gelatinous ozonide with hydrogen peroxide) were steam distilled. The reaction products from each portion were isolated and identification undertaken.

The distillate was extracted with ethyl ether and subsequent removal of the ether yielded 89 mg. of a slightly colored oil, with a definite mint-camphor odor. The Infrared Spectrum 17, page 97 indicated differences at 1070, 1240, 1580, 1630, 1660-1690 and 1390 cm^{-1}, when compared with Spectrum 16, the spectrum of the parent fraction. No selective absorption was observed in the ultraviolet region (spectrum not given). Attempts to prepare a 2, 4-dinitrophenylhydrazone were not successful although a carbonyl group was definitely indicated by the absorption peaks at 1700 cm^{-1}.

Extraction of the non-distillable residue with ethyl ether yielded a quantity of colorless oil, which possessed a pungent odor, similar to acetic acid. In an attempt to remove a few drops of water from the residue, it was found that some of the material was insoluble in petroleum ether. The fraction was separated into petroleum ether-soluble and petroleum ether-insoluble fractions.

The petroleum ether-soluble portion (identical with fraction previously described as indicated by Infrared Spectra 17 and 18, page 97) consisted of a viscous oil, and the entire fraction of 182 mg. was used in an attempt to prepare a semicarbazone. The work up of the reaction mixture in the usual manner gave only an oily residue. Recrystallization from methanol and from benzene was not successful. It was not possible to determine if a semicarbazone derivative had formed. Preparation of a 2, 4-dinitrophenylhydrazone was not successful.

The infrared spectrum (Spectrum 19, page 97) of the petroleum ether insoluble material indicated the presence of either a hydroxy acid or keto acid. Unfortunately it is difficult to distinguish between the two by infrared absorption studies. Attempts to prepare a semicarbazone and 2, 4-dinitrophenylhydrazone (respectively) were unsuccessful. The small quantity prevented further examination except verification of its acid-character. It was extracted from an ether solution with dilute aqueous sodium bicarbonate.

Examination of Chloroform Fraction 3

Purification

This fraction (obtained as described on page 82) was light-brown, insoluble or only slightly soluble in hot acetone. However, the fraction was soluble in ace-

tone-methanol mixture. Even after three recrystallizations from the acetone-methanol mixture the solution was hazy. Yellowish crystalline clumps were obtained which melted between 70-80°.

The fraction was boiled in benzene with a small amount of methanol added. A very hazy solution was produced, and the solution remained hazy even after filtering through Celite and filter paper. However, the solution was clarified by filtering through a fritted glass filter, giving a clear yellow filtrate. A small amount of precipitate remained on the filter (discarded). The filtrate crystallized as a gel and after recrystallization from acetone-methanol mixture yielded tan, semispheres melting between 80-88°.

One-half of the fraction was decolorized by heating with charcoal in hot benzene. However, the results were not very satisfactory as the solution had a tendency to gel while filtering. Recrystallization from methanol yielded white granules melting between 82-87°.

Recrystallization from chloroform-methanol mixture was unsatisfactory. The fraction formed a gel at room temperature with chloroform but was readily soluble in warm chloroform.

Recrystallization from hot 95 per cent ethanol, yielded white granules melting between 82-88°, and recrystallization from methanol yielded white spheres with a melting point of 84-86°.

There were 1.142 grams used for the preparation of an acetate (pyridine, (CH_3CO_2O). The resulting product after several recrystallizations from acetone or acetone-methanol mixture yielded white microcrystals melting at 103-104°. After extensive recrystallization and drying for 48 hours over phosphorus pentoxide (under water-pump aspirator vacuum), the product had a melting point of 105-107°. Found: C, 74.45, 74.70; H, 11.78, 11.87.

A sample of the acetate, 436 mg., was saponified 30 minutes under an atmosphere of nitrogen with 10 ml. of 5 per cent alcoholic potassium hydroxide. The reaction mixture was distributed between water and ethyl ether. The ether phase was washed to neutrality and removed, leaving 185 mg. of a colorless solid. Recrystallization from methanol gave white granules, melting between 89-91°.

The aqueous phase was very hazy. It was acidified and extracted with ether. The ether solution was washed with water, and the ether removed leaving a white solid. Recrystallization from methanol gave white granules melting between 89-91°. The substance appeared to be hydrophilic and had a tendency to remain suspended in water.

No depression of melting point was observed when the two above low melting solids were mixed. The solids were then combined and several recrystallizations from methanol gave transparent granules melting between 89-91°. After extensive recrystallizations and drying 48

hours (in vacuum over phosphorus pentoxide), irregular granules with a sharp melting point of 91-92° were obtained. The compound was analyzed and found to have the following composition: C, 77.15, 77.06; H, 12.76, 12.78.

By saponification of the acetate, 2.226 grams of the free compound were obtained. Recrystallization of the free compound from methanol gave transparent granules melting between 90-91°. A portion purified by recrystallization four times from methanol was found to have the following composition: C, 76.90, 77.02; H, 12.58, 12.70; m. p. 90-91°. These values agreed very well with the values for the free compound obtained without previous conversion to the acetate. The compound was found to have a molecular weight of 670, therefore, a provisional formula of $C_{43}H_{84}O_4$ was established.

Preparation of an Acetate

An acetate was prepared using 232 mg. of the pure compound (obtained by preparation of the acetate and subsequent saponification). The resulting derivative was purified by recrystallization from methanol and dried in the usual manner. Irregular crystals melting between 106-107° were obtained. Found: C, 74.63, 74.40; H, 11.86, 11.72; a molecular weight of 800 was obtained. Calculations using the above data gave the provisional formula of $C_{50}H_{94}O_7$.

Preparation of a Benzoate

An attempt was made to prepare a benzoate and was not successful.

