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STEROLS AND FATTY ACIDS OF ORGAN PIPE

CACTUS (LEMAIREOCEREUS THURBERI)

by

Harold Leslie Bird, Jr.

A Dissertation Submitted to the Faculty of the
COMMITTEE ON AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my
direction by Harold Leslie Bird, Jr.
entitled STEROLS AND FATTY ACIDS OF ORGAN PIPE
CACTUS (LEMAIREOCEREUS THURBERI)
be accepted as fulfilling the dissertation requirement of the
degree of Doctor of Philosophy

Henry W. Kircher
Dissertation Director

March 30, 1974
Date

After inspection of the final copy of the dissertation, the
following members of the Final Examination Committee concur in
its approval and recommend its acceptance:*

Antoine H. Vogel
W. F. McCaughey

April 1, 1974
April 2, 1974

*This approval and acceptance is contingent on the candidate's
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ABSTRACT

The fatty acid compositions of organ pipe cactus (Lemaireocereus thurberi), saguaro cactus (Carnegiea gigantea), cardon cactus (Pachycereus pringlei), and china cactus (Rathbunia alamosensis) were determined by gas liquid chromatography of the methyl esters of crude fatty acid fractions from lipid extracts of dried cactus tissue.

Organ pipe cactus showed no long chain fatty acids in tissue from mature stems, and only extremely small amounts of the long chain acids in tissue from young stems and growing tips. An abundance of short chain acids (6 to 12 carbons in length) was present. Saguaro and cardon showed predominantly the normal long chain acids with only small amounts of shorter chain acids, while china showed a more even distribution of short and long chain acids.

Organ pipe showed a much greater content of extractable lipid material than saguaro, cardon, or china. Differences in the type of solvent showing maximum extraction indicated a difference in the site or type of lipids in organ pipe from those in the other cacti.

The sterols in the nonsaponifiable fractions of organ pipe, saguaro, cardon, and china were investigated. Unusual sterols were found only in organ pipe and china. Saguaro and cardon had only normal phytosterols, apparently unesterified in the tissues. Organ pipe had extremely small amounts of the normal phytosterols in the nonsaponified fraction, appearing to occur as sterol esters in the tissues. The

predominant sterols in organ pipe were three dihydroxysterols, identified as peniocerol, macdougallin, and a new sterol, thurberol. These sterols were present in the tissues esterified with short chain fatty acids. Cina appeared to have some sitosterol and at least three unidentified sterols. One of the GLC peaks was at the same retention time as the single peak shown by the dihydroxysterols of organ pipe.

The yields of nonsaponifiables from organ pipe ranged from 11.2 to 17.8 g/100 g dried cactus, representing 55 to 93% of the crude lipids extracted. In contrast, the yields of nonsaponified fractions ranged from 0.8 g/100 g of dried cardon to 2.0 g/100 g of dried cina, representing only 11 to 20% of the crude lipid extracts.

Triterpenes were found in organ pipe in larger amounts than the dihydroxysterols and some appeared to be present in cina. None were found in saguaro or cardon.

No alkaloids were found in organ pipe. In cina the alkaloid fraction was less than 0.02% of the dried cactus; in saguaro, 1%; in cardon epidermis, 2.5%; and in cardon cortex, 4%.

The dihydroxysterols were separated from the other materials in the nonsaponifiable fraction by silica gel chromatography. Peniocerol and thurberol were separated from macdougallin with digitonin, and from each other by preparative thin-layer chromatography of their diacetates on layers of silver nitrate-silica gel G with chloroform as the developing solvent.

A new method of cleavage of sterol digitonides was discovered. The precipitates were heated in methanol and digitonin precipitated by the addition of large volumes of acetone.

Two of the dihydroxysterols were identified as peniocerol and macedougallin by reactions, analytical data, and direct comparison with very small samples of peniocerol diacetate, macedougallin diacetate, and the 3(14) isomer of peniocerol diacetate supplied by Dr. Carl Djerassi.

The structure of the third dihydroxysterol was determined to be 8,14-cholestadien-3 β ,6 α -diol, a previously undescribed compound for which the trivial name thurberol is proposed.

Some biochemical implications of the results of this research on the Drosophila nigrospiracula-organ pipe relationship are discussed.

CHAPTER 1

INTRODUCTION

The earliest report of the essential role of dietary sterols for insects was that of Hobson (1) in 1935, in which he showed that sterols were necessary for growth and development of the larvae of a blow-fly Lucilia sericata Mg. Later work, thoroughly reviewed in 1964 by Clayton (2), showed the complete dependence upon dietary sterols for the growth, development, and fecundity of many kinds of insects. In addition, all of the insects in those studies were able to utilize cholesterol as a dietary sterol for all of the processes for which sterols were needed. In the following year, however, Heed and Kircher (3) reported that a fruit fly, Drosophila pachea, common to the Sonoran Desert region, could not utilize cholesterol for its life processes but instead needed a very unusual sterol which it obtained from a cactus. More recently, Chu, Norris, and Kok (4) found a beetle, Xyloborus ferrugineus that also needed sterols other than cholesterol for reproduction.

General Insect-Host Plant Relationships

Since phytophagous insects get the necessary sterols they ingest from the plants they eat and/or microorganisms associated with those plants, more or less specific insect-host plant relationships emerge depending upon the availability of certain sterols in certain

plants and how specific the requirement for certain sterols is in the insect. This specificity can, of course, be modified even further by specific nutritional requirements in addition to sterols. The specificity of the insect-host plant alliance is also very definitely modified by the presence in some plants of substances that attract, repel, or poison certain insects. Further modifying factors are the physical structure of the plant (moisture content, waxy protective coating, etc.) and competition with other insects for the same host plant. Most plants have the common phytosterols and most insects can utilize these for their sterol requirement if present in a form the insect can use. Plant sources become much more limiting when the insect requires unusual sterols.

Drosophila-Cactus Host Relationships

The insect-host plant relationships of several species of cactiphilic *Drosophila* to several genera of cacti of the subfamily Cereinae common to the Sonoran Desert region have been the subject of extensive study at The University of Arizona. Most of the reports of that work were reviewed by Kircher and Heed (5) in 1970.

The common and scientific names of the cacti most frequently mentioned in the present work are: senita, *Lophocereus schottii* (Engelmann) Britton and Rose (6); saguaro, *Carnegiea gigantea* (Engelmann) Britton and Rose; cardon, *Pachycereus pringlei* (S. Watson) Britton and Rose; agria, *Machaerocereus gummosis* (Engelmann); organ pipe *Lemaireocereus thurberi* (Engelmann) Britton and Rose; cina, *Rathbunia alamosensis* (Coulter) Britton and Rose; and hecho, *Pachycereus pecten-aboriginum*

(Engelmann) Britton and Rose. The common names are used in all the work that follows.

Drosophila pachea-Senita Cactus Studies

The very extensive study of this relationship showed that Drosophila pachea Patterson and Wheeler is completely dependent upon senita cactus which has at least four unusual sterols. One of the sterols, 7-stigmasten-3 β -ol (schottenol), is essential for complete development of D. pachea. In addition, senita has three isoquinoline alkaloids (lophocereine, pilocereine, and piloceredine) that are toxic to other Drosophila species but relatively innocuous to D. pachea. This prevents other species of Drosophila from using rotting senita as a breeding ground. Thus the monophagous relationship between D. pachea and senita cactus is well explained by the unusual sterol and the alkaloids in the plant.

Other Drosophila-Cactus Studies

Specific relationships between other Cereus cacti and Drosophila are equally interesting but not yet as fully explained as that of the D. pachea-senita dependence. These relationships were reported by Heed, Russell, and Ward (7), Kircher and Heed (5), and Fellows (8).

Drosophila nigrospiracula Patterson and Wheeler is a member of the desert adapted repleta species group and is oligophagous. It inhabits primarily saguaro and the closely related cardon, and hecho. These are considered as the host plants of this species. Drosophila mojavensis Patterson and Crow, whose usual host plant in Mexico is

agria, also breeds on organ pipe, in Organ Pipe Cactus National Monument, Arizona, and other areas where agria is absent.

Fellows (8) found D. mojavensis breeding and feeding on saguaro, the host plant for D. nigrospiracula, during the summer months in that location when organ pipe rots were unavailable. This shows that D. mojavensis can utilize saguaro cactus. However, D. nigrospiracula is never found feeding and breeding in organ pipe. In fact, Fellows (8), working with artificially rotted organ pipe, showed that D. nigrospiracula had only very limited ability to reproduce on organ pipe. There were many eggs and developing larvae but mass mortality occurred in the first and second instars and few larvae continued development to the imago stage. When the rotting organ pipe was injected with live Baker's yeast, Saccharomyces cerevisiae, the yield of imagos was 10 times the number on the nonyeasted organ pipe but still small compared to yields of D. nigrospiracula on its host plant, saguaro. There was also evidence that the yeast was unable to multiply on the organ pipe cactus. This relative nonemergence of D. nigrospiracula on organ pipe cactus and the apparent inhibition of yeast growth by organ pipe are unexplained.

Alkaloids of Organ Pipe and Saguaro

Because of the D. pachea-senita situation, alkaloids might be considered as logical inhibitors of both yeast growth and breeding of D. nigrospiracula on organ pipe. However, Djerassi, Geller, and Lemin (9) reported a total absence of alkaloids in an alcoholic extract of organ pipe cactus. On the other hand, saguaro and cardon are known

to contain at least two alkaloids, carnegine, found by Heyl (10), and gigantine, found by Hodgkins, Brown, and Massengill (11). Neither of these alkaloids is apparently toxic to D. nigrospiracula or D. mojaven-sis since both species can feed and breed on saguaro. Neither is apparently inhibitory to Saccharomyces cerevisiae because this is one of the yeasts found in cardon rots (12).

Previous Published Work
on Organ Pipe Cactus

A search of the literature revealed that only two papers had been published reporting work with organ pipe cactus. The paper by Djerassi et al. (9) reported the isolation of oleanolic acid and a new triterpene, thurberogenin, both occurring as glycosides. Jolad and Steelink (13) reported the isolation of thurberogenin, betulin, and another new triterpene which they called "thurberin." However, later, "thurberin" was found to be identical to a compound, calenduladiol, previously isolated from flowers of the marigold, Calendula officin-ales, by Kasprzyk and Pyrek (14). Both groups agreed it should be named calenduladiol (15). None of these papers mentioned any work with sterols or fatty acids in organ pipe cactus.

CHAPTER 2

THE PURPOSE OF THE CURRENT RESEARCH

Is the inability of D. nigrospiracula to utilize organ pipe cactus due to a direct effect of a substance, or substances, in the cactus that inhibit the development of the fly or is some substance, or substances (sterol? fatty acid?), lacking that is needed for its full development? Why then does organ pipe support D. mojavenis?

Is this nonsupport an indirect effect due to inhibition by organ pipe on the growth of a yeast essential for the full development of D. nigrospiracula? If so, what is causing this inhibition of the yeast growth?

The answers to these questions will require, at least, a closer look at organ pipe cactus and how it compares or contrasts with saguaro and perhaps some other *Cereus* cacti. This closer look at organ pipe cactus and some comparisons of it with other cacti common to the Sonoran Desert region prompted the research reported in this dissertation. A study of the sterols and fatty acids was thought to offer possible answers to the above questions.

The purpose of this research was fourfold:

1. To determine the fatty acid profile and general sterol profile of organ pipe cactus.
2. To compare the fatty acid profile and sterol profile with saguaro and other *Cereus* cacti if possible.

3. To isolate any unusual sterols and characterize them if possible.

4. To analyze the results for leads to answering the questions posed and for any other biochemical implications.

CHAPTER 3

GENERAL MATERIALS AND METHODS

Most of the materials, methods, and instruments used in this research are described in this chapter, but some very specific methods are given in the chapters describing the phase of work for which they were used.

Instruments

Ultraviolet spectra were run on a Perkin-Elmer model 202 ultraviolet-visible spectrophotometer, using a 1 cm cell and 95% ethanol as the solvent.

Infrared spectra were run on a Perkin-Elmer Infracord 137B spectrophotometer, using 0.5 mm sodium chloride cells. Acetylated sterols were dissolved in carbon disulfide, carbon tetrachloride, or chloroform. Some work was done with the free sterols in potassium bromide disks.

Nuclear magnetic resonance (NMR) spectra were obtained on both the Varian A60 and HA100 instruments. Carbon tetrachloride and deuterated chloroform were used as solvents.

Mass spectrometry was done on a double focusing Hitachi Perkin-Elmer RMU-6E mass spectrometer.

Melting points were all obtained on a Thomas-Hoover capillary melting point apparatus. Some were done in air and some in vacuum. All were uncorrected.

Evaporation of all solvents was done, unless otherwise noted, using a Buchi Rotavapor R rotary vacuum evaporator with a water aspirator as the vacuum source.

Solvents

All solvents used for chromatography or crystallizations were analytical reagent grade or redistilled. Skellysolve B used for extraction was distilled (60-90°C boiling fraction) before use.

Cactus Materials

All cactus samples used in this research were collected in Arizona or in Mexico by Dr. H. W. Kircher, Dr. W. B. Heed, or myself and were sliced, air and/or vacuum-dried, and ground to small particle size in Waring blenders.

Organ Pipe Cactus

The organ pipe cactus tissue used in this study was obtained, by special permission, from one large plant that had been uprooted by the wind in Organ Pipe Cactus National Monument, Arizona. Mature and young stems of this columnar cactus were collected and dethorned. The tissue was divided into the following categories: epidermis, cortex, woody skeleton, and pith. The tissue classified as "epidermis" was all of the green epidermis and included some cortex tissue. The nature of the convoluted surface of the stems of organ pipe, as seen in Figure 1, make an absolute separation of the epidermis from cortex tissue nearly impossible. The cortex tissue was always free of any epidermal tissue except in the case of the cortex from growing tips of young stems,

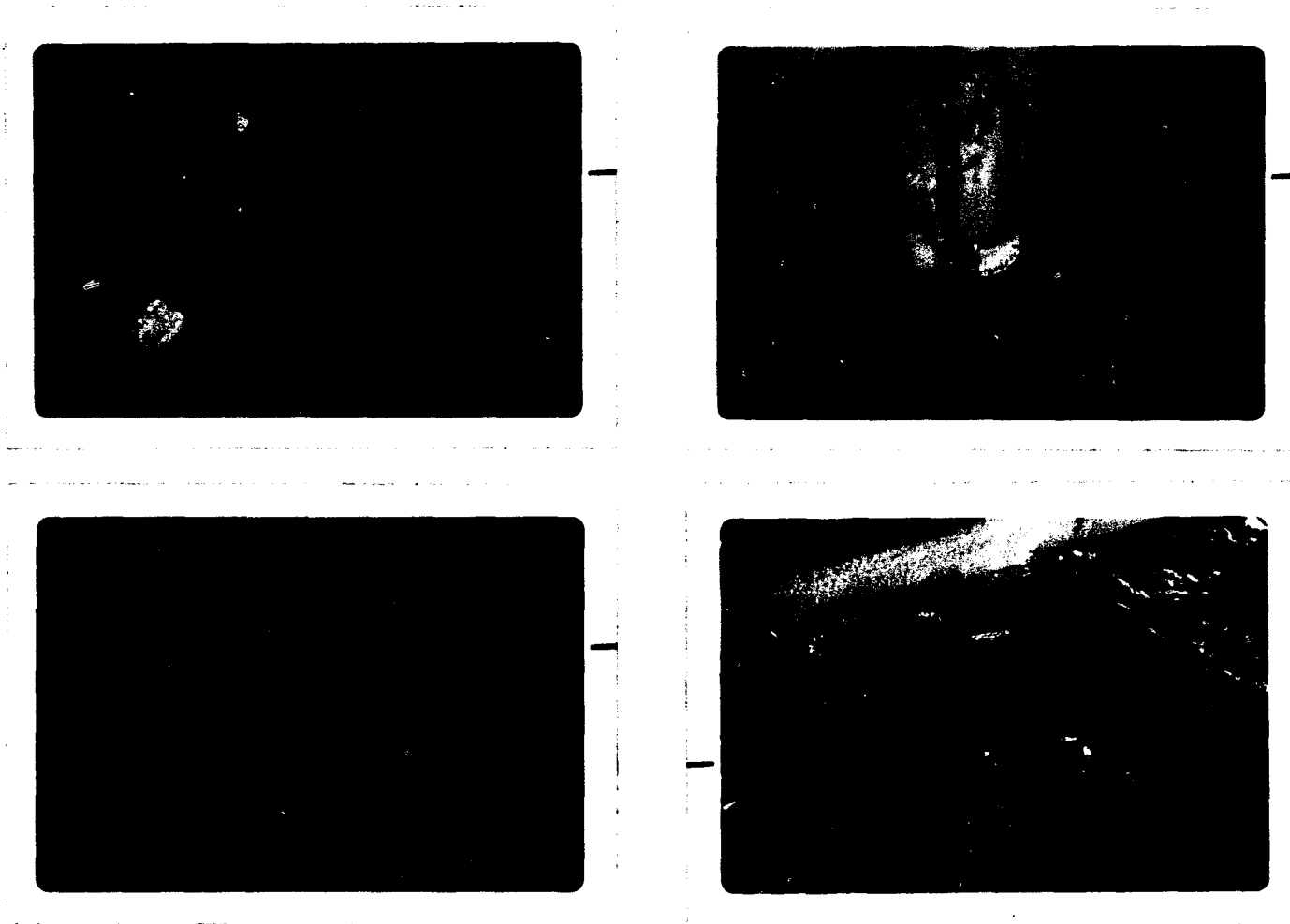


Figure 1. Organ pipe cactus tissue.

where some of the pith and soft woody tissue were included. The cortex tissue of mature organ pipe was yellow with a spongy, not very wet texture, very different from saguaro or cardon. All tissue was sun-dried to crispness, then vacuum-dried before grinding.

Saguaro Cactus

The saguaro tissue used in this research was all obtained from the growing end of an arm obtained from a very old specimen which had been downed in Tucson to make way for a large department store. The arm was approximately one foot in diameter. The tissue was sliced and dried without separating epidermis, cortex, pith, or woody type tissue. The tissue was sun-dried, but dried much slower than organ pipe tissue, and was finally dried in a vacuum oven before grinding.

Cardon Cactus

The sample of cardon was the growing end of an arm about 10 inches in diameter and 18 inches long collected in Sonora, Mexico, by Dr. H. W. Kircher. This tissue was separated into epidermis and cortex tissue and air-dried in the laboratory before drying in a vacuum oven. Again, the epidermis contained some cortex tissue but because of the nature of the cactus, a much better separation could be made than in the case of organ pipe.

Cina Cactus

The cina was supplied as dried, ground, complete tissue by Dr. W. B. Heed.

Extraction of Cactus Lipids

The dried, ground cactus powder (25-600 g batches) was extracted overnight with occasional stirring in 250-600 ml of Skellysolve B, acetone, chloroform-methanol mixtures, or chloroform per 100 g of dry cactus. The extract was filtered off, and the residue resuspended, at least once more in the same solvent, for another overnight extraction. All extracts of the same solvent were pooled and evaporated on a rotary evaporator to give the crude lipids. The cactus residue after Skellysolve B extraction was often reextracted with chloroform or a chloroform-methanol mixture, and these second extracts were evaporated separately to get a second crude lipids fraction.

Saponification of Crude Lipids

The lipids were saponified by refluxing them 3-4 hours with 10 ml of 10% KOH in 95% ethanol per gram of material. After cooling, two volumes of water were added to the saponification mixture.

Extraction of Nonsaponifiables, Fatty Acids, and Alkaloids

The diluted saponification mixtures were extracted with diethyl ether; the ether extracts were washed with water, then with 10% sulfuric acid and water again. The first water washes were added to the previous aqueous phase. The 10% sulfuric acid and following water washes were combined and saved for later extraction of nonphenolic alkaloids. The washed ether extracts were dried over anhydrous sodium or magnesium sulfate and evaporated to yield the nonsaponifiable fractions used for the sterol work.

The ether extracted, aqueous saponification mixtures were acidified with sulfuric acid and extracted with ether. This ether extract was washed with water and evaporated. Water was removed by adding absolute ethanol, followed by evaporation to leave the dry, crude fatty acid fractions.

The 10% sulfuric acid washings and following water washes were made basic with 15 M aqueous ammonia, and any alkaloids present were extracted with chloroform. The chloroform extracts were evaporated to leave a crude alkaloid fraction if the cactus had alkaloids. In the case of organ pipe cactus, nothing appeared in this fraction, so the 10% sulfuric acid wash and subsequent water washes were discarded. This alkaloid fraction (in g/100 g dry cactus) was 0.17 for cina, 0.95 for saguaro, 2.55 for cardon epidermis, and 4.15 for cardon cortex. Thin-layer chromatography (TLC) showed one additional alkaloid spot in cardon that was not in saguaro, and cina showed many different spots that gave an orange reaction with Dragendorff reagent. No further work was done with these alkaloids.

Thin-Layer Chromatography

Thin-layer chromatography was accomplished on 0.25 mm layers of silica gel G or silica gel G with 10% silver nitrate added before spreading. The plain silica gel G layers were usually used without heat activation, but the AgNO_3 -silica gel G layers were usually activated at 120°C for 30-45 minutes before use. Various developing solvents were used, and these will be specified where any TLC results are given. Visualization of sterols was accomplished by spraying the finished

chromatogram with a 30% (v/v) aqueous solution of sulfuric acid followed by heating the sprayed plate in an oven at 120°C until colored spots were observed or until charring was produced.

Preparative thin-layer chromatography was carried out on 1 mm layers and visualization of the bands was accomplished by spraying the dried layer with a very fine spray of distilled water. The sterol areas did not wet as well with the water and appeared as white areas on a translucent background, allowing these areas to be marked for scraping. Elution of the sterol material was accomplished by transferring the scraped material to a small chromatographic tube of appropriate size and plugged by a small wad of glass wool. The appropriate eluting solvent was poured in the top of the column, and the eluate collected. This elution by chromatography was continued until the eluate showed a negative test for sterol with the Liebermann-Burchardt reaction, performed on a few drops of the eluate. Very little solvent was needed for complete elution by this method.

Liebermann-Burchardt Reaction

The Liebermann-Burchardt reaction was used to detect the sterols, triterpenes, and digitonin in various fractions. Solids were dissolved or suspended in 5-10 drops of chloroform and an equal number of drops of acetic anhydride was added. Liquid samples, if not already in chloroform or ethyl acetate, were evaporated to dryness and chloroform was added as with solid samples. The same number of drops of a 10% (v/v) solution of 18 M H₂SO₄ in glacial acetic acid was then added. Different colors were produced depending upon the type of sterol or

triterpene present. The time of formation of the color and the change in color were also characteristic when working with purer materials. The colors are described in the section where the use of the reaction is noted.

Silation and GLC of Sterols, Triterpenes, and Sterol Digitonides

Silation of sterols and triterpenes was carried out by adding a few drops of bis(trimethylsilyl)acetamide to a small amount of the material in a small vial. The capped vial was warmed for several minutes on a steam bath and a sample of the solution was injected into the GLC instrument as such or after dilution with a few drops of benzene. The same procedure was used on sterol digitonides, but it was necessary to heat for a longer period of time.

Gas liquid chromatography of sterols, triterpenes, acetylated sterols, silated sterols, and silated triterpenes was accomplished on a Model 600 Research Specialties Co. instrument with an Sr^{90} detector, using argon as the carrier gas. Three different types of columns were used at different times during this work, 3% QF-1 at 235°C and 40 psig argon, 3% SE 52 at 255°C and 80 psig argon, and 5% OV 101 at 260°C and 60 psig argon. The last column gave the best separations and was used for most of the GLC.

Methylation and GLC of Fatty Acids

Formation of the methyl esters of fatty acids was carried out by the addition of 10 ml of 7% BF_3 in methanol to approximately 1 g or 1 ml of fatty acid or fatty acid fraction and allowing the mixture to

stand overnight at room temperature. After the addition of 20 ml of water, the methyl esters were extracted with Skellysolve B or hexane, the solvent evaporated and the methyl esters dissolved in benzene for GLC.

Gas liquid chromatography of the methyl esters of fatty acids was performed on an A90P Aerograph instrument using an 8' x $\frac{1}{8}$ " column packed with 15% diethylene glycol succinate (DEGS) on Chromasorb W at 195°C and 30 psig helium.

CHAPTER 4

FATTY ACIDS OF ORGAN PIPE CACTUS; A COMPARISON WITH OTHER CEREUS CACTI

The types of fatty acids found in higher plants and their distribution within the plant have been extensively investigated for many families and innumerable genera of plants, but little has been reported on fatty acids in the Cactaceae family. In their rather extensive monograph, Hitchcock and Nichols (16) present data of the fatty acids in 106 genera, in 50 families, of higher plants but do not include the Cactaceae. Nichols (17), in a personal communication, revealed that they have looked at a "variety of cacti" and found only "normal" plant fatty acids and that "the major lipids present were the galactosyl diglycerides and phospholipids common to green tissue rather than triglycerides." In an earlier review, Nichols and James (18, p. 42) expressed the view that "the evidence available shows that all photosynthetic apparatus which performs the Hill reaction have the same basic acyl lipid composition," but that the relative stoichiometry and individual fatty acid composition could vary. They indicated that in all the major acyl lipids of the chloroplast lamellae the major fatty acid was α -linolenic.

Gellerman and Schlenk (19) found, in a quantitative GLC study, that the predominant fatty acids in the saguaro cactus were oleic, α -linolenic, palmitic, linoleic, behenic, and lignoceric acids in

decreasing order from 25.4% to 5.3%. They also found nine other fatty acids (12 carbons and longer) in amounts from 3% down to 0.2%. No mention was made of the fatty acids shorter than 12 carbons, but the lauric (12 carbons) and myristic (14 carbons) were present at only 2.9% and 1.6%, respectively.

Kircher (20) reported that the predominant fatty acids of another *Cereus* cactus, *senita*, were palmitic, oleic, linoleic, and linolenic acids. Samples of mature stem tissue, especially from plant material collected in the southern end of Sonora, Mexico, were richer in short chain (shorter than 16 carbons) fatty acids as well. Samples of mature stems collected from the northern part of Sonora, and all younger stems collected, showed only very small amounts of the shorter chain acids. Epidermal tissue of younger stems was richer in α -linolenic acid than the cortex.

Kircher (21) also found in a single sample of pitaya agria, the host plant of *D. mojavensis* in Mexico, an abundance of short chain fatty acids along with the "normal" palmitic, oleic, linoleic, and α -linolenic acids.

The purpose of this part of the current study was to determine the fatty acid pattern of organ pipe cactus and to see how it compared with other *Cereus* cacti, particularly with the fatty acid patterns of saguaro and cardon which support the growth of *D. nigrospiracula* so well.

Specific Materials and Methods

Samples of crude fatty acid fractions obtained from various extracts of organ pipe, saguaro, cardon, and cina cactus were methylated according to the procedure described in Chapter 3. Benzene solutions of the methyl esters were injected into the Aerograph GLC instrument described in Chapter 3.

Methyl esters of caproic (6:0), caprylic (8:0), capric (10:0), and lauric (12:0) acids were prepared for GLC standards. The methyl esters of myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1,9c), linoleic (18:2,9c 12c) and linolenic (18:3,9c 12c 15c) acids were already available.

Results and Discussion

The first sample of methyl esters of fatty acids submitted to gas liquid chromatography was from a Skellysolve B extract of the cortex of a mature organ pipe cactus. No long chain fatty acids were present even when a large sample was injected. Only caproic, caprylic, capric, and lauric acids were observed and capric appeared to be the predominant one. This result was rather startling in the light of the fatty acid patterns previously obtained with saguaro, senita, and agria. This prompted a more extensive investigation of the fatty acid pattern of organ pipe cactus than originally planned.

It now became important to determine whether epidermal tissue would show a different pattern, whether more immature tissue would give a different pattern than the mature stems, and whether a different solvent would extract more of the long chain acids. It was also important

to compare the fatty acid pattern of other cacti extracted with the same solvents and the extracts processed in the same manner as the organ pipe extracts and to examine the yields of crude lipids and fatty acid fractions of extracts of organ pipe and other cacti to determine how they relate to type of cactus, type of tissue, and type of solvent used for the extraction of the lipids.

Types of Fatty Acids

Table 1 shows the common fatty acids found in the fatty acid fractions of various extracts of organ pipe, cina, saguaro, and cardon. The study was limited to the most common acids, and only their presence or absence is shown although, in some cases, some additional unidentified acids were present. The only attempt at quantitation was to show what appeared to be the predominant acid, or acids, with a large X and to indicate small amounts and trace amounts with an s and t, respectively. The chloroform extract shown on the third line of the table was obtained from the residue of the preceding incomplete, single, Skellysolve B extract. The acetone extract was performed on a fresh sample in a Soxhlet extractor overnight to get a very complete extraction.

Regardless of the solvent used for extraction, the extracts of mature organ pipe stem tissue, whether epidermis or cortex, gave no indication of any long chain fatty acids. Only chloroform:methanol extracts of young organ pipe stem and growing tip (including meristematic tissue) showed any of the common long chain fatty acids. However, even

Table 1. Common fatty acids in fatty acid fractions of organ pipe cactus and other cereus cacti.

Cactus tissue		Solvent	Fatty acids ^a									
			6:0	8:0	10:0	12:0	14:0	16:0	18:1	18:2	18:3	
Organ pipe	Mature	Epidermis	Skelly B	x	x	X	x	t				
Organ pipe	Mature	Epidermis	Skelly B	x	x	X	s					
			Chloroform	x	x	X	x					
Organ pipe	Mature	Cortex	Acetone	x	x	X	x					
Organ pipe	Mature	Cortex	Skelly B	x	x	X	x					
Organ pipe	Mature	Cortex	Skelly B	x	x	X	x					
Organ pipe	Mature	Cortex	3:2 C:M	X	x	x	x					
Organ pipe	Young	Epidermis	Skelly B		X	X	X	s				
			2:1 C:M ^b	x	X	x	x	x	x	s	s	s
Organ pipe	Young tip	Epidermis	Skelly B		X	X	X	s	t			
			2:1 C:M ^b		X	X	x	s	s	s	s	s
Organ pipe	Mature	Complete	2:1 C:M	x	x	X	x					
Cina	?	Complete	Skelly B	x	x	X	x	x	x	x	x	x
			2:1 C:M ^b		x	X	x	x	x	x	x	x
Saguaro	Mature	Complete	Skelly B		s	s	x	s	X	X	X	X
			2:1 C:M ^b				s	t	s	X	X	X
Cardon	Mature	Epidermis	Skelly B				t		t			
			2:1 C:M ^b				x	x	X	x	x	x
Cardon	Mature	Cortex	Skelly B				x	x	X	x		
			2:1 C:M ^b				s		x	x	x	x

a. "s" means small amount; "t" means trace amount; "X" means major fatty acid.

b. (v/v) Chloroform:methanol after Skelly B extraction.

here they were present in very small amounts compared to the short chain acids.

Extracts of saguaro and cardon, processed in the same manner as the young organ pipe tissue, showed a predominance of the long chain acids and little or no short chain acids. This confirms qualitatively the quantitative data of Gellerman and Schlenk for saguaro (19). A difference between saguaro and cardon was that the fatty acid fractions of the Skellysolve B extracts of cardon appeared to have less of the long chain fatty acids while chloroform: methanol extracts were richer in these. Palmitic appeared predominant in cardon while oleic, linoleic, and α -linolenic appeared predominant in saguaro. Cina fatty acid fractions had both long chain and short chain fatty acids, appearing similar to agria and the mature senita collected from the southern part of Sonora as previously mentioned. Capric acid appeared to be predominant.

Lipid and Fatty Acid Yields

The yields of lipids and fatty acid fractions from various extracts of organ pipe, cina, cardon, and saguaro cactus tissues are shown in Table 2. Where two successive extracting solvents were used on the same batch of dried cactus tissue, both individual lipids and fatty acid yields are given. Also total lipids and total fatty acids figures are given. The last column shows the percentage of the total lipids represented by the total fatty acids.

Several conclusions can be drawn from the data of Table 2. First, organ pipe has more lipid than cina, saguaro, or cardon. Second,

Table 2. Lipid and fatty acid fractions from cactus tissue extracts (g/100 g dry cactus).