Preparation of a 3, 5-Dinitrobenzoate

The preparation of a 3, 5-dinitrobenzoate was undertaken using 3, 5-dinitrobenzoyl chloride. Recrystallization from acetone-methanol, from acetone alone, and from acetone-methanol mixture yielded brown crystals melting between 80 and 90°. After decolorizing by charcoal, recrystallization from acetone gave a yellow crystalline solid which melted between 115 and 120° with decomposition. After several crystallizations from acetone-methanol mixture a product was obtained which consisted of tan irregular crystals melting from 135 to 148°. As the product could not be brought to a sharp melting point, it was not analyzed.

Treatment with Digitonin

Preparation of a digitonide was not successful, as no precipitate was produced in an ethanol solution of 311 mg. of the sample and 519 mg. of digitonin in ethanol, even after standing several days in the refrigerator.

Preparation of a Bromide

A grayish gum resulted when 102 mg. of the compound were used to prepare a bromine derivative, after recrystallization from methanol. A white solid had preci-

pitated from the mother liquors from the methanol recrystallization. This white solid was removed, but it also dried to a grayish gum. Work on the bromine derivative was discontinued.

Preparation of a Semicarbazone

To establish the identity of the remaining oxygen atom in the compound, an attempt was made to prepare a semicarbazone. This attempt also was unsuccessful as only a white granular product melting between 82-86° was obtained. This was the original product and so work on this derivative was discontinued.

Oxidation Studies

Oppenauer Oxidation. The compound was subjected to the Oppenauer oxidation as follows:

831 mg. of the compound, m. p. 84-86°
4 grams of alumin isopropoxide
20 ml. of dry benzene
10 ml. of dry acetone

The system was flushed with nitrogen, then the mixture was refluxed under anhydrous conditions for seven hours. The reaction mixture was distributed between ethyl ether and water, strongly acidified with hydrochloric acid. The ether was removed, washed to neutrality and removed by distillation. Crystallization of the white solid from methanol-acetone gave white granules melting between 84 and 90°. When mixed with a sample of the original compound a melting point of 85-89° was obtained. The

compound appeared to be resistant to oxidation under the above conditions.

Chromic Acid Oxidation. With slight heating in 10 ml. of glacial acetic acid, 577 mg. of the compound were dissolved. Five drops at a time were added from a separatory funnel[1] with ice cooling. When the addition of the chromic acid solution no longer caused the solution to become warm, the reaction mixture was allowed to stand 10 minutes and diluted with 40 ml. of distilled water. The mixture was colored green.

The reaction mixture was distributed between ether and water, and the latter discarded. The ether solution was washed with water, aqueous 5 per cent sodium bicarbonate solution, and with water respectively and then the ether was removed leaving 291 mg. of a greenish oil. This was soluble in benzene and low boiling petroleum ether, but insoluble in methanol and acetone.

The greenish oil was placed on a 50-gram alumina column with the aid of low boiling petroleum ether and 30 ml. fractions were collected with petroleum ether as elutent:

> Fraction 1 contained a small quantity of white oil
> Fractions 2 through 10 did not contain any material.

Two per cent ethanol-petroleum ether was used as elutent and the following fractions collected:

---

[1] A solution of 50 grams of chromic oxide dissolved in 35 ml. of water diluted with 35 ml. of glacial acetic acid.

Fractions 1 and 2 did not contain any material

Fractions 3 through 9 contained a small quantity of green oil

Fractions 10 through 25 contained some white solid, recrystallization yielded white flakes melting between 90-97° (probably the original compound).

No identifiable compounds or fractions were obtained from the reaction of chromic oxide and the compound.

Refluxing with Alcoholic Hydrochloric Acid

The compound was subjected to treatment as follows:

206 mg. of the compound, m. p. 90-91°
15 ml. of 95 per cent ethanol
3 drops of concentrated hydrochloric acid

The above mixture was refluxed for 2 hours, cooled and distributed between ether and water. Removal of the ether after washing three times with water yielded 7.0 mg. of a yellowish oil.

Addition of 5 per cent sodium bicarbonate solution to the aqueous liquor caused appreciable solid to precipitate. The solid was removed by ether extraction. The ether was washed with water, and removed, yielding 196 mg. of a yellowish wax. Recrystallization from methanol gave whitish, irregular crystals melting between 85-88°. Crystallization from methanol gave yellowish irregular crystals melting between 86-88°. Mixing with the original sample melting between 90-91° gave a melt-

ing point of 87-90°. The compound was stable to acid hydrolysis in ethanol.

The compound did not form any color with the Liebermann-Burchard color reagent.

DISCUSSION

Plant Investigation

Ursolic and oleanolic acids have been shown to be widely distributed in nature (155). The results of the investigation of lipid fractions of the plant tissues indicated that Plantago rugelii contained both of these carboxylated triterpenes and Lamb's Quarters contained oleanolic acid. The function of these substances in plants is not known, and the function of sitosterol is not known. Anderson and Moore (156) have commented on the fact that although the phytosterols occur in all parts of plants their relatively great abundance in seeds and pollen would seem to justify the assumption that they play an important role in the life of the plant cells.

The constant association of ursolic acid and sitosterol would suggest the possibility that ursolic acid may be closely related to the biosynthesis of sitosterol. While no work was undertaken to demonstrate that ursolic acid is related to the biosynthesis of sitosterol, other considerations, in addition to the distribution mentioned above, are given to support the proposed relationship.

Rings A, B, C, and D of the triterpenes have the same configuration as the sterols (see page 54). It does not seem likely that two separate biosynthetic mechanisms would be used to synthesize compounds which are similar in structure. The presence of the methyl groups on ursolic and oleanolic acids could indicate that they had been formed from small molecules, possibly by a scheme similar to the one proposed by Bonner and Arreguin (90). Then the changes such as the elimination of the methyl groups, the breaking of ring E to facilitate the establishing of the sitosterol side chain and the conversion of a six-membered ring D to a five-membered ring D. This still leaves the introduction of a methyl group at carbon-13 of the sitosterol. There are examples of conversion of a six-membered ring to a 5-membered ring under very mild conditions (157).