Cactus sample	Solvent	Lipids	Total lipids	Fatty acids	Total fatty acids	$\frac{TFA}{TL}\%$
Organ pipe, mature cortex	SkB	13.7	13.7	2.1	2.1	15
Organ pipe, mature, epidermis	SkB Chloroform	17.4 2.1	19.5	0.6 1.9	2.5	13
Organ pipe, mature, epidermis	Acetone	18.7	18.7	6.0	6.0	33
Organ pipe, young, epidermis	SkB C:M ^a	15.9 5.6	21.5	4.5 2.6	7.1	33
Organ pipe, young tip, epidermis	SkB C:M ^a	17.5 4.4	21.9	5.3 1.5	6.8	31
Cina, complete	SkB C:M ^a	2.7 8.0	10.7	1.0 2.8	3.8	36
Cardon, cortex	SkB C:M ^a	0.5 6.2	6.7	0.3 0.6	0.9	13
Cardon, epidermis	SkB C:M ^a	0.6 5.3	5.9	0.6 0.6	1.2	20
Saguaro, complete	SkB C:M ^a	0.4 9.1	9.5	0.4 0.1	0.5	5

a. 2:1 (v/v) Chloroform:methanol extraction after Skellysolve B extraction.

most of the organ pipe lipid extracts with Skellysolve B--not so with cina, saguaro, or cardon. Chloroform-methanol extracts more lipid than Skellysolve B from cina, saguaro, and cardon, but a smaller percentage of this lipid material is fatty acid than that of the lipid extracted by Skellysolve B. Third, a greater amount of lipids are extractable, by ordinary room temperature methods, from younger organ pipe tissue than from the more mature stems. However, the lipid material extracted by Soxhlet extraction of mature stems with acetone seems to have the same content of fatty acids as the extracts of younger tissue by the normal extraction procedure.

The data in Table 2 suggest that mature epidermis from organ pipe has more extractable lipid than the cortex. However, several other Skellysolve B extracts of the cortex from mature organ pipe stems showed lipid contents in the same range as those from the epidermal tissue.

Conclusions

The results, taken as a whole, show that organ pipe is a very different cactus from the other *Cereus* cacti investigated in its lipid content and in the types of fatty acids present. The difference in extractability with Skellysolve B would indicate that the lipids were in a different form in organ pipe than in the other cacti studied. The greater extractability or content of fatty acids in the younger organ pipe tissue might point to a different form of lipids in younger tissue than in more mature tissue.

The lack of long chain fatty acids in the epidermis of mature organ pipe, a tissue one would expect to be photosynthetic, is an anomaly. Nichols (17), in a personal communication, wrote "if your system is essentially a photosynthetic one then a predominance of acids of chain length C_{12} and below is quite unique." The epidermal tissue of younger stems only shows a tendency to adhere to the expected pattern of photosynthetic tissue in having at least small amounts of the longer chain fatty acids, and yet it does not seem likely that the green epidermal tissue of the mature organ pipe cactus stem is not photosynthetically active tissue. If photosynthesis does indeed take place, very different lipids must be involved in the chloroplast lamellae. These usually have monogalactosyl diglyceride, digalactosyl diglyceride, sulfolipid, and phosphatidyl glycerol as the major lipids and the major fatty acid in these lipids is normally α -linolenic acid.

The different type of lipids, the large content of short chain fatty acids, and the lack of long chain fatty acids are characteristic differences between organ pipe cactus which does not support the growth of D. nigrospiracula and saguaro or cardon which are both hosts to this fly. How, or if, these differences are important in the D. nigrospiracula-organ pipe relationship is not known but must certainly be taken into consideration.

CHAPTER 5

PHYTOSTEROLS OF ORGAN PIPE CACTUS; A COMPARISON WITH SAGUARO AND OTHER CEREUS CACTI

Two unusual sterols, lophenol (4α -methyl-7-cholesten- 3β -ol) and schottenol (7-stigmasten- 3β -ol), were isolated from senita cactus by Djerassi et al. (22). A structure was assigned to lophenol and only a tentative structure was given for schottenol because some discrepancies existed in its physical constants when compared to synthetic material. Heed and Kircher (3) further established the structure of schottenol as tentatively proposed by Djerassi et al. (22). Kircher (20) found three additional unidentified sterols in senita cactus and reported that preliminary evidence suggested that two of them may be 8, 14-dienes.

Kircher (23) determined (TLC and GLC evidence) that saguaro cactus had sitosterol as its primary sterol and smaller quantities of stigmasterol. He also had some data which showed that organ pipe cactus contained a single sterol that had the retention time of campesterol on GLC but an R_f value on TLC similar to one of the unknown sterols found in senita cactus.

The preceding data were all the facts known about the sterols of the *Cereus* cacti of the Sonoran Desert before the current study was started.

Specific Materials and Methods

One sample of dried, ground saguaro cactus tissue was extracted with 2:1 (v/v) chloroform:methanol, and the extract was saponified by refluxing in 10% ethanolic potassium hydroxide solution for 45 minutes. Only nonsaponifiables and alkaloids were extracted from this particular saponification mixture. Later extracts of saguaro tissue by Skellysolve B followed by 2:1 (v/v) chloroform:methanol were saponified by the longer saponification described in Chapter 3.

Samples of dried, ground epidermis and cortex from a mature stem of organ pipe cactus were extracted with 3:2 (v/v) chloroform:methanol, and the crude extract obtained from each type of tissue was saponified by refluxing in 10% methanolic potassium hydroxide solution for one hour. The nonsaponifiables were extracted and from these no alkaloids could be obtained, thus confirming the findings of Djerassi et al. (9) that organ pipe cactus does not have alkaloids. Most of the work reported in this chapter on the general sterol pattern in organ pipe cactus was done on these nonsaponifiables from cortex and epidermis, but work done with two other extracts is also reported here, one, a Skellysolve B extract of epidermis, and the other a 2:1 (v/v) chloroform:methanol extract of cortex tissue. The saponification on these later extracts was done as described in Chapter 3.

Dried, ground epidermal and cortex tissues from cardon cactus and complete tissue from cina cactus were extracted first with Skellysolve B and then with 2:1 (v/v) chloroform:methanol and saponification done as described in Chapter 3.

Digitonides were obtained, unless otherwise stated, by dissolving the nonsaponifiables in a minimum volume of hot 95% ethanol, cooling to room temperature, and removal of any precipitate. The solution was then reheated and a 1% solution of digitonin in hot 90% ethanol was added in excess to precipitate the possible sterols present. The solution was allowed to stand at least overnight, and the digitonides were filtered off, washed, and dried. Silation for GLC of digitonides was performed as given in Chapter 3 by heating for at least one-half hour.

Samples of thurberogenin, calenduladiol, and oleanolic acid were obtained from Dr. C. Steelink, Department of Chemistry, The University of Arizona. Sterol standards were available from Dr. H. Kircher.

Results and Discussion

Table 3 shows the relationship between the yields of lipids and the yields of nonsaponifiables from these lipids for extractions of organ pipe, cina, saguaro, and cardon. The higher yields of lipids from organ pipe than from the other cacti was already observed in Table 2, so the higher yields of total nonsaponifiables from organ pipe would be expected. The last column in Table 3 shows the percentage of the total lipids represented by the nonsaponifiables. This column shows the high range of 55-93% for organ pipe contrasted with less than 20% for the other cacti, and demonstrates still another case where organ pipe is very different from the other *Cereus* cacti of the Sonoran Desert.

Table 3. Lipid and nonsaponifiable fractions from cactus tissue extracts.

Cactus tissue	Solvent	(g/100 g dry cactus) ^a				%TN
		L	TL	N	TN	
Organ pipe, mature cortex	Skelly B	15.4	15.4	11.2	11.2	73
Organ pipe, mature cortex	Skelly B	13.7	13.7	12.8	12.8	93
Organ pipe, mature cortex	Skelly B	16.7	16.7	12.5	12.5	74
Organ pipe, mature cortex	3:2 C:M	19.2	19.2	12.0	12.0	63
Organ pipe, mature epidermis	Skelly B	17.1	17.1	14.4	14.4	84
Organ pipe, mature epidermis	Skelly B	20.2	20.2	13.6	13.6	67
Organ pipe, mature epidermis	Skelly B Chloroform	17.4 2.1	19.5	17.2 0.6	17.8	90
Organ pipe, mature epidermis	Acetone	18.7	18.7	12.7	12.7	68
Organ pipe, mature epidermis	3:2 C:M	24.9	24.9	13.7	13.7	55
Organ pipe, young epidermis	Skelly B 2:1 C:M	17.5 4.4	21.9	14.7 0.3	15.0	68
Cina, complete tissue	Skelly B 2:1 C:M	2.7 8.0	10.7	1.4 0.6	2.0	19
Saguaro, complete tissue	Skelly B 2:1 C:M	0.4 9.1	9.5	0.4 0.6	1.0	11
Cardon, mature epidermis	Skelly B 2:1 C:M	0.6 5.3	5.9	0.5 0.3	0.8	14

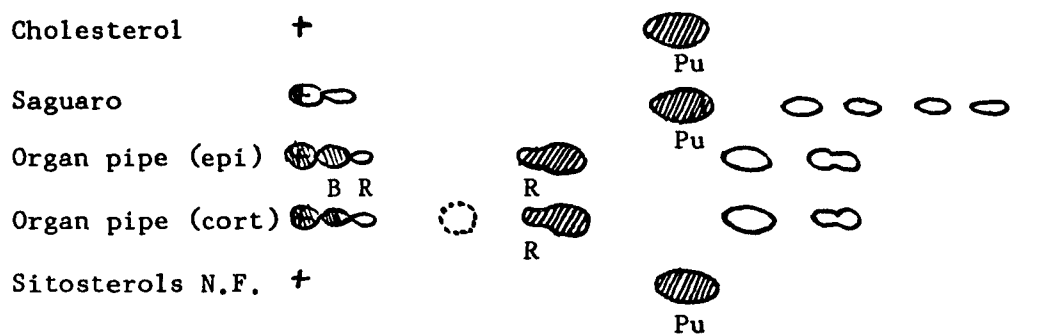
a. L = lipid, TL = total lipid, N = nonsaponifiables, and TN = total nonsaponifiables.

Sterols in Saguaro

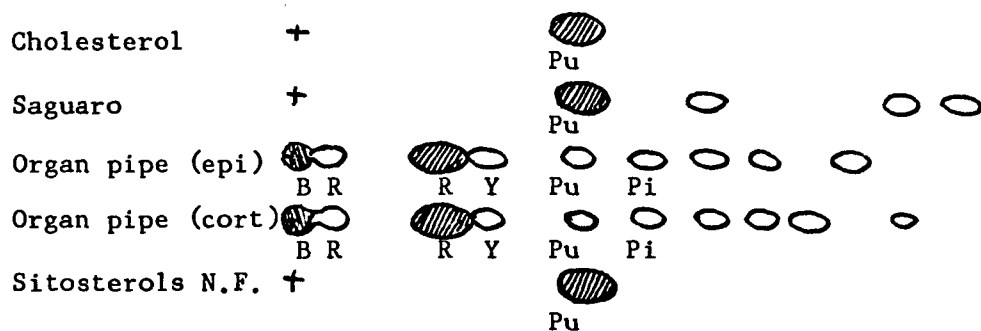
Thin-layer chromatography of saguaro nonsaponifiabiles showed the major component with essentially the same mobility as cholesterol and giving the same color with the 30% sulfuric acid spray. In addition, some material at, or near, the origin and several lesser components more mobile than cholesterol were present (Figure 2).

The GLC on the 3% QF-1 column showed two peaks in the sterol region with retention times identical to the two peaks of sitosterols N.F. (sitosterol and campesterol). The larger was sitosterol and appeared to be about 2.5 times the amount of the other sterol which was determined to be campesterol rather than stigmasterol as proposed by Kircher (23). Stigmasterol and campesterol have the same retention time on 3% QF-1 but very different retention times on 1% SE 52 and on 5% OV-101. The saguaro nonsaponifiabiles run on the 1% SE 52 column showed the minor sterol was definitely campesterol and confirmed that the major component was sitosterol.

The crude lipids in a Skellysolve B extract of saguaro showed the same components in approximately the same ratio as in the nonsaponifiabiles already shown, with a small amount of cholesterol in addition (Figure 3). Also, a small shoulder on the sitosterol peak toward the higher retention time side indicates a small amount of some unidentified material, probably sterol. The presence of the sterols as such, in the crude lipid extract, indicates that sitosterol and campesterol are present as free sterols in saguaro cactus tissue.



AgNO₃-Silica gel G, 95:5 (v/v) chloroform:methanol



Silica gel G, 4:1 (v/v) Skellysolve B:ethyl acetate

Figure 2. Thin-layer chromatography of organ pipe and saguaro nonsaponifiable fractions.

B = blue
R = red
Y = yellow

Pu = purple
Pi = pink

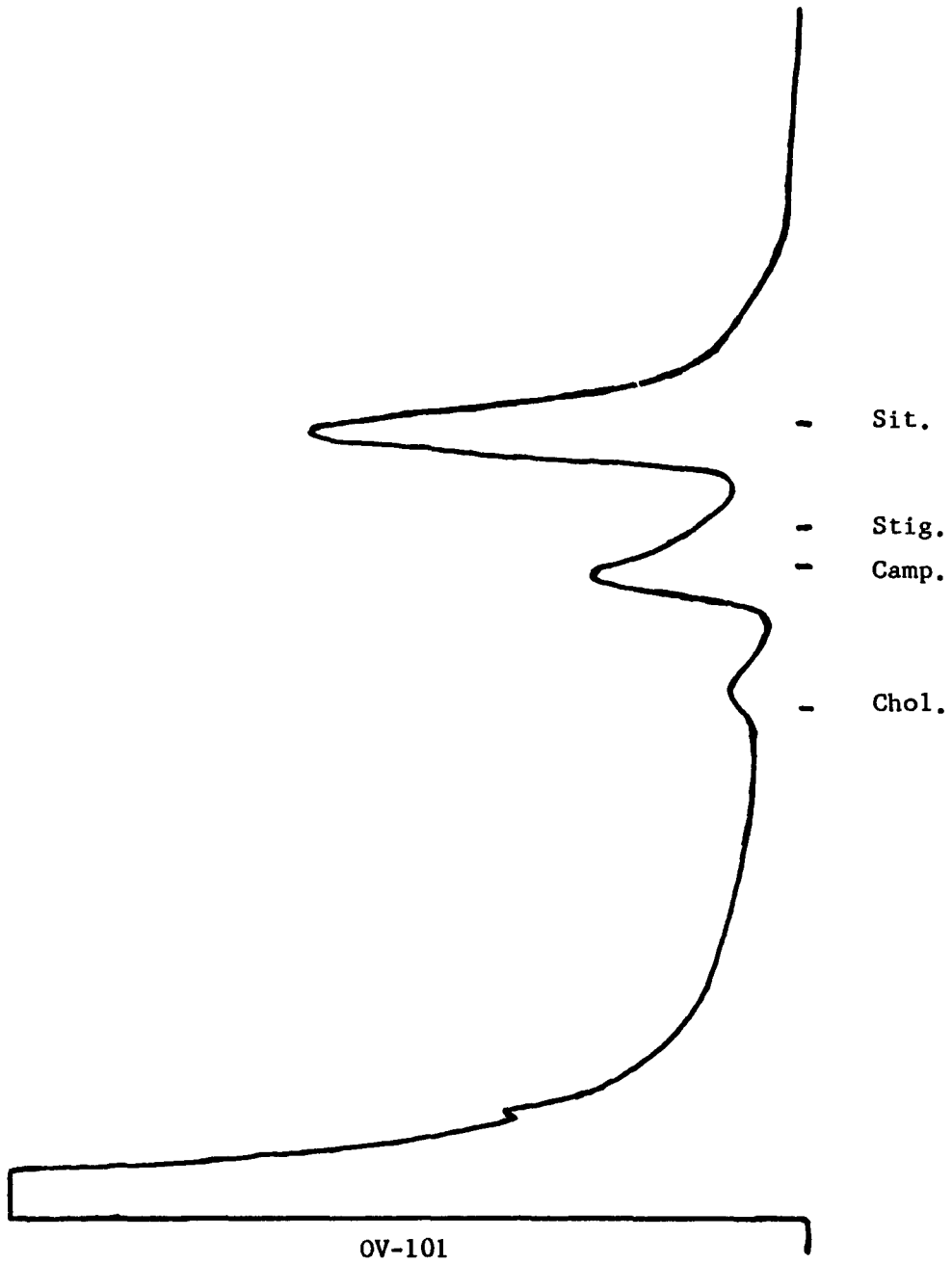


Figure 3. GLC: Skellysolve B extract of saguaro.

Sterols in Organ Pipe

Thin-layer chromatography of the nonsaponifiables of the chloroform-methanol extract of epidermis and cortex from a mature stem showed essentially the same pattern (Figure 2). The predominant components were more polar than the common phytosterols and consisted of at least two substances less mobile than cholesterol that reacted red to the sulfuric acid spray. In addition there were some very polar materials on or close to the origin that reacted blue to the spray and several more mobile minor components. The TLC with schottenol, lo-phenol, lupeol, phytol, and cycloartenol as standards indicated that none of these compounds were the major components.

GLC of Organ Pipe Nonsaponifiables. A silylated sample of epidermal nonsaponifiables run on 3% QF-1 showed two main peaks (Figure 4). Only one of the peaks was within the normal range of retention times for sterols and it had a retention time similar to that of campesterol or stigmasterol. This finding agreed with Kircher's unpublished data. A minor shoulder on the sterol peak was at the retention time of cholesterol.

Correlation of TLC Components with GLC Peaks. After preparative TLC of a sample of nonsaponifiables in 4:1 (v/v) Skellysolve B: ethyl acetate on a silica gel plate, several zones were scraped, eluted, silylated, and run on GLC using a 3% QF-1 column. The zones scraped corresponded to the blue-reacting zone at the origin, both red-reacting zones, and the purple zone at the mobility of cholesterol, as shown in Figure 2. The highly polar blue-reacting material was the

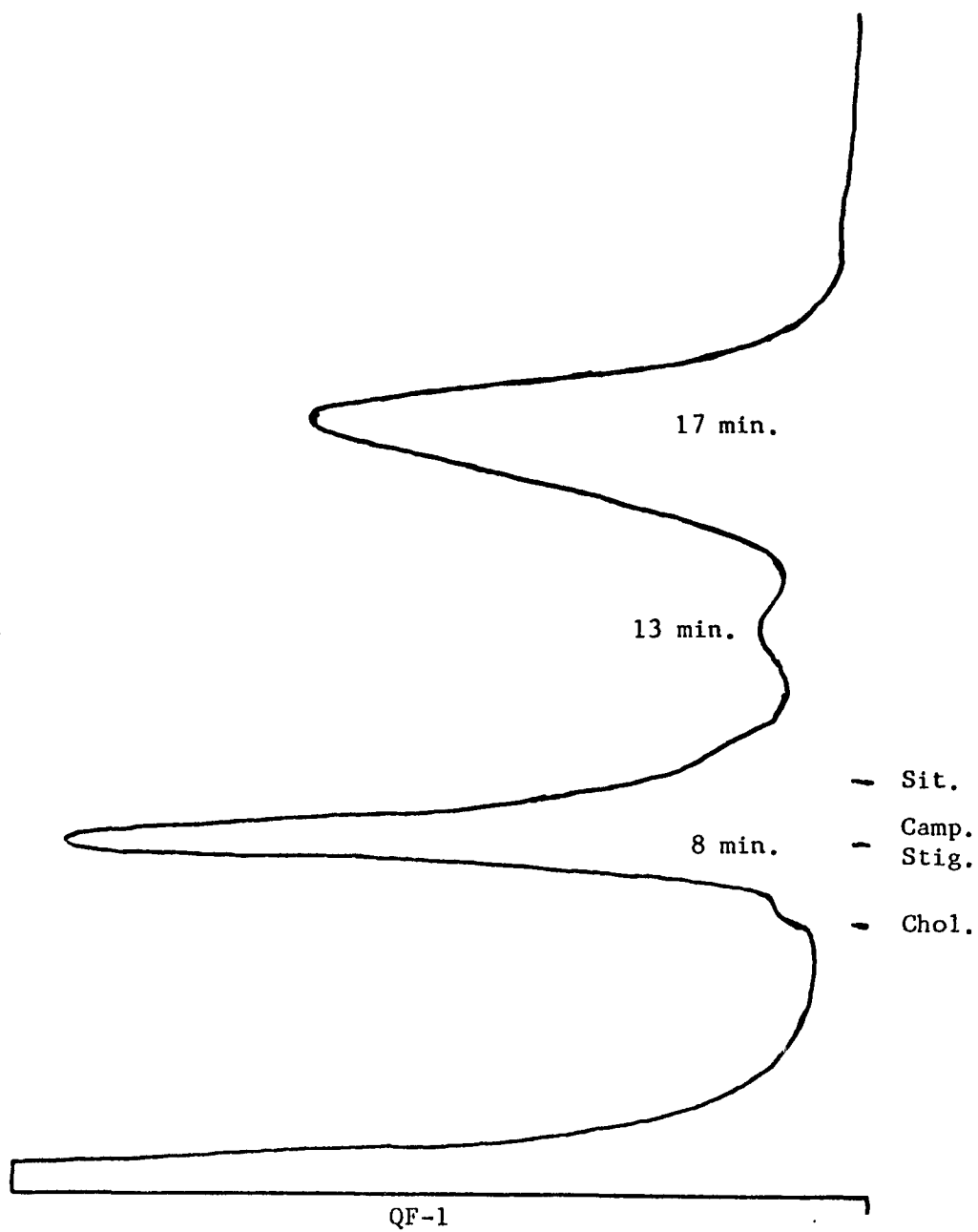


Figure 4. GLC: Organ pipe epidermis nonsaponifiable fraction (silated).

substance causing the main sterol peak (Figure 4), both red-reacting materials appeared as part of the main longer retention time peak, and the substance with an R_f of cholesterol on TLC showed a small peak at the retention time of cholesterol on GLC.

Another preparative plate on silica gel G of organ pipe epidermis nonsaponifiables was developed with 9:1 (v/v) chloroform:acetone and showed the components illustrated in Figure 5. The designation of the spots on the drawing are for the purpose of the following discussion. Very little, if any, component W actually showed on the particular preparative plate described. When the scraped bands were eluted and evaporated to dryness, band X contained the largest amount of material (based upon appearance), and this was followed by Z, Y, V, Y', and Z' in decreasing order. The eluted materials rechromatographed true, showing that no decomposition products had formed during the chromatography. When chromatographed on a silver nitrate-silica gel G plate, X and V showed a tendency to split into two spots.

Correlation of eluted components with the GLC pattern on the QF-1 column is shown in Table 4. The highly polar, blue-reacting Z material corresponded to the GLC peak within the sterol range and thus was the component of most interest in this study, although it was not known at the time that it was a sterol.

The TLC of thurberogenin (9), calenduladiol (13-15), and oleanolic acid in two systems with the organ pipe nonsaponifiables showed that V was apparently thurberogenin, X was calenduladiol, and Y probably oleanolic acid or an unknown triterpene. Although some

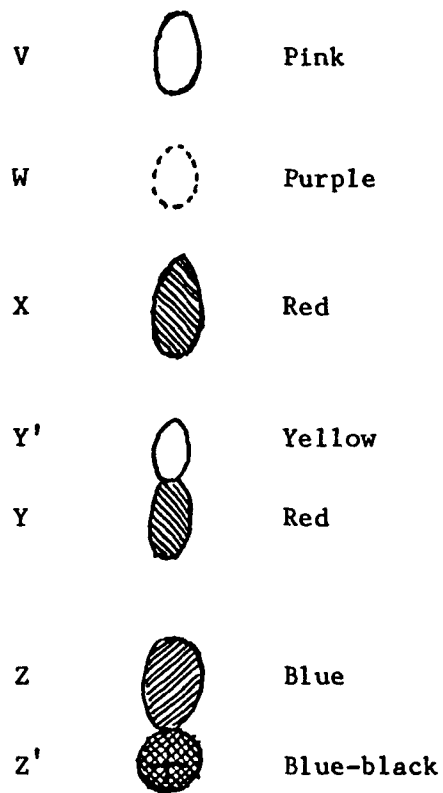


Figure 5. TLC of organ pipe epidermis nonsaponifiable fraction.

Developing solvent: 9:1 (v/v) chloroform:methanol
Plate: silica gel G

Table 4. Correlation of TLC and GLC data.

TLC Spot ^a	GLC Ret. time ^b	Probable compound
9:1 (v/v) Chloroform:acetone Silica gel G	3% QF-1, 235°C 40 psig argon	
V	13 minutes	Thurberogenin
X	17 minutes	Calenduladiol
Y'	No peak	
Y	17 min. 13 min.	Oleanolic acid
Z	8 minutes	
Z'	Trace of 8 min. (contaminant)	

a. See Figure 5.

b. See Figure 4.

interesting data on the triterpenes were obtained as a by-product of the sterol work, in the interest of limiting this discussion to sterols, these compounds will be referred to as "triterpenes" rather than individually. They occur at longer retention time, so they will not be shown on any of the GLC records used as figures.

Evidence of Common Phytosterols. The GLC on OV-101 of crude lipids from many different solvent extracts of organ pipe cactus tissue showed little or no evidence of common phytosterols or Z components. The sterol retention time region of a typical Skellysolve B extract of epidermal tissue is shown in Figure 6. The single main peak running at a retention time slightly longer than the retention time of cholesterol was never identified. A free sterol as obvious as this in the lipids would be expected to be even more prominent in the nonsaponifiables, and sterol esters would run at much longer retention times.

The nonsaponifiable fraction from the lipid extract in Figure 6 is shown in Figure 7. Only a small peak appeared at the same retention time as the main peak in Figure 6 and the main peak is that of the highly polar Z material. Very little if any of the common phytosterols could be seen. When this nonsaponifiable fraction was silylated before GLC, the single Z peak split into two peaks, as shown in Figure 8, thus showing that Z was composed of at least two components. The peak with the longer retention time was designated as Z-1 and the one with the shorter retention time as Z-2.

When epidermis nonsaponifiable material was fractionated into Skellysolve B solubles and insolubles, the phytosterols concentrated in

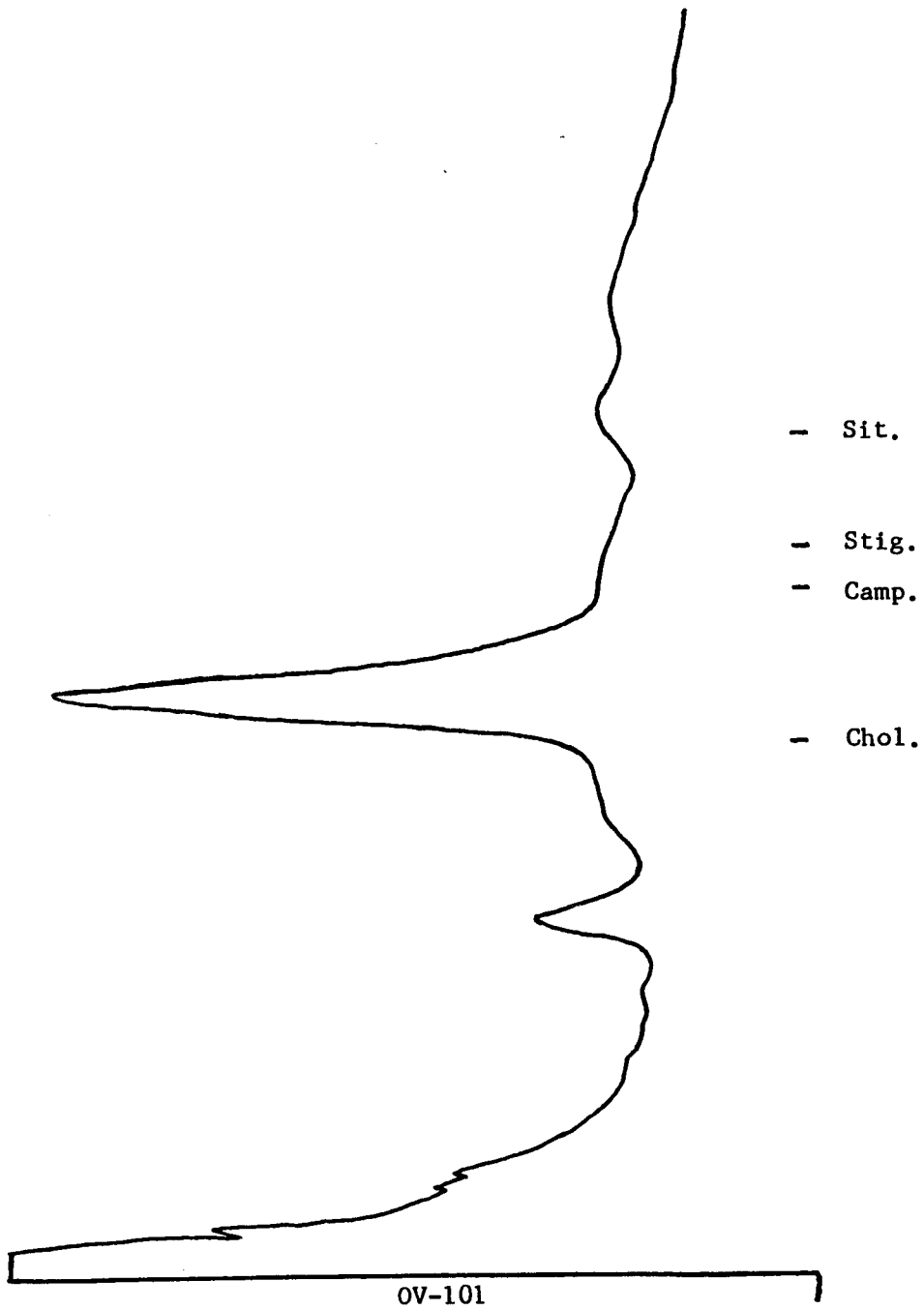


Figure 6. GLC: Skellysolve B extract of organ pipe epidermis.

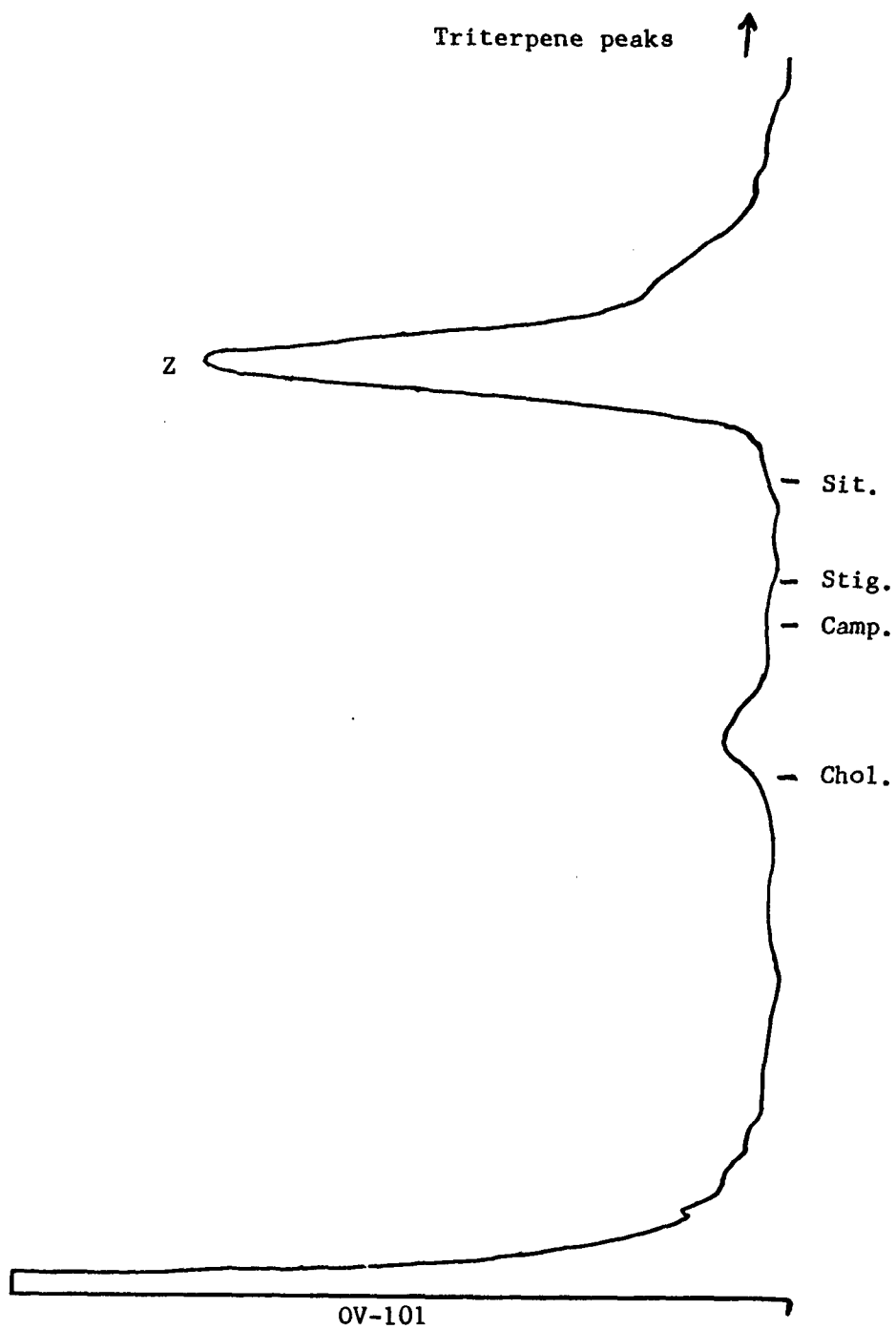


Figure 7. GLC: Organ pipe epidermis nonsaponifiable fraction (Skellysolve B extract).