Wadkins (158) observed that after the addition of ursolic acid to the incubation medium containing surviving leaf tissue, from Plantago rugelii, there was an increase in oxygen uptake. The conventional Warburgh technique was used, with the leaf tissue in the medium without ursolic acid as controls. The results have not, as yet, been evaluated, but suggest a close relationship of ursolic acid to the metabolism of Plantago rugelii.

Wadkins and Nicholas (159) have developed a method, using chromatographic techniques, for the detection

of ursolic and oleanolic acids in very small amounts. They have applied their technique to a study of wet, green plants, and their results indicated that ursolic and oleanolic acids were not present in green, wet Plantago rugelii plants. They have not, as yet, carried out sufficient experiments to confirm the preliminary observations.

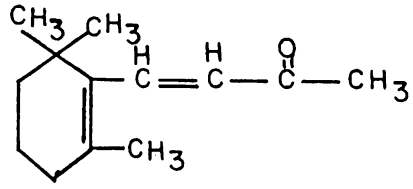
The above observation does not invalidate the hypothesis as proposed. With the harvesting of the mature plants, the biosynthetic processes would be disrupted to such an extent that the biosynthesis of sitosterol would no longer be required. The biosynthetic processes, however, could continue for some time, and the intermediates in the sitosterol, would accumulate. With this viewpoint, the finding of quantities of the triterpene acids in the dry but not in the wet plant would also suggest that they might be precursors of sitosterol, or allied to a precursor.

Brain Tissue Investigation

The examination of the neutral constituents of the lipid fractions of beef brain tissue revealed the presence of several substances which have not been previously reported in brain tissue lipid extracts. They are: hydrocarbon material, a low molecular weight fragrant oil, containing two oxygen functional groups, and a low

melting, high molecular weight compound.

The red oil was not completely characterized. The identity of the hydroxyl and carbonyl groups indicated by absorption bands at 3500 and 1700 cm^{-1} regions respectively could not be verified by obtaining a solid derivative. However, the fact that solid derivatives were not obtained does not necessarily mean that the reagents did not attack the specified groups. It is known that the α,β unsaturated carbonyl compounds do not readily form carbonyl derivatives. The low molecular weight terpenes likewise do not form solid carbonyl derivatives. Also it is possible that the two groups may be hindered. The fragmentary evidence indicated that the fraction obtained may be related to the low molecular weight terpenes, or a similar type compound. The data suggested a close similarity to β -ionone, a $C_{13}H_{20}O$ compound. The structure of β-ionone is given below (160).



β-ionone

The low melting high molecular weight compound appeared to form a acetate. A provisional formula of $C_{43}H_{84}O_4$ was prepared for this compound, however, the formula should be accepted with some reservation. No

elemental analyses other than carbon and hydrogen were performed on this compound.

Three fractions were isolated from the hydrocarbon mixture. Two were very unstable, as they rapidly changed color and polymerized in a few days even on standing under nitrogen. The third fraction was partially characterized as a $C_{27}H_{52}$ compound, and gave a green color with the Liebermann-Burchard color reagent. Attempts to locate the double bond were not successful. On the basis of the number of carbon atoms, and the color formation with acetic anhydride-sulfuric acid, it would suggest a close structural relationship of this compound to cholesterol. The compound appeared to contain a branched carbon chain, which would also support such a possible relation to cholesterol.

The isolation of unsaturated hydrocarbon compounds have been reported many times. Ditmer (161) isolated a $C_{45}H_{76}$ compound from the liver of adult humans, and called the compound hepene. Hepene was not found in the liver of the foetus. Channon and Marrian (162) reported the presence in the livers of man, ox, sheep, horse, and pig of high molecular weight unsaturated hydrocarbons, however, the hydrocarbons have not been completely characterized, even after extensive work on the hydrocarbon found in pig liver. Toyama (163) reported the presence of another hydrocarbon, pristane, in fish-liver

oil. The presence of hydrocarbons in other fish-liver oils has been mentioned previously.

The recent evidence of Langdon and Block (99) indicated that natural squalene was a precursor of cholesterol, in the sense that C^{14} biosynthetically labeled squalene, supplied carbon atoms for the biosynthesis of cholesterol in mice. Other work by these authors, indicated that the liver of the rat contained squalene, at least on the basis of the Sobel (150) colorimetric determination for squalene. Other investigators, Mac Kenna, Wheatley, and Wormal (164) reported the presence of squalene in the human, and very recently the human body has been shown to have the ability to synthesize labeled squalene from C^{14} labeled acetone (165). Thus on the basis of this evidence, it appears that unsaturated hydrocarbons may have important physiological roles, which remain to be elucidated. Also considering the wide distribution of squalene in plants (166), the squalene may have some role in the biosynthesis of a compound such as sitosterol, or other sterols.

SUMMARY

1. Hentriacontane, ursolic acid, oleanolic acid and sitosterol were isolated and identified from the lipid extracts of Plantago rugelii. Ursolic acid and sitosterol were found in all parts of the plant examined. Oleanolic acid was found only in the aereal parts of the whole mature plants.

2. A large number of additional plants were investigated for ursolic acid and oleanolic acid. Of the plants investigated only Lamb's Quarters yielded oleanolic acid.

3. Three and possibly four terpene-like oils were isolated from beef brain tissue. Some initial operations were performed to characterize the substances. One of these was characterized as a $C_{27}H_{52}$ hydrocarbon.

4. A high-molecular weight low melting compound tentatively characterized as a $C_{43}H_{84}O_4$ has been isolated from beef brain tissue.

BIBLIOGRAPHY

1. Windaus, A., Einige Bemerkungen über die Konstitution des Cholesterins und des Ergosterins, Nachr. Ges. Wissensch. Goettingen, Math.-physik, Klasse, 92-102 (1933)

2. Wieland, H. and E. Dane, Untersuchungen über die Konstitution der Gallensauren, Ztschr. physiol. Chem. 210:268-281 (1932)

3. Rosenheim, O. and H. King, The ring-system of sterols and bile acids, J. Soc. Chem. Ind. 51:464-466 (1932)

4. Windaus, A. and A. Lüttringhaus, Ueber die Zahl der Kohlenstoffatome in Molekül der Sterine, vor allem des Ergosterins, Nachr. Ges. Wissensch. Goettingen, Math-physik. Klasse, 4-7 (1932)

5. Gerard, E., Sur les cholesterines vegetales, Compt. rend. 114:1544-1546 (1892)

6. Gerard, E., Sur les cholesterines des cryptogames, Compt. rend. 121:723-726 (1895)

7. Pruess, L. M., W. H. Peterson, H. Steenbock, and E. B. Fred, Sterol content and antirachitic activatability of mold mycelia, J. Biol. Chem. 90: 369-384 (1931)

8. Birkinshaw, J. H., R. K. Callow, and C. F. Fischman, Studies in the biochemistry of microorganisms. XXII The isolation and characterization of ergosterol from Penicillium puberulum Bainier grown on a synthetic medium with glucose as sole source of carbon, Biochem. J. 25:1977-1980 (1931)

9. Pruess, L. M., W. H. Peterson, and E. B. Fred, Isolation and identification of ergosterol and mannitol from Aspergillus fischeri, J. Biol. Chem. 97:483-489 (1932)

10. Bills, C. E., O. N. Massengale, and P. S. Prickett, Factors determining the ergosterol content of yeast. I Species, J. Biol. Chem. 87:259-264 (1930)

11. Massengale, O. N., C. E. Bills, and P. S. Prickett, Factors determining the ergosterol content of yeast. II Carbohydrate sources, J. Biol. Chem. 94:213-219 (1931)

12. Sumi, M., On the ergosterin content of various edible mushrooms in Japan, Sci. Papers Inst. Phys. Chem. Research (Tokyo) 20:254-258 (1933). Cited by Bills, (25).

13. MacLean, I. S., and D. Hoffert, The carbohydrate and fat metabolism of yeast. III The nature of the intermediate stages, Biochem. J. 20:343-357 (1926)

14. Terroine, E. F., R. Bonnet, G. Kopp, and J. Vechot, La formation des sterols est-elle liee au metabolism des matiere grasse? Bull. Soc. Chim. Biol. 9:678-691 (1927).

15. Hesse, O., Ueber phytosterin and cholesterin, Ann. 192: 175-179 (1878)

16. Windaus, A., and A. Hauth, Ueber stigmasterin, ein neues phytosterin aus Calabar-Bohnen, Ber. 39: 4378-4384 (1906)

17. Fieser, L. F., and M. Fieser, Natural Products Related to Phenanthrene, Reinhold Publishing Corp., New York (1949) 3rd ed., p. 94, 285.

18. Lindenmeyer, O., Beiträge zur Kenntniss des Cholesterins, J. prakt. Chem. 90:321-331 (1863). Cited by Bills, (25).

19. Beneke, F. W., Ueber das Cholestearin, Arch. d. Vereins f. wissenschaftl. Heilk. 2:432-446 (1866). Cited by Bills, (25).

20. Schulze, E., and J. Barbieri, Zur Kenntniss der Cholesterine, J. prakt. Chem., N.F., 25:159-180 and 458-462 (1882). Cited by Bills, (25).

21. Schulze, E., Bilden sich Cholesterine in Keimpflanzen, welche bei Lichtabschluss sich entwickeln? Ztschr. physiol. Chem. 14:491-521 (1890). Cited by Bills, (25).

22. Minovici, S., Contribution a l'etude du cholesterol au point de vue chimique et physiologique, Bull. soc. chem. biol. 9:1129-1164 (1927). Cited by Bills, (25).

23. Schreiner, O., and E. C. Shorey, The presence of cholesterol substance in soils; agrosterol, J. Am. Chem. Soc. 31:116-118 (1909)

24. Marker, R. E., R. B. Wagner, P. R. Ulshafer, E. L. Wittbecker, D. P. J. Goldsmith, and C. H. Ruof, Steroidal sapogenins, J. Am. Chem. Soc. 69:2167-2230 (1947)

25. Bills, C. E., Physiology of sterols, including vitamin D, Phys. Rev. 15:1-97 (1935), p. 21.

26. Bills, C. E., (25), p. 23.

27. Couerbe, J. P., Du cerveau, considere sous le point de vue chimique et physiologique, Ann. Chim. phys., series 2, 56:160-193 (1834). Cited by Bills, (25).

28. Flint, A., Experimental researches into a new excretory function of the liver; consisting in the removal of cholesterine from the blood, and its discharge from the body in the form of stercorine. (The seroline of Boudet.) Am. J. Med. Sci. new series, 44:305-365 and 3 plates (1862). Cited by Bills, (25).

29. Beneke, F. W., Ueber den Cholestearinge halt des menschlichen Gehirns, Sitzungs berichte d. gesammten Naturwissensch. zu Marburg, 25-28 (1880) Cited by Bills, (25).