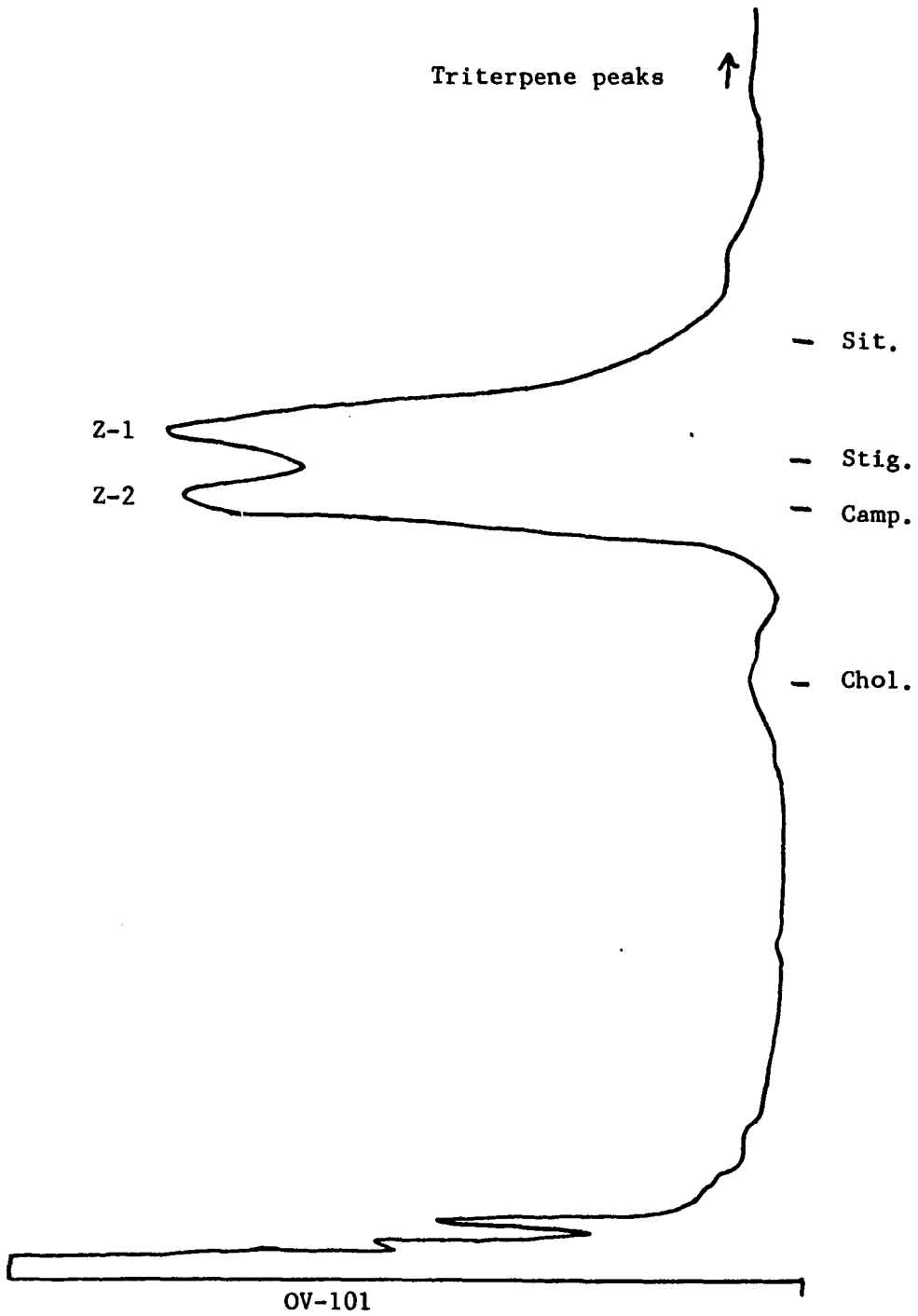


Figure 8. GLC: Organ pipe epidermis nonsaponifiable fraction (Skellysolve B extract) (silated).

the solubles fraction and became evident in the GLC records of these fractions after silation, as shown in Figures 9 and 10. The Skellysolve B solubles shown in Figure 10, having a large peak of cholesterol, came from nonsaponifiables of a 3:2 (v/v) chloroform:methanol extract of epidermis, while the one in Figure 9 came from nonsaponifiables of a Skellysolve B extract of epidermis.

The greater amount of cholesterol in nonsaponifiables from the chloroform-methanol extract than in those from a Skellysolve B extract is interesting in the light of the findings of Knights (24). He extracted chloroplasts from eight plant species sequentially with petroleum ether, then with acetone, and found much more cholesterol in the acetone extracts than in the petroleum ether extracts, while most of the other phytosterols had been extracted by the petroleum ether. He attributed this to a difference in binding of the cholesterol in the chloroplasts. This could enter into the results reported here on organ pipe extracts.

Figures 11 and 12 show the GLC of digitonides from the Skellysolve B solubles shown in Figures 9 and 10, respectively. Three peaks that appear to be sitosterol, campesterol, and cholesterol show in both digitonide preparations; the small peak in Figure 12, between the sitosterol and campesterol peaks, was probably stigmasterol. The digitonide precipitate, shown in Figure 12, gave a typical phytosterol type Liebermann-Burchardt reaction (slow reaction through blue to green).

A similar fractionation of a chloroform-methanol extract of cortex also showed the common phytosterols.

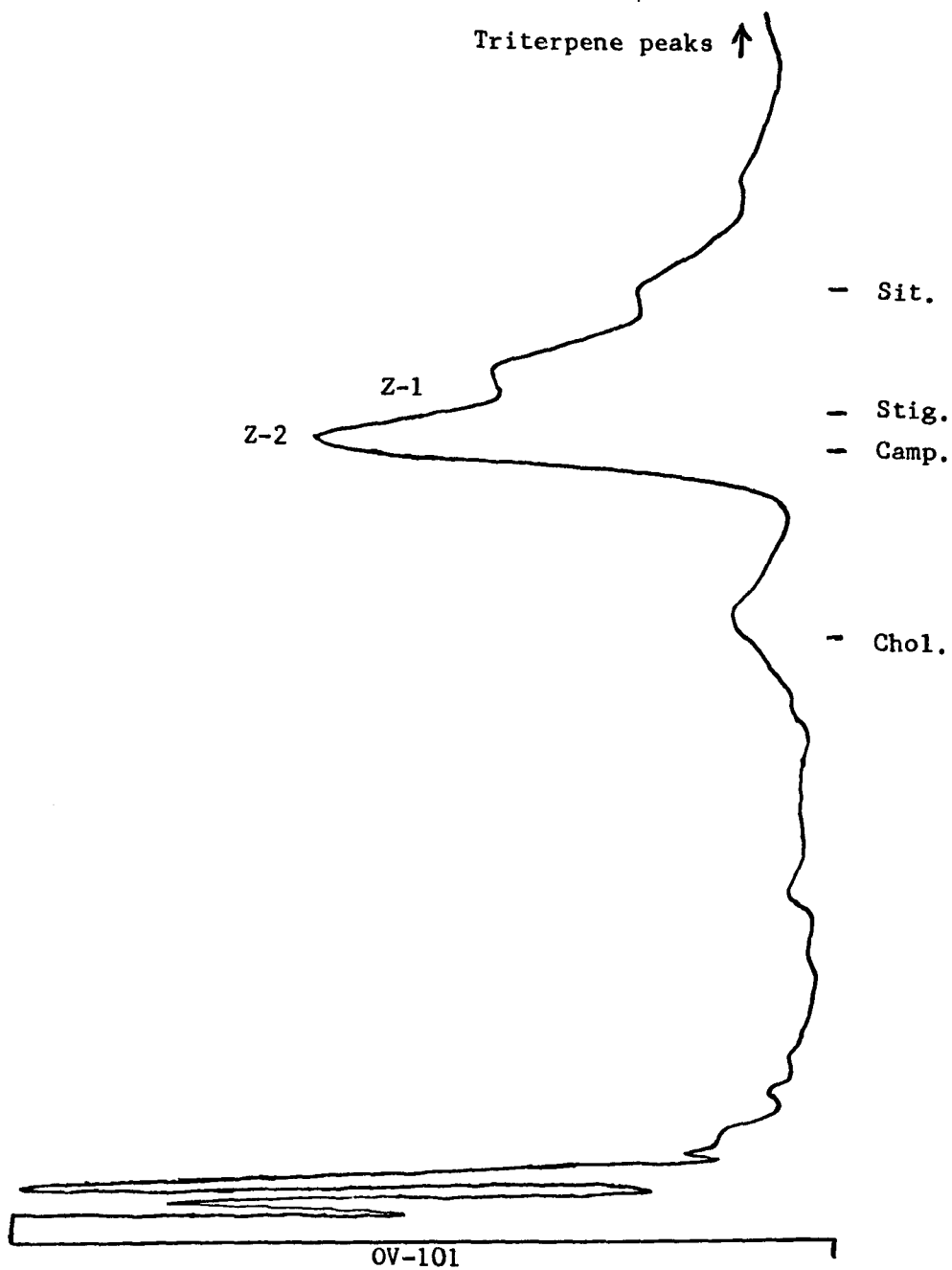


Figure 9. GLC: Skellysolve B solubles of organ pipe epidermis nonsaponifiable fraction (Skellysolve B extract) (silated).

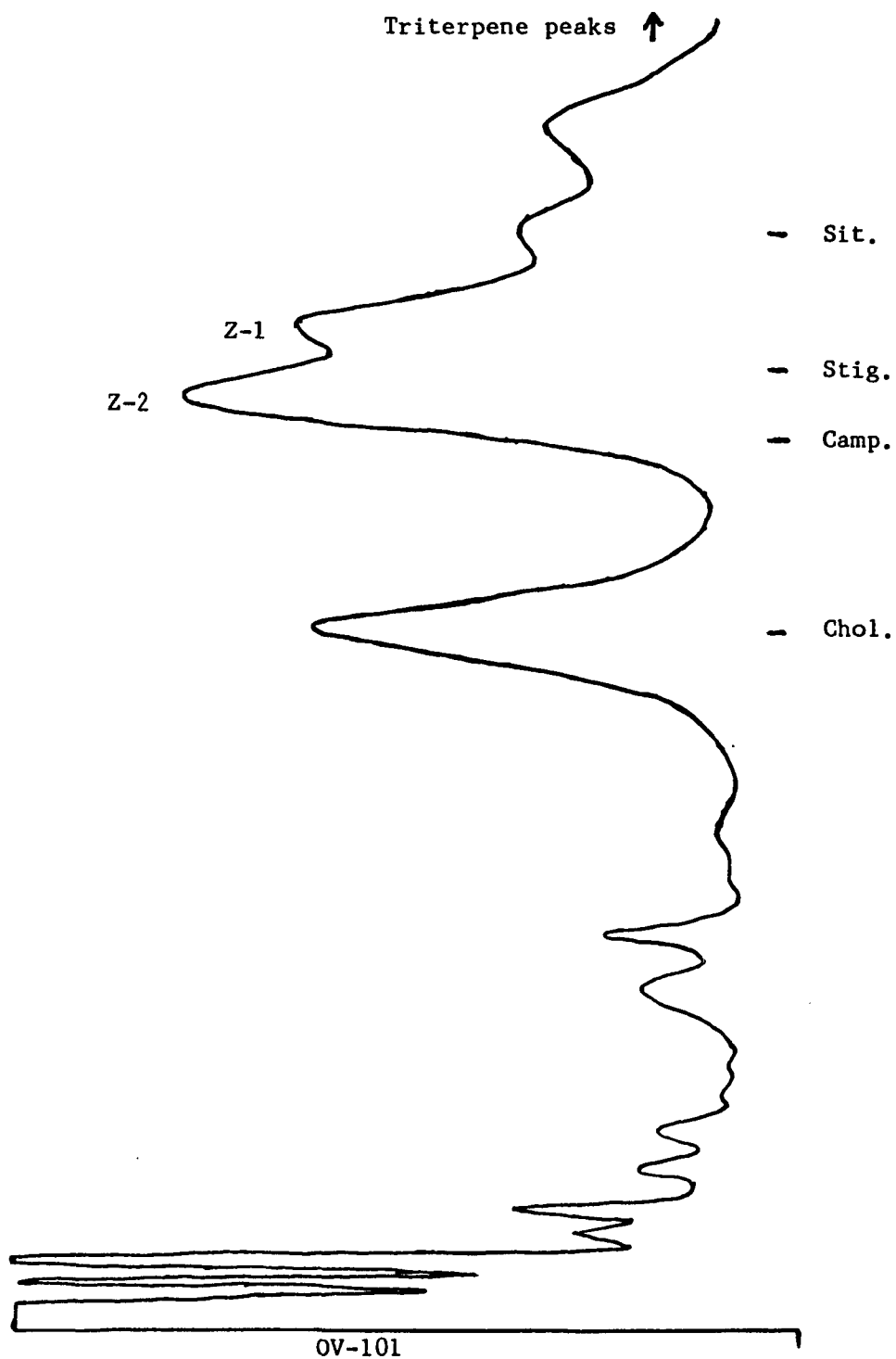


Figure 10. GLC: Skellysolve B solubles of organ pipe epidermis (chloroform-methanol extract) (silated).

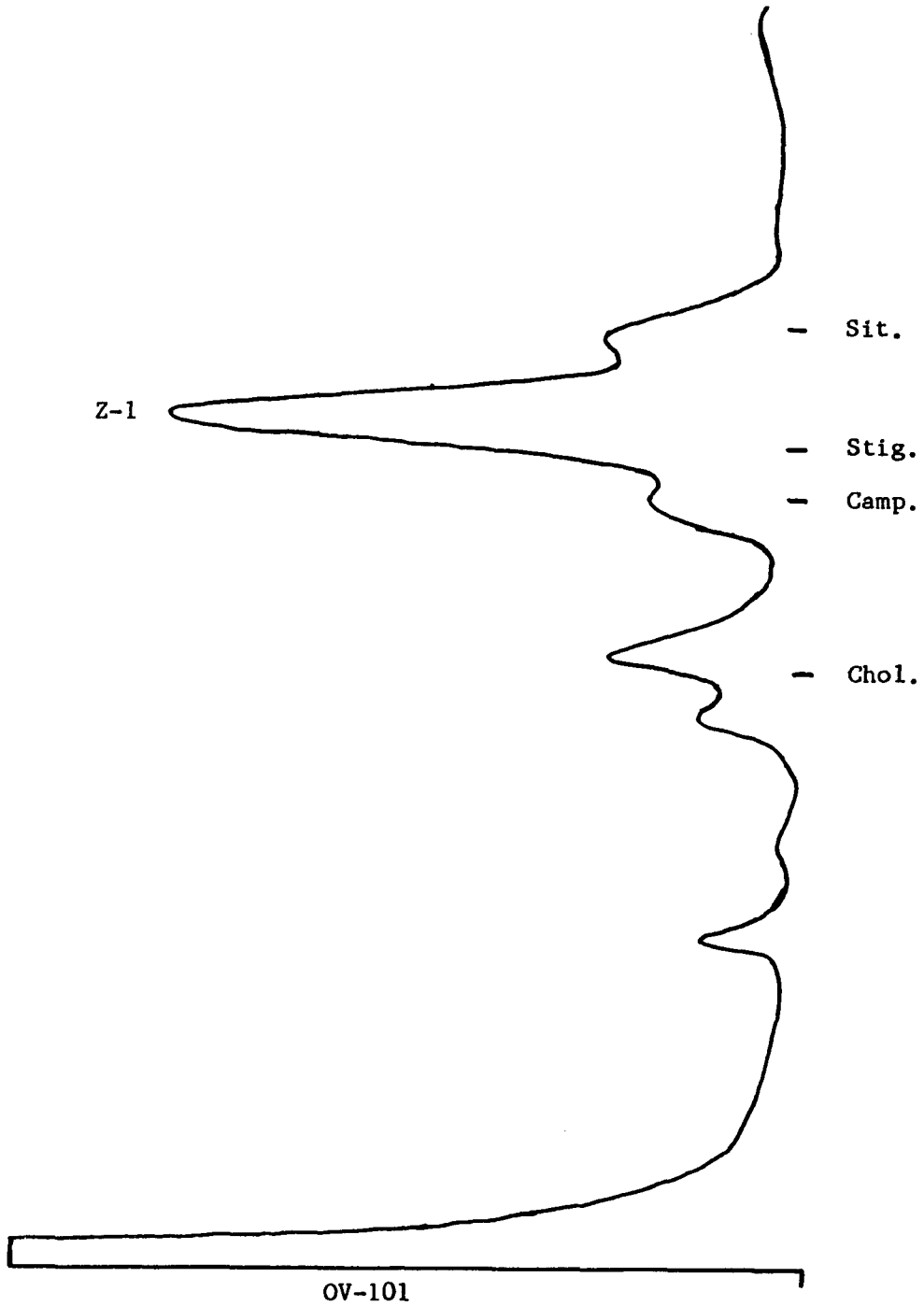


Figure 11. GLC: Digitonide from organ pipe epidermis nonsaponifiable fraction (Skellysolve B extract) (silated).

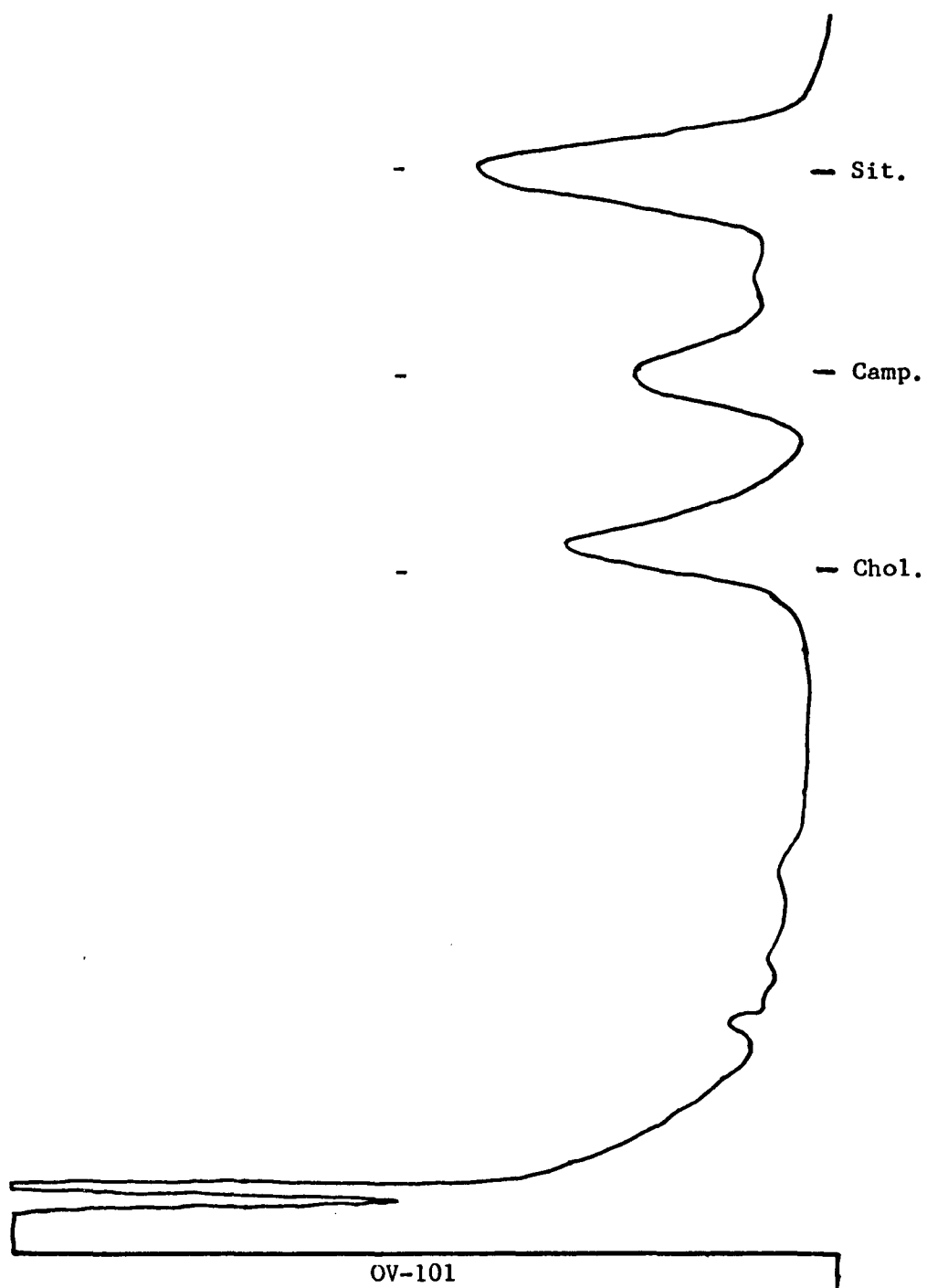


Figure 12. GLC: Digitonide from Skellysolve B solubles of organ pipe epidermis nonsaponifiable fraction (chloroform-methanol extract) (silated).

There were other fractions obtained from time to time throughout this research that also showed small peaks on GLC corresponding to the common sterols. The data already presented show that the common plant sterols, sitosterol, campesterol, stigmasterol, and probably cholesterol are present in organ pipe cactus tissue but in very much smaller concentrations than the Z substances, identified later as dihydroxysterols. The common sterols probably exist in the plant tissue as esters, as do the Z substances and some of the triterpenes. However, no information is available at present that can prove that unequivocally.

Sterols in Cardon

Cardon cactus showed the common plant sterols, sitosterol, campesterol, cholesterol, and stigmasterol. They appeared in various ratios in Skellysolve B extracts and subsequent 2:1 (v/v) chloroform:methanol extracts from cortex and epidermis tissue. These were also seen in the nonsaponifiables and were precipitated by digitonin from ethanolic solutions of the nonsaponifiables. Figure 13 shows the GLC pattern of a Skellysolve B extract of cardon cortex. Sitosterol is the major component followed by campesterol, stigmasterol, and cholesterol in decreasing order. The lipids from epidermis tissue showed a similar pattern but with an increased amount of cholesterol. The GLC pattern of the silylated digitonide precipitate from the nonsaponifiables of a Skellysolve B extract of cardon epidermis is shown in Figure 14. The digitonide from the nonsaponifiables of the Skellysolve B extract of cortex showed a similar pattern.

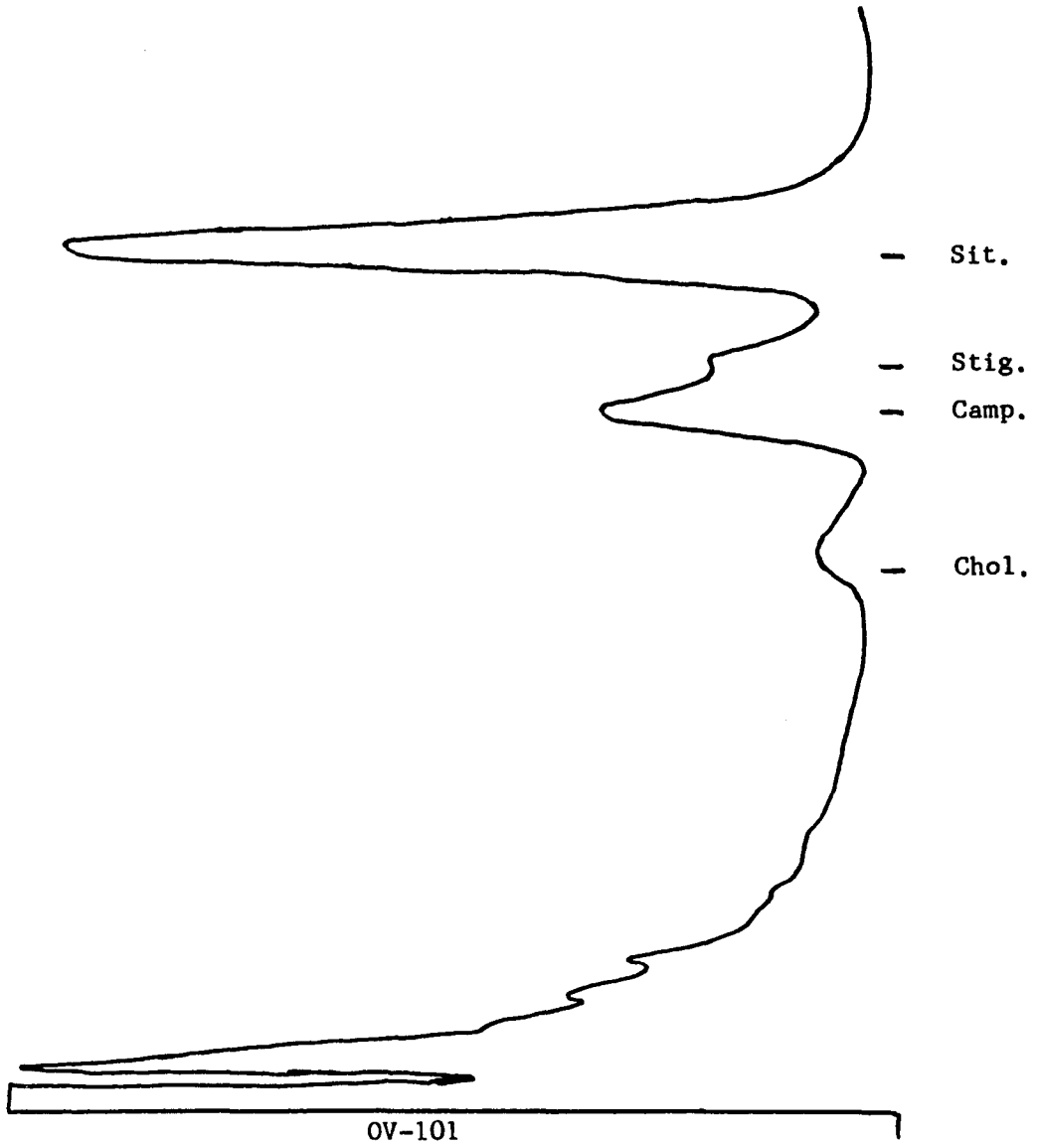


Figure 13. GLC: Skellysolve B extract of carbon cortex.

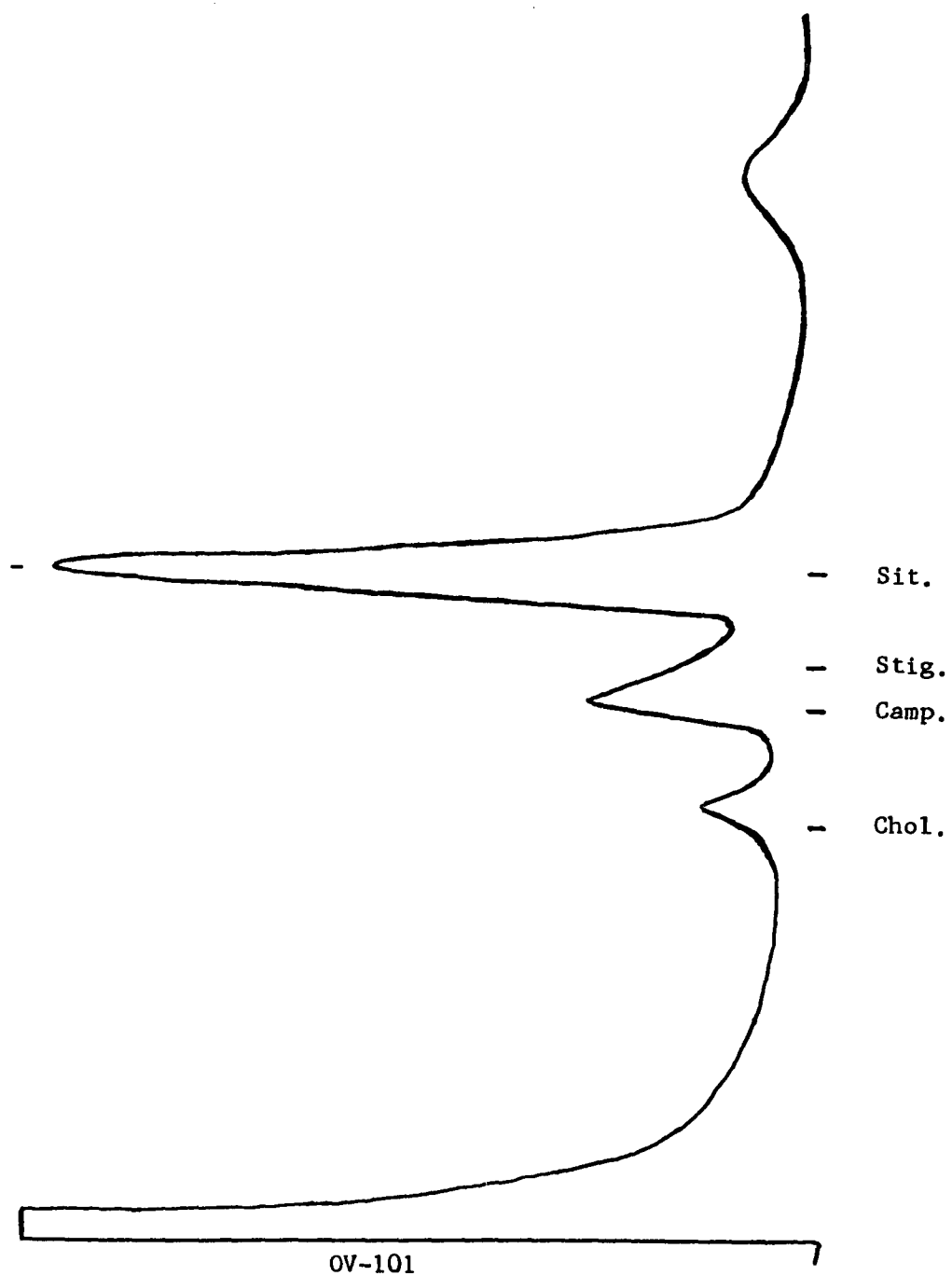


Figure 14. GLC: Digitonide from cardon epidermis nonsaponifiable fraction (Skellysolve B extract) (silated).

The GLC of nonsaponifiabiles from the 2:1 (v/v) chloroform:methanol extract, as shown in Figure 15, showed a greatly increased amount of cholesterol while the corresponding nonsaponifiabiles from cortex showed no greater ratio of cholesterol than that shown in the nonsaponifiabiles from the Skellysolve B extract.

In cardon, as in organ pipe, the chloroform-methanol extracts of epidermis showed a greater ratio of cholesterol to the other phytoosterols than the Skellysolve B extract. Chloroform-methanol extracts of the cortex, however, did not exhibit this increased ratio of cholesterol over the Skellysolve B extract. This showed some probable connection with the chloroplasts and thus to the work of Knights (24) referred to earlier.

The relationship between cardon and saguaro was shown in the sterols present as well as the fatty acids and alkaloids. The sitosterol and cholesterol levels were higher in the particular sample of cardon than in the sample of saguaro, but this might not prove to be true if many samples of each type were examined. Age of the sample might be a factor.

The sterols of both saguaro and cardon appear to be free sterols, since they appear in the lipid extracts.