30. Dezani, Arch. farm. 17:4 (1914). Cited by Gamble et al., (31).

31. Gamble, J. L., and K. D. Blackfan, Evidence indicating a synthesis of cholesterol by infants, J. Biol. Chem. 42:401-409 (1920)

32. Gardner, J. A., and F. W. Fox, On the origin and destiny of cholesterol in the animal organism. XII On the excretion of sterols in man, Proc. Roy. Soc. London, series B 92:358-367 (1921)

33. Channon, H. J., Cholesterol synthesis in the animal body, Biochem. J. 19:424-432 (1925)

34. Randles, F. S., and A. Knudson, Studies on cholesterol. I Synthesis of cholesterol in the animal body, J. Biol. Chem. 66:459-466 (1925)

35. Schoenheimer, R., Versuch einer Sterinbilanz an der legenden Henne, Ztschr. physiol. Chem. 185: 119-122 (1929)

36. Schoenheimer, R., and F. Breusch, Synthesis and destruction of cholesterol in the organism, J. Biol. Chem. 103:439-448 (1933)

37. Schoenheimer, R., New contributions in sterol metabolism, Science 74:579-584(1931)

38. Schoenheimer, R., Speicherungsversuche mit sitosterin, Ztschr. physiol. Chem. 180:5-16 (1929). Cited by Schoenheimer, (37).

39. Schoenheimer, R., Uber die Sterine des Kaninchenkotes, Ztschr. physiol. Chem. 180:32-37 (1929). Cited by Schoenheimer, (37).

40. Behring, v. and R. Schoenheimer, 5. Mitteilung: Sind gesattigte Sterine resorbierbar? Ztschr. physiol. Chem. 192:97-102 (1930)

41. Schoenheimer, R. and H. v. Behring, Klin. Woch. 9:1309 (1930). Cited by Schoenheimer, (37).

42. Page, I. H., and W. Menschick, Ueber die Wirkung von Ergosterinacetateverfutterung bei Kaninchen, Biochem. Ztschr. 221:6-10 (1930)

43. Schoenheimer, R. and H. Dam, Ueber Ergosterin-Resorption bei der legenden Henne, Ztschr. physiol. Chem. 211:241-245 (1932)

44. Tsujimoto, M., An unsaturated hydrocarbon in shark liver oil, J. Chem. Ind. Japan, 19:277-281 (1916). C.A. 10:1602 (1916)

45. Chapman, A. C., Spinacene: A new hydrocarbon from certain fish liver oils, J. Chem. Soc. 111:56-69 (1917)

46. Chapman, A. C., Spinacene: A new hydrocarbon from certain fish liver oils, J. Chem. Soc. 111:56-69 (1917)

47. Chapman, A. C., Spinacene: Its oxidation and decomposition, J. Chem. Soc. 123:769-779 (1923)

48. Chapman, A. C., Spinacene and some of its derivatives, J. Chem. Soc. 113:458-466 (1918)

49. Heilbron, I. M., E. D. Kamm, and W. M. Owens, The unsaponifiable matter from the oils of Elasmobranch fish. Part I A contribution to the study of the constitution of squalene (spinacene). J. Chem. Soc. 129:1630-1649 (1926)

50. Heilbron, I. M., T. P. Hilditch, and E. D. Kamm, The unsaponifiable matter from the oils of Elasmobranch fish. Part II The hydrogenation of squalene in the presence of nickel. J. Chem. Soc. 129: 3131-3140 (1926)

51. Channon, H. J., The biological significance of the unsaponifiable matter of oils. I. Experiments with the unsaturated hydrocarbon squalene (spinacene) Biochem. J. 20:400-408 (1926)

52. Vanghelovici, M., Structure of cholesterol, J. Soc. Chem. Ind. 53:998 (1934)

53. Robinson, R., Structure of cholesterol, J. Soc. Chem. Ind. 53:1062-1063 (1934)

54. Robinson, R., Constitution of cholesterol, Nature 130:540-541 (1930)

Robinson, R., Constitution of cholesterol, Nature 130:665-666 (1930)

55. Bryant, W. M. D., A relationship between cholesterol and carotene structures, J. Soc. Chem. Ind. 54: 907-908 (1935)

56. Spring, F. S., Polyterpenoid nature of sterols, J. Soc. Chem. Ind. 54:972-973 (1935)

57. Bryant, W. M. D., Carotenoid origin of cholesterol, J. Soc. Chem. Ind. 54:1082-1083 (1935)

58. Andre, E., and H. Canal, Contribution a l'etude des huiles d' animaux marins. Recherches sur l'huile de centrophore granuleux (centrophorus granulosus Müller et Henle). Etude comparei des matieres in saponifiable retirees de l'oeuf, du foie du foetus et du foie des animaux adultes. Bull. Soc. Chim. de Fr. series 4;45:-511-524 (1929)

59. Windaus, A., Nachr. v. d. Gesell. d. Wiss. zu Goettingen, 1:59 (1935) Cited by Needham, (65).

60. Reichstein, T., Uber Bestandtiele der Nebennieren-Rende (XI) Zur Konstitution der $C_{21}O_5$-Gruppe, Helv. Chim. Acta. 20:978-991 (1937)

61. Du Feu, F., J. McQuillin, and R. Robinson, Experiments on the synthesis of substances related to the sterol. Part XIV. A simple synthesis of certain octalones and ketotetrahydrohydrindenes, which may be angle-methyl-substituted type. A theory of the biogenesis of the sterols. J. Chem. Soc. 140: 53-60 (1937)

62. Marker, R. E., R. B. Wagner, P. R. Ulshafer, E. L. Wittbecker, D. P. J. Goldsmith, and C. H. Ruof, Steroidal sapogenins, J. Am. Chem. Soc. 69:2167-2230 (1947)

63. Miescher, K., and P. Wieland, Uber Steroide. 100 Mitteilung. Zur Biosynthese der Steroide. Helv. Chim. Acta. 33:1847-1864 (1950)

64. Ruzicka and Stepp, Ergebnisse der Vitamin und Hormonforschung. Akademische Verlagsgesellschaft Leipzig. (1938) p. 347. Cited by Marker, et al. (62).

65. Needham, J., Biochemistry and Morphogenesis, Cambridge University Press, London (1942) p. 250.

66. Hall, J. A., A system of structural relationships in phytochemistry, Chem. Rev. 20:305-344 (1937)

67. Channon, H. J., G. R. Tristam, The effect of the administration of squalene and other hydrocarbons on cholesterol metabolism in the rat. Biochem. J. 31:738-747 (1937)

68. Kimizuka, T., Biochemische untersuchgen über die unversiefbaren substanzen. I. Mitteilung. Die Verteilung der unverseifbaren substanzen in der Geweben der mit denselben substanzen gefutterten Tiere. J. Biochem. Japan 27:469-488 (1938)

69. Sonderhoff, R., and H. Thomas, Die enzymatische dehydrierung der trideutero-essigsaure, Ann. Chem. 530:195-213 (1937)

70. Block, K., and D. Rittenberg, The biological formation of cholesterol from acetic acid, J. Biol. Chem. 143:297-298 (1942)

71. Block, K. and D. Rittenberg, On the utilization of acetic acid for cholesterol formation, J. Biol. Chem. 145:625-636 (1942)

72. Block, K. and D. Rittenberg, An estimation of acetic acid formation in the rat, J. Biol. Chem. 159:45-58 (1945)

73. Ponticorvo, L., D. Rittenberg, and K. Block, The utilization of acetate for the synthesis of fatty acids, cholesterol and protoporphyrin, J. Biol. Chem. 179:839-842 (1950)

74. Little, H. N., and K. Block, Studies on the utilization of acetic acid for the biological synthesis of cholesterol, J. Biol. Chem. 183:33-46 (1950)

75. Block, K. and D. Rittenberg, Sources of acetic acid in the animal body, J. Biol. Chem. 155:243-254 (1944)

76. Block, K. and D. Rittenberg, On the utilization of acetic acid for cholesterol formation, J. Biol. Chem. 145:625-636 (1942)

77. Block, K., Metabolism of leucine and valine, J. Biol. Chem. 155:255-263 (1944)

78. Block, K. and D. Rittenberg, On the utilization of acetic acid for cholesterol formation, J. Biol. Chem. 145:625-636 (1942)

78a. Zabin, I. and K. Block, The utilization of butyric acid for the synthesis of cholesterol and fatty acids, J. Biol. Chem. 192:261-266 (1951)

79. Zabin, I. and K. Block, The utilization of isovaleric acid for the synthesis of cholesterol, J. Biol. Chem. 185:131-138 (1950)

80. Zabin, I. and K. Block, Studies on the utilization of isovaleric acid in cholesterol synthesis, J. Biol. Chem. 192:267-273 (1951)

81. Block, K., Borek, E, and D. Rittenberg, Synthesis of cholesterol in surviving liver, J. Biol. Chem. 162:441-449 (1946)

82. Brady, R. O. and S. Gurin, The biosynthesis of radioactive fatty acids and cholesterol, J. Biol. Chem. 186:461-468 (1950)

83. Brady, R. O. and S. Gurin, The synthesis of radioactive cholesterol and fatty acids in vitro, J. Biol. Chem. 189:371-377 (1951)

84. Brady, R. O. and S. Gurin, The synthesis of radioactive cholesterol and fatty acids in vitro, J. Biol. Chem. 189:371-377 (1951)

85. Curran, G. S., Utilization of acetoacetic acid in cholesterol synthesis by surviving rat liver, J. Biol. Chem. 191:775-782 (1951)

86. Anker, H. S., Some aspects of the metabolism of pyruvic acid in the intact animal, J. Biol. Chem. 176:1337-1352 (1948)

87. Block, K., The biological synthesis of lipids, Cold Spring Harbor Symposia Quant. Biol. 29: 13-34 (1948)

88. Brady, R. O., J. Rabinowitz, J. V. Baalen, and S. Gurin, The synthesis of radioactive cholesterol and fatty acids in vitro, J. Biol. Chem. 193:137-143 (1951)

89. MacKenzie, C., J. P. Chandler, E. B. Keller, J. R. Rachele, N. Cross, and V. du Vigneaud, The oxidation and distribution of the methyl group administered as methionine, J. Biol. Chem. 180:99-111 (1949)

90. Bonner, J. and B. Arreguin, The biochemistry of rubber formation in the guayule. I. Rubber formation in seedling. Arch. Biochem. 21:109-124 (1949)

91. Little, H. N., and K. Block, Studies on the utilization of acetic acid for the biological synthesis of cholesterol, J. Biol. Chem. 183:33-46 (1950)

92. Wuersch, J., R. L. Huang, and K. Block, The origin of the isooctyl side chain of cholesterol, J. Biol. Chem. 195:439-446 (1952)

93. Bonner, J. and B. Arreguin, The biochemistry of rubber formation in the guayule. I. Rubber formation in seedling. Arch. Biochem. 21:109-124 (1949)

94. Cornforth, J. W., G. D. Hunter, and G. Popjak, Studies of cholesterol biosynthesis. I. A new chemical degradation of cholesterol, Biochem. J. 54: 590-597 (1953)

Cornforth, J. W., G. D. Hunter, and G. Popjak, Studies of cholesterol metabolism. II. Distribution of acetate carbon in the ring structure. Biochem. J. 54:597-601 (1953)

95. Ottke, R. C., E. L. Tatum, and K. Block, Isotopic acetate and isovalerate in the synthesis of ergosterol by neurospora, J. Biol. Chem. 189:429-433 (1951)

96. Hanahan, D. J. and S. J. Wakil, The origin of some of the carbon atoms of the side chain of C^{14} ergosterol, J. Am. Chem. Soc. 75:273-275 (1953)

97. Srere, P. A., Dissertation, Univ. of Calif. (1951)

98. Langdon, R. G. and K. Block, The biosynthesis of squalene, J. Biol. Chem. 200:129-134 (1953)

99. Langdon, R. G. and K. Block, The utilization of squalene in the biosynthesis of cholesterol, J. Biol. Chem. 200:135-144 (1953)

100. Tomkins, G. M., I.L. Chaikoff, W. G. Dauben, and H.L. Bradlow, Synthetic C^{14}"squalene": Concerning its incorporation into cholesterol by liver, J. Am. Chem. Soc. 74:6145-6146 (1952)