Sterols of Cina

The GLC pattern of the nonsaponifiabiles of a Skellysolve B extract of cina cactus can be seen in Figure 16. The highest peak is probably the same Z sterol peak found in organ pipe cactus. The other peaks and shoulders appear to be sitosterol, stigmasterol, and

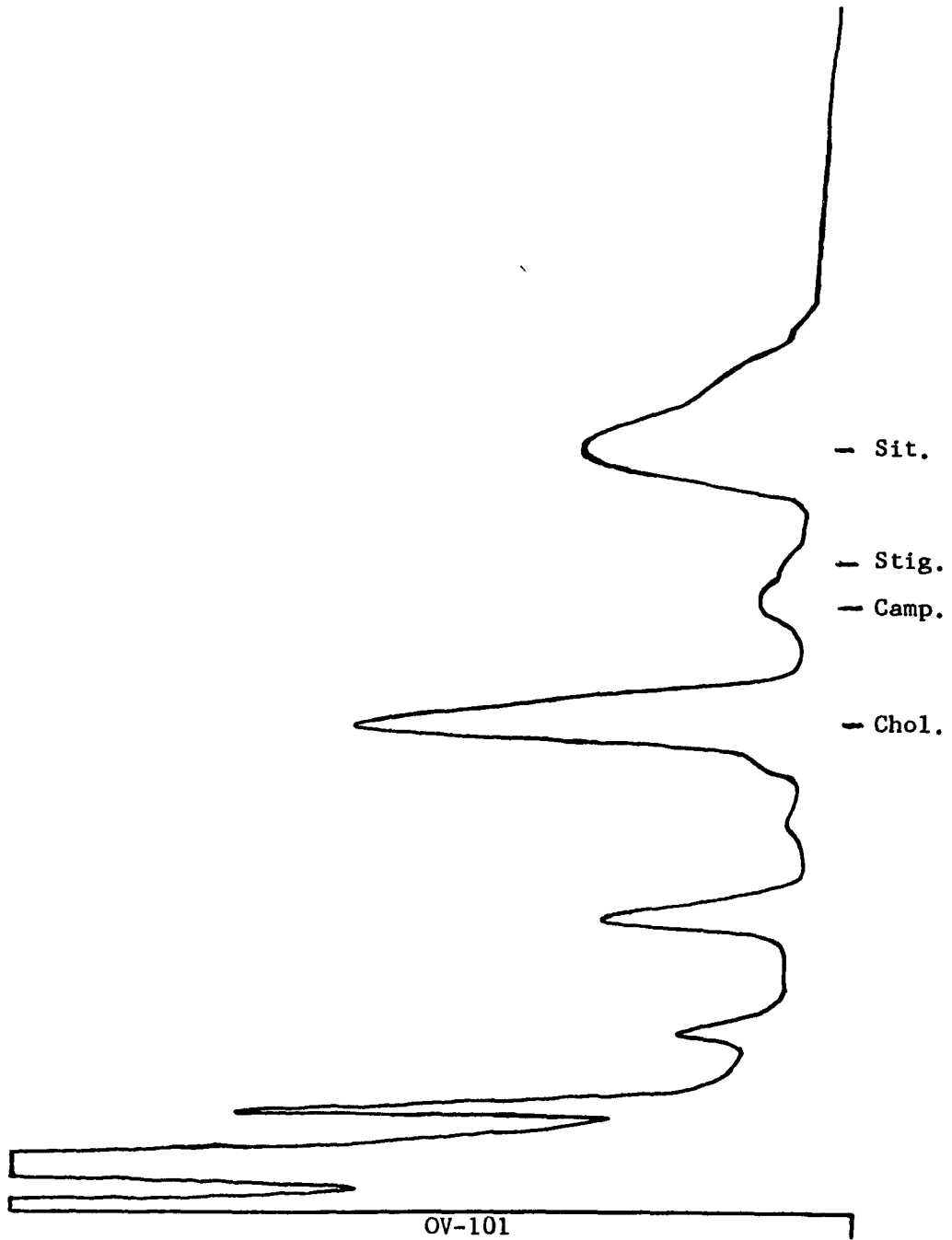


Figure 15. GLC: Cardon epidermis nonsaponifiable fraction (chloroform-methanol extract).

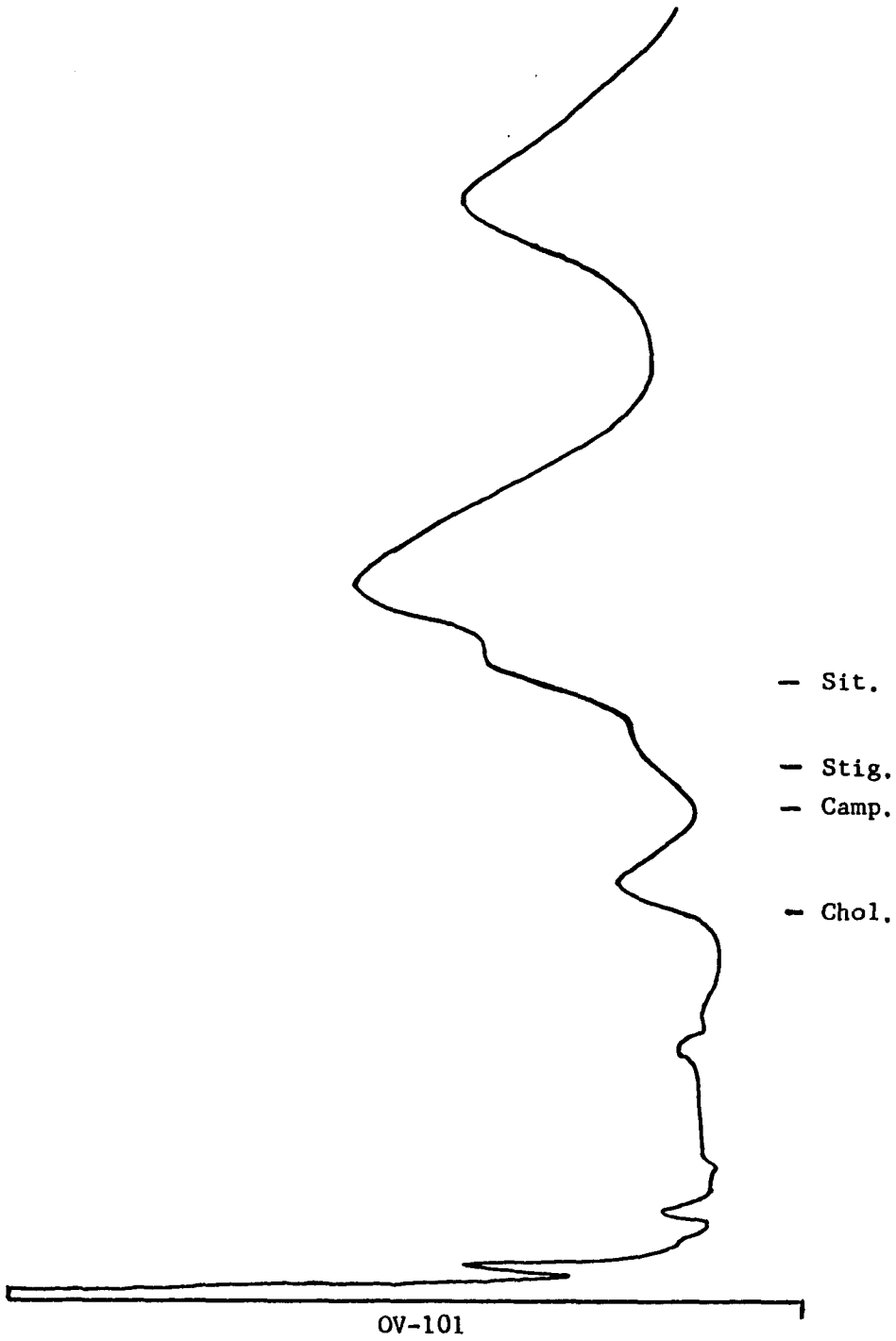


Figure 16. GLC: Cina nonsaponifiable fraction (Skellysolve B extract).

cholesterol. Peaks in the triterpene area were also present. The GLC of the silylated digitonide prepared from the nonsaponifiables as shown in Figure 17 shows essentially only peaks of probably Z-1 sterol and sitosterol. This would tend to rule out cholesterol and stigmasterol in the nonsaponifiables, although peaks of close to the proper retention values were present and it seems reasonable in the face of this data to classify those two peaks as unidentified compounds. Essentially the same results were obtained with the nonsaponifiables from the subsequent 2:1 (v/v) chloroform:methanol extract of cina and the digitonide prepared from them. The GLC pattern of the nonsaponifiables is shown in Figure 18 with the highest peak appearing to be sitosterol. The digitonides showed only the sitosterol and Z-1 peaks (similar to Figure 17).

In summary, cina cactus appears to have sitosterol, the Z-1 dihydroxysterol found in organ pipe, and possibly two unidentified sterols. This would be an interesting cactus for future work.

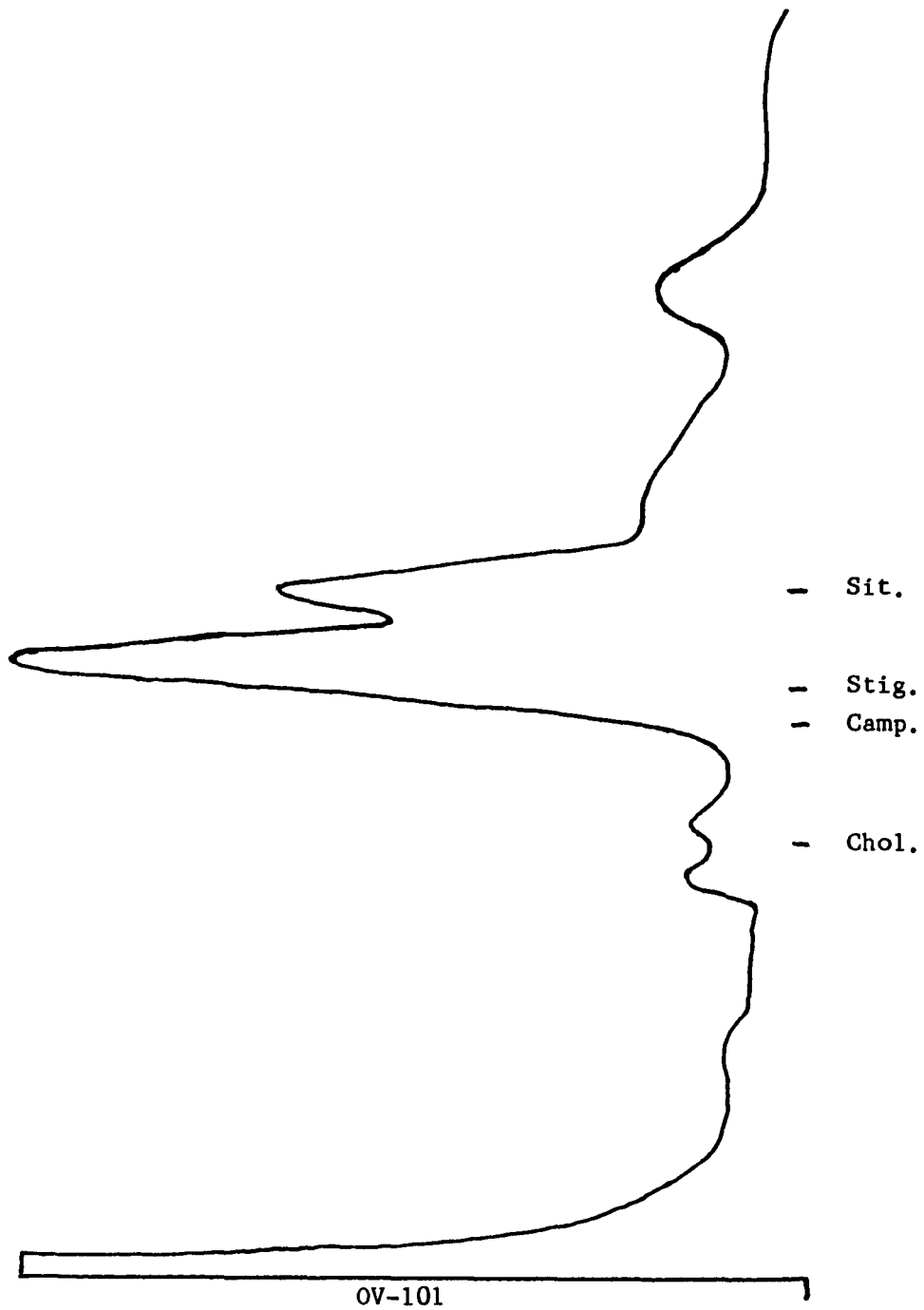


Figure 17. GLC: Digitonide of cina nonsaponifiable fraction (Skellysolve B extract) (silated).

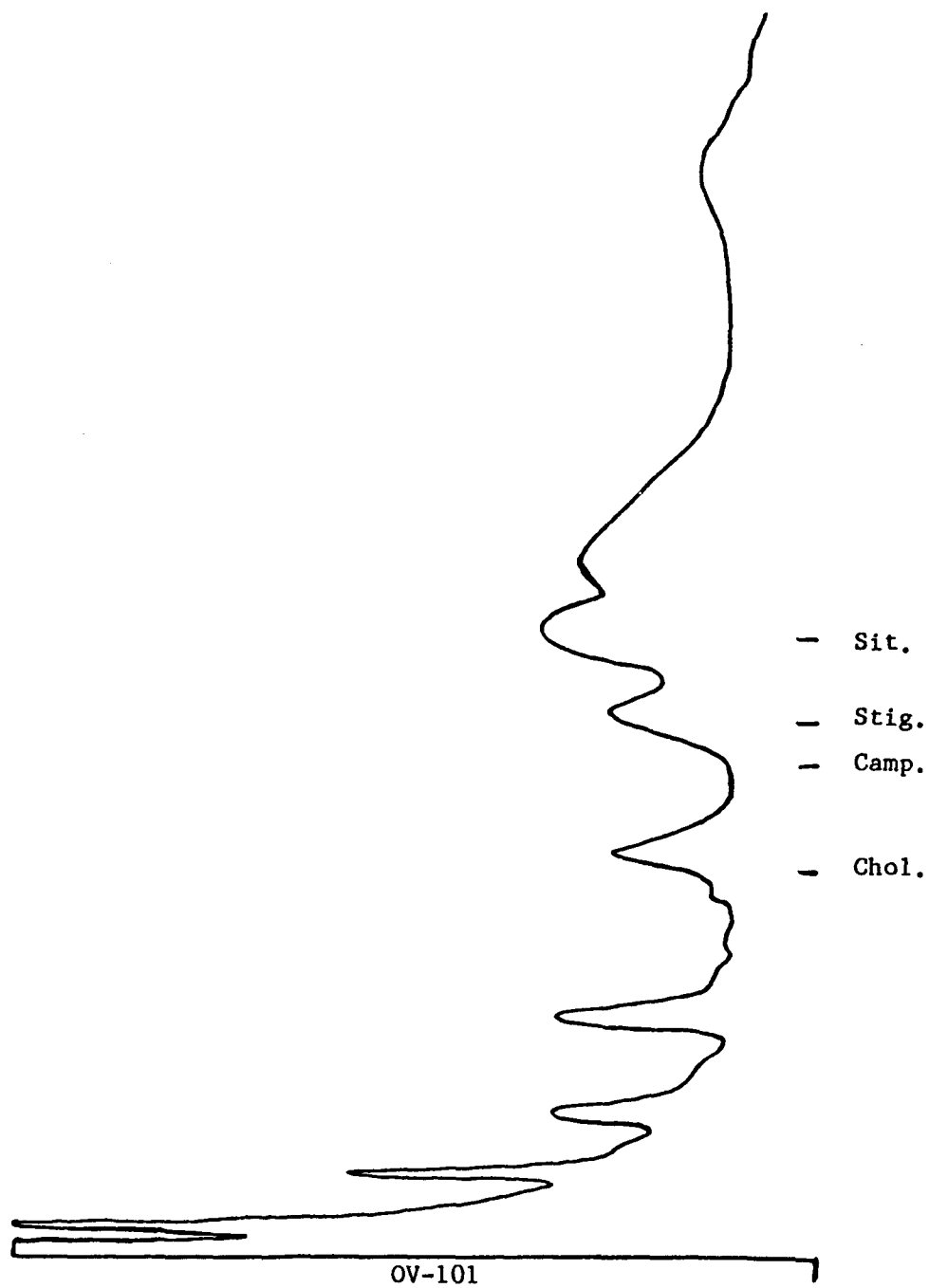


Figure 18. GLC: Cina nonsaponifiable fraction (chloroform-methanol extract).

CHAPTER 6

ISOLATION AND IDENTIFICATION OF THE POLAR STEROLS OF ORGAN PIPE; A NEW STEROL, THURBEROL

In the preceding chapter the general sterol pattern of organ pipe cactus was described and comparisons were made with the sterol patterns of some other *Cereus* cacti. Some common plant sterols were found in organ pipe in very small concentrations when compared to those present in saguaro and cardon, and it is doubtful if they are present as free sterols. In saguaro and cardon the sterols are definitely present as the free sterols in the dried cactus tissue.

Organ pipe, however, showed larger quantities of more polar materials, assumed to be sterols, and even larger amounts of several triterpenes. These polar materials were tentatively assumed to be sterols because of their reaction to the Liebermann-Burchardt reagent, their reaction on TLC to the sulfuric acid spray, and their retention time on GLC, it being within the range of the retention times of known sterols.