101. Tomkins, G. M., W. G. Dauben, H. Sheppard, and I. L. Chaikoff, Squalene was a precursor of cholesterol in liver, J. Biol. Chem. 202:487-489 (1953)

102. Dauben, W. G., H. L. Bradlow, N. K. Freeman, D. Kritchevsky, and M. Kirk, The regeneration of squalene from its solid hexahydrochloride, J. Am. Chem. Soc. 74:4321-4323 (1952)

103. Wald, G., The biochemistry of vision, Ann. Rev. of Biochem. 22:497-526 (1953)

104. Block, K. and R. B. Woodward, The cyclization of squalene in cholesterol synthesis, J. Am. Chem. Soc. 75:2023-2024 (1953)

105. Dauben, W. G., S. Abraham, S. Hotta, I.L. Chaikoff, H. L. Bradlow, and A. H.Soloway, On the incorporation of acetate into cholesterol, J. Am. Chem. Soc. 75:3038 (1953)

106. Cornforth, J. W., Steroids, Ann. Reports Prog. of Chem. 49:190-202 (1952)

107. Gilman, H., Organic Chemistry, John Wiley & Sons, Inc., New York (1953) Vol. 4, p. 690.

108. Klein, H. P., Relation of coenzyme A to steroid and total lipid synthesis in yeast, Fed. Proc. 10:209 (1951)

109. Hanahan, D. J. and S. J. Wakil, The biosynthesis of ergosterol from isotopic acetate, Arch. Biochem. 37:167-171 (1952)

110. Klein, H. P., and F. Lipman, The relationship of coenzyme A to lipid synthesis. I. Experiments with yeast, J. Biol. Chem. 203:95-100 (1953)

111. Klein, H. P. and F. Lipman, The relationship of coenzyme A to lipid synthesis. II. Experiments with rat liver, J. Biol. Chem. 203:101-108 (1953)

112. Guehring, R. R., L.S. Hurley, and A. G. Morgan, Cholesterol metabolism in pantothenic acid deficiency, J. Biol. Chem. 197:485-493 (1952)

113. Becker, R. R., H. B. Burch, L. L. Salomon, T. A. Venkitasubramanian, and C. G. King, Ascorbic acid deficiency and cholesterol synthesis, J. Am. Chem. Soc. 75:2020 (1953)

114. Heinrich, M. R. and H. A. Mattill, Lipids of muscle and brain in rats deprived of tocopherol, Pro. Soc. Exptl. Biol. and Med. 52:344-346 (1943)

115. Callazo, J. A., I. Torres, and S. Rodriquez, Das A Vitamin und der Cholesterinstoffweschsel, Klin. Woch. 13:1678-82 (1934)

116. Taylor, C. B. and R. G. Gould, Effect of dietary cholesterol on rate of cholesterol synthesis in the intact animal measured by means of radioactive carbon, Circulation 2:467-468 (1950)

117. Langdon, R. G. and K. Block, The effect of some dietary additions on the synthesis of cholesterol from acetate in vitro, J. Biol. Chem. 202:77-81 (1953)

118. Schwenk, E., M. T. Werthessen, H. Rosenkrantz, Studies on the biosynthesis of cholesterol. Isolation of 3β , 5 α dihydroxy-6-ketocholestane from cholesterol, Arch. Biochem. 37:247-249 (1952)

119. Tomkins, G. M., H. Sheppard, and I. L. Chaikoff, Cholesterol synthesis by liver. III. Its regulation by ingested cholesterol, J. Biol. Chem. 201: 137-141 (1953)

120. Tomkins, G. M., H. Sheppard, and I. L. Chaikoff, Cholesterol synthesis by liver. II. Suppression by steroid administration, J. Biol. Chem. 203: 781-786 (1953)

121. Anker, H. S. and K. Block, On the metabolism of $\Delta^{4,5}$ cholestenone, J. Biol. Chem. 178:971-976 (1949)

122. Langdon, R. G. and K. Block, Accumulation of Δ^{7} cholestenol after squalene feeding, Fed. Proc. 12: 235-236 (1953)

123. Bucher, N. L. R., The formation of radioactive cholesterol and fatty acids from C^{14} labeled acetate by rat liver homogonates, J. Am. Chem. Soc. 75:498 (1953)

Rabinowitz, J. L. and S. Gurin, The biosynthesis of radioactive cholesterol by particle-free extracts of rat liver, Biochem. Biophys. Acta. 10: 345-346 (1953)

124. Popjak, G. and M. L. Beeckmans, Extrahepatic lipid synthesis, Biochem. J. 47:233-238 (1950)

125. Srere, P. A., I. L. Chaikoff, and W. G. Dauben, The in vitro synthesis of cholesterol from acetate by surviving adrenal cortical tissue, J. Biol. Chem. 176:829-833 (1948)

126. Block, K., E. Borek, and D. Rittenberg, Synthesis of cholesterol in surviving liver, J. Biol. Chem. 162:441-449 (1946)

127. Srere, P. A., I. L. Chaikoff, S. S. Treitman, and L. S. Burstein, Extrahepatic synthesis of cholesterol, J. Biol. Chem. 182:629-634 (1950)

128. Siperstein, M. D., I. L. Chaikoff, and S. S. Chernick, Significance of endogenous cholesterol in arteriosclerosis: Synthesis in arterial tissue, Science 113:747-749 (1951)

129. Thudichum, J. L. W., Chemical Constitution of the Brain, London (1884). Cited by Page, (130).

130. Page, I.H., Chemistry of Brain, C. C. Thomas, Springfield, Ill. (1937)

131. Block, K., B. N. Berg, and D. Rittenberg, The biological conversion of cholesterol to cholic acid, J. Biol. Chem. 149:511-517 (1943)

132. Waelsch, H., W. M. Sperry, and V. A. Stoyanoff, A study of the synthesis and deposition of lipids in brain and other tissues with deuterium as an indicator, J. Biol. Chem. 135:291- 296 (1940)

133. Waelsch, H., W. M. Sperry, and V. A. Stoyanoff, Lipid metabolism in brain during myelination, J. Biol. Chem. 135:297-302 (1940)

134. Waelsch, H., W. M. Sperry, V. A. Stoyanoff, The influence of growth and myelination on the deposition and metabolism of lipids in the brain, J. Biol. Chem. 140:885-897 (1941)

135. Gordon, A. H., A. J. P. Martin, and R. L. M. Synge, Partition chromatography in the study of protein constitutents, Biochem. J. 37:79-86 (1943)

136. Clenshaw, E. and Smedley-MacLean I, The nature of the unsaponifiable fraction of the lipoid matter extracted from green leaves, Biochem. J. 23:107-109 (1929)

137. Fieser, L. F. and M. Fieser, Natural Products Related to Phenanthrene, Reinhold Publishing Corp., New York, (1949) 3rd ed p. 285.

138. Shriner, R. L. and R. C. Fuson, The Systematic Identification of Organic Compounds, John Wiley & Sons, Inc., New York (1948) 3rd ed p. 228.

139. King, N. M., A. Chatterjee, and L. M. Parks, A note on the isolation of ursolic acid from Verbena stricta, Vent., J. Am. Pharm. Ass. 39:595-597 (1950)

140. Winterstein, A. and G. Stein, Uber das Guajacsaponin und ein Saponin aus Calendula officinalis, Z. Physiol. Chem. 199:64-74 (1931)

141. Obata, Y., The component of Viscum album. II. Free resin acids and unsaponifiable matter of resin wax contained in the woody portions, J. Agr. Chem. Soc. Japan 17:219-21 (1941). C. A. 45:3921c (1951)

142. Rowe, E. J., J. E. Orr, A. H. Uhl, and L. M. Parks, Isolation of oleanolic acid and ursolic acid from Thymus vulgaris, L., J. Am. Pharm. Assoc. 38:122-124 (1949)

143. Sando, C. E., Ursolic acid, J. Biol. Chem. 90:477-495 (1931)

144. Jacobs, W. A. and E. E. Fleck, The partial dehydration of ursolic acid, J. Biol. Chem. 92:487-494 (1931)

145. Goodson, J. A., The occurrence of ursolic acid in Escallonia tortuosa. Conversion of ursolic acid into α-amyrin, J. Chem. Soc. 999-1001 (1938)

146. Birch, A. J., Homocyclic compounds, Ann. Reports Progress Chem. 47:177-219 (1950)

147. Haworth, R. D., The triterpenes, Ann. Reports Progress Chem. 34:327-342 (1937)

148. Fieser, L. F. and M. Fieser, Natural Products Related to Phenanthrene, Reinhold Publishing Corp., New York (1949) 3rd ed p. 285.

149. Anderson, R. J., Properties of cholesterol from different sources, J. Biol. Chem. 71:407-418 (1926-1927)

150. Sobel, H., Squalene in sebum and sebum-like materials, J. Invest. Dermatology 13:333-338 (1949)

151. Ysauda, M., The determination of the iodine number of lipids, J. Biol. Chem. 94:401-409 (1931-1932)

152. Fitselon, J., Detection of olive oil in edible oil, J. Assoc. Official Agr. Chem. 26:499-506 (1943)

153. Kuhn, R. and H. Roth, Mikro-Bestimmung von Acetyl-, Benzoyl-, and C-Methylgruppen, Ber. dtsch. Chem. Ges. 66:1274-1278 (1933)

154. Carr, F. H., and E. A. Price, Colour reactions attributed to vitamin A., Biochem. J. 20:497-501 (1926)

155. Djerassi, C., L. E. Giller, and A. J. Lemin, Terpenoids: I. The triterpenes of the cactus Lemaineocereus thurberi, J. Am. Chem. Soc. 75:2254 (1953)

Gilman, H., Organic Chemistry, John Wiley & Sons, Inc., New York, Vol. 4 p. 692, (1953)

156. Anderson, R. J. and M. G. Moore, A study of the phytosterols of corn oil, cottonseed oil, and linseed oil, J. Am. Chem. Soc. 45:1944-1953 (1923)

157. Glasebrook, A. L. and W. G. Lovell, The isomerization of cyclohexane acid methyl-cyclopentane, J. Am. Chem. Soc. 61:1717-1720 (1939)

158. Wadkins, C. L., Personal communication.

159. Wadkins, C. L., H. J. Nicholas, Personal communication.

160. Gilman, H., Organic Chemistry, John Wiley & Sons, Inc., New York (1953) Vol. 4, p. 706.

161. Ditmer, A., Untersuchungen uber das Unverseifbare. II. Mitteilung. Das Unverseifbare aus dem fetalen Lebern und den Lebern Erwachsener, Z. Physiol. Chem. 271:293-315 (1941)

162. Channon, H. J. and G. F. Marrian, The biological significance of the unsaponifiable matter of oils. II. An unidentified unsaturated hydrocarbon present in mammalian liver, Biochem. J. 20:409-418 (1926)

163. Toyma, Chem. Umschau. 30:181 (1923). Cited by Channon and Marrian, (162).

164. MacKenna, R. M. B., V. R. Wheatley, and A. Wormal, Studies of sebum. Some constitutents of the unsaponifiable matter of human sebum, Biochem. J. 52:161-168 (1952)

165. Eidinoff, M. L., R. S. Rosenfeld, J. E. Knoll, B. J. Marano, and L. Hellman, The biosynthesis of squalene from acetate in the human, Abstracts of 124th National A.C.S. Meetings.

166. Fitselon, J., The occurrence of squalene in natural fats, J. Assoc. Official Agr. Chem. 26:506-511 (1943)