These polar sterols, collectively designated as Z (Figure 5), apparently exist in the dried cactus tissue esterified with short chain fatty acids; a conclusion based upon the facts that they were extracted easily by Skellysolve B, they did not appear on GLC separation diagrams of the crude lipids but did appear in the nonsaponifiable fractions of the lipids, and that only short chain fatty acids were present in organ

pipe. Also, the TLC of crude lipids showed primarily components more mobile than common sterols while the TLC of the nonsaponifiables showed these polar sterols to be much more polar than common sterols.

As they existed in the nonsaponifiables, these polar sterols showed the following properties:

1. They reacted to the Liebermann-Burchardt reagents with an immediate dark blue turning slowly to green.
2. They were only very slightly soluble in Skellysolve B.
3. By TLC they are quite polar, run as one spot, and react dark blue to 30% sulfuric acid spray after heating.
4. By GLC on an OV-101 column, injected as the free sterol, they gave one peak with a retention time slightly longer than that of sitosterol. When injected after silylation they gave two peaks. One of these (Z-1) had a retention time just slightly longer than silylated stigmasterol, and the second (Z-2) had a retention time slightly shorter than silylated stigmasterol and slightly longer than silylated campesterol. (Silylation thus decreased the retention time of the polar sterols, whereas silylation increases the retention time of normal sterols on OV-101.)

Since the polar sterols exist in organ pipe cactus in concentrations greater than the concentrations of the normal phytosterols, their isolation and identification were important for a knowledge of the chemistry of this cactus. This knowledge is needed to assist in answering the question, "Why does organ pipe cactus act as a host plant for D. mojavenis but not support the growth of D. nigrospiracula?"

The procedures devised for isolation of these polar sterols and the data which led to their identification are reported in this chapter.

Special Materials and Methods

Some of the work reported in Chapters 4 and 5 and here was done on nonsaponifiable fractions from chloroform-methanol extracts of organ pipe cactus. Most of the work reported in this chapter, however, was performed on nonsaponifiables from Skellysolve B extracts of epidermis or cortex tissue from mature stems of organ pipe cactus.

Skellysolve B extracted the polar sterols as well as did chloroform-methanol mixtures because these sterols are esterified but did not remove saponins and other polar materials that were extracted by the more polar solvent. The Skellysolve B extracts were thus easier to handle. Saponification of these produced fewer problems with emulsions than the chloroform-methanol extracts and the solubility properties of the nonsaponifiables were better for subsequent chromatography. All extractions and saponifications were done as given in Chapter 3.

Acetone Fractionation of Nonsaponifiables

Some of the triterpenes could be removed by solution of the nonsaponifiables in a minimum volume of hot acetone and allowing the solution to cool to room temperature. The precipitate was mainly triterpenes with a small amount of Z sterols. The sterols could be separated by a recrystallization of the precipitate from acetone; the sterol-rich mother liquor was then added to the filtrate from the

first precipitate. The acetone was evaporated from this combined filtrate to leave a residue for chromatography.

In some cases the nonsaponifiable fraction was chromatographed directly without preliminary acetone fractionation.

Chromatography of Nonsaponifiables on Silica Gel

Except for some small scale trial runs, chromatography to separate the Z sterols from the triterpenes was carried out with dry 1:1 (w/w) silica gel:celite placed in a fritted-glass, Buchner-type, filtering funnel. The layer of adsorbent was packed by vibration, and a circle of filter paper, cut to the diameter of the filter funnel, was placed on the top of the silica gel-celite layer to prevent roiling of the top portion of the adsorbent when liquid was added.

The total nonsaponifiable fractions, their Skellysolve B insoluble portions, or the acetone soluble portions of the nonsaponifiables were dissolved in a minimum volume of chloroform, 1:1 (v/v) chloroform:benzene, or 8:2 (v/v) chloroform:benzene and poured on the top of the filter paper disk in a manner to allow the solution to distribute itself evenly in the adsorbent. Development was then carried on as described in the Results and Discussion section of this chapter. Eluates were monitored closely by the Liebermann-Burchardt reaction on 5-10 drop portions taken at intervals. The L.B. reaction for eluates containing triterpenes only was pink, mixtures of Z sterols and triterpenes appeared reddish purple, those with "pure" Z showed an immediate blue turning fairly slowly to green, and, as the most polar materials began to elute, the blue reactions turned more rapidly to green. The

validity of the L.B. reaction, as a reliable monitor for the collection of eluates and pooling, was verified by TLC in all early column work and, at intervals, in later larger scale separations. All Z materials from chromatography were checked by GLC before and after pooling for the next step in purification.

Acetone Purification of Z Sterols

The pooled Z sterols from chromatography (free of triterpenes, common sterols, and relatively free of more polar impurities) were recrystallized from acetone. Cooling to room temperature gave a first crop, and cooling the mother liquor in a refrigerator gave a second crop of precipitate. Further precipitate was obtained by evaporation and cooling. This procedure was carried out until precipitates obtained were obviously impure as shown by yellow color. Each crop was checked by GLC before pooling for further isolation steps.

Digitonide Precipitation

The Z-1 was obtained free from Z-2 by precipitation of the former as a digitonide. A 1% solution of acetone-purified Z sterols in hot 95% ethanol was added to at least 3.5 times as much of a 1% solution of digitonin in hot 90 or 95% ethanol and allowed to stand at room temperature overnight. The first crop of Z-1 digitonide was removed and the filtrate evaporated to one-half its volume to get a second crop. The latter usually had a small amount of Z-2 present but could be recrystallized from hot 80% ethanol to get pure Z-1 digitonide. The mother liquor from this recrystallization contained mostly Z-2

digitonide and could be added to the filtrate from the second crop of Z-1 digitonide. These combined filtrates were then processed by a series of evaporations, filtrations, recrystallizations, pooling and reworking to obtain more Z-1 digitonide and some Z-2 digitonide free of Z-1 digitonide. All steps were monitored by GLC of silylated digitonide precipitates.

The Z-1 digitonide did not precipitate as rapidly as common sterol digitonides, e.g., cholesterol digitonide, and is probably more soluble than that of cholesterol. The Z-2 digitonide is even more soluble than that of Z-1. Digitonide of Z-1 contaminated with small amounts of Z-2 could be purified by recrystallization from hot 95% ethanol leaving the Z-2 in the mother liquor. All recrystallizations were monitored by GLC of silylated digitonides.

Cleavage of Digitonides; A New Method

Published procedures by Schoenheimer and Dam (25), using pyridine and ether, and by Issidorides, Kitagawa, and Mosettig (26), using dimethyl sulfoxide (DMSO) were tried for cleavage of the digitonides. The DMSO procedure did not work with these polar sterol digitonides because the free sterol was too polar to be extracted from the DMSO by hexane or Skellysolve B. The pyridine-ether method worked, but had the disadvantage of requiring special handling due to the hazards and unpleasant odor of pyridine.

A new method of cleavage of digitonides was discovered while attempting to recrystallize a Z-2 rich digitonide precipitate from hot methanol by addition of a large volume of acetone at room temperature.

The precipitated material proved to be digitonin itself, not the Z sterol digitonides which gave a blue L.B. reaction. This showed that at least some of the Z sterol digitonides had cleaved in the hot methanol. The Z sterols were found as free sterols along with some uncleaved Z digitonide in the acetone-methanol solution. (Note: Although pure digitonin theoretically should not react with L.B., all digitonin used and precipitated had an initial red color turning to yellow.)

The method, as further developed, was as follows: The digitonide was dissolved in a minimum volume of hot methanol and the solution was kept hot (almost boiling) for one-half hour. (Refluxing or keeping the solution hot for a longer period of time might work even better, but was not tried. A more thorough examination of this method is needed to establish optimum conditions for complete cleavage without undesirable effects on the sterol.) The hot methanol solution was then mixed with at least 10 volumes of acetone at room temperature, stirred well, and refrigerated for at least one hour. (Overnight or several days did no harm and perhaps permitted more digitonin to precipitate in this stage.)

The precipitated digitonin was filtered off and its L.B. reaction tested. The filtrate was evaporated to a smaller volume and a second precipitate of digitonin was removed. The filtrate was evaporated to dryness and the residue extracted several times with boiling acetone. The acetone insoluble material, found to be uncleaved digitonide and any digitonin not previously precipitated, was resubmitted to another methanol-acetone cleavage or discarded. (The amount of

uncleaved digitonide usually represented a very small amount of sterol.)

The acetone solution was refrigerated overnight to get a first crop of free sterol precipitate. The remainder of the free sterol was precipitated in stages by repeated evaporations to smaller volumes and refrigeration of each filtrate to avoid contamination by yellow decomposition products. Each precipitate was checked by GLC and/or TLC before pooling for the next stage of purification.

This new method has the advantages of working with volatile solvents so that the recovery of digitonin was simplified, eliminating special handling, because of the odors and hazards of pyridine. A typical recovery from 6.00 g of digitonide was: 1.21 g free sterol, 3.90 g digitonin, and 0.19 g uncleaved digitonide. Cholesterol digitonide was also cleaved by this method. (Note: Where dienes are involved the hot methanol and hot acetone solutions should be handled under nitrogen to minimize air oxidation of the dienes.)

Acetylation

Several methods were used to obtain acetylated Z-1 and Z-2 during the course of this research: 1) Heating with acetic anhydride overnight in an open container on a steam bath. 2) Refluxing for one-half hour with acetic anhydride followed by refrigeration to get acetylated sterol crystals, which were filtered off and washed with water and cold acetone. 3) Acetylation with pyridine and acetic anhydride at room temperature overnight, followed by addition of water, and subsequent extraction of the acetylated sterol with hexane or Skellysolve B. The

hexane extract was washed 2 X with 2 M HCL, 2 X with water, 2 X with saturated NaHCO₃, and 3 X with water before being filtered through filter paper, and evaporated to dryness. The clear glassy acetylated product was crystallized from acetone or methanol. 4) Direct acetylation of digitonides with pyridine and acetic anhydride followed by extraction with Skellysolve B was also successful when recovery of digitonin was unimportant. Method 3 was selected as the one least likely to cause decomposition.

Hydrogenation

Unless otherwise stated, hydrogenations were carried out at room temperature in ethyl acetate at atmospheric pressure over 5% Pd/C, platinum oxide, or Raney nickel for various times. At the end of the hydrogenation the catalyst was removed, the solvent evaporated, and the product crystallized from an appropriate solvent. Most of the hydrogenations were carried out on acetylated sterols.

Synthesis of 8,14-Cholestadienyl Acetate

The 8,14-cholestadienyl acetate, used as a model compound for some comparisons, was synthesized by modification of a method described by Fieser and Ourisson (27). A mixture of acetic anhydride (3 ml) and 12 M HCL (0.2 ml) was added slowly to 7-dehydrocholesteryl acetate (1g) in acetic acid (17 ml), benzene (5 ml), 12 M HCL (0.3 ml). This mixture was refluxed for one hour after which 0.3 g sodium acetate was added, the reaction mixture cooled, and water added. The mixture was

ether extracted, the ether evaporated, and the residue dried by repeated evaporations with absolute ethanol.

The 8,14-cholestadienyl acetate crystallized from methanol in elongated plates, m.p. 92-93.5° (vac). After chromatography on silica gel-celite, material was obtained with m.p. 96-97° (vac). Fieser and Ourisson gave m.p. 99-100° for their purest material. The GLC showed a very small amount of impurity at a lower retention time than the main peak. The TLC showed essentially one spot with a trace of less mobile impurity. Ultraviolet spectrum gave ϵ_{248} 19,400. Infrared spectrum (in CS₂) showed strong absorption at 803 cm⁻¹. Mass spectroscopy: 426 (M⁺ and base), 411 (M⁺-CH₃), 366 (M⁺-CH₃COOH), 351 (M⁺-CH₃COOH-CH₃), 313 (M⁺-C₈H₁₇), 253 (M⁺-CH₃COOH-C₈H₁₇), and 238 (M⁺-CH₃COOH-C₈H₁₇-CH₃) m/e.

Results and Discussion

Even before the Z sterols were freed from the triterpenes and other materials in the nonsaponifiable fractions, it was obvious that at least two components were involved although they moved as one spot near the origin on TLC. The GLC showed that this was not a homogeneous material; two peaks were shown by the silylated product.

Separation of Z from Other Nonsaponifiables

The first separation of the Z sterols from the triterpenes and other materials with which they were associated in the nonsaponifiables was done on 1:1 (w/w) silica gel:celite columns. Because the silica gel plates, developed with mixtures of chloroform-acetone and

chloroform-methanol, were able to accomplish the separation needed, it was reasonable to go to this combination for column chromatography to obtain "pure" Z material.

Some experimentation was done with a batch-wise separation. A silica gel:celite mixture was stirred for 2 hours in a chloroform solution of nonsaponifiables, then filtered, and washed with chloroform. The Z material and other more polar materials were adsorbed on the silica gel, leaving the triterpenes in the chloroform solution. The Z material and other polar materials were then eluted from the silica gel with 9:1 (v/v) chloroform:methanol. The TLC and GLC showed the eluted Z material and more polar substances to be free of the triterpenes.

Several small size columns of silica gel-celite were run with chloroform, chloroform-acetone mixtures, and chloroform-methanol mixtures used as the eluting solvents. The columns were monitored by testing the eluate with the Liebermann-Burchardt (L.B.) reaction mentioned earlier. The validity of this use of the L.B. reaction was confirmed by TLC.

As larger amounts were separated, improvements were made on the method of development. In the case of the first larger columns, the nonsaponifiable material was placed on the column in chloroform and eluted with chloroform until all triterpene material had been removed, then developed with 9:1 (v/v) chloroform:acetone to remove Z. Materials more polar than Z were eluted with 9:1 (v/v) chloroform: methanol.

A more complete separation between the triterpenes and Z sterols was achieved by elution of the column with 1:1 (v/v)

chloroform:benzene, gradually increasing the proportion of chloroform to pure chloroform, and then gradually adding methanol in increasing proportions up to 80:20 (v/v) chloroform:methanol. Columns eluted with 8:2 (v/v) chloroform:benzene until all triterpenes were removed, then chloroform and 98:2 (v/v) chloroform:methanol to remove the Z sterols also worked well.

Separations of triterpenes from the Z sterols on columns were better when acetone reprecipitation had been applied to the nonsaponifiables before the chromatography. A smaller quantity of the triterpenes was present in samples treated in this manner.

Acetone Crystallized Z

The Z material from columns was crystallized from hot acetone to remove yellow color which also enriched the Z-1 content of the white materials obtained in these recrystallizations. The Z-2 seemed to be more soluble in acetone, as was the yellow color.

The first acetone crystallized Z sterols obtained in sufficient quantity to get some analytical data gave essentially one spot on TLC (traces only of 3 impurities) with 95:5 (v/v) chloroform:methanol on silica gel G and showed a ratio of about 3:1 of Z-1 to Z-2 by GLC. This material had a melting range of 167-170° in air with some decomposition, but appeared to melt at 175-176° in vacuum. It had an ultraviolet absorption at 247-248 m μ and an end absorption at 205 m μ . The infrared spectrum showed no carbonyl but had a stronger absorption at 3300 cm⁻¹ than normal sterols show in this region. An absorption at 807 cm⁻¹ was present.

The UV spectrum indicated the presence of heteroannular conjugated double bonds and the IR band at 807 cm^{-1} is in the region where trisubstituted carbon to carbon double bonds show absorption. The extra strong 3300 cm^{-1} absorption indicated a polyhydroxy compound which explains the polar nature of the Z sterols. A rough comparison with 3 β -hydroxy sterols indicated twice as much OH was present in the Z sterols as in normal sterols. Dilution of the sample did not shift the OH absorptions, which would indicate intramolecular hydrogen bondings. The material also gave a positive Tortelli-Jaffe reaction, which indicates double bonds at the 7, 8(9), or 8(14) position. Mass spectroscopy showed a mixture of at least three compounds. There were strong ion peaks at 402, 401, 400, and a weaker peak at 416 m/e, and this was tentatively interpreted to be caused by a mixture of compounds having molecular weights of 416, 402, and 400. The 401 m/e peak was probably the $416^+ - \text{CH}_3$. Another strong ion peak at 247 m/e could be $\text{M}^+ - 155$ for the compound of MW 402. Some indication of larger MW impurities was evident when run at greater sensitivity. The molecular weights were within reason for sterols.

The data so far suggested that Z was probably a mixture of dihydroxysterols of molecular weights 400, 402, and 416. At least one of these had heteroannular conjugated double bonds. Since the 400 and 402 differed by 2, the 400 compound probably had the heteroannular conjugated double bonds and the other, one double bond. The third compound, MW 416, had a molecular weight 14 larger than the 402 compound. This would indicate an extra methyl group.

Separation of Z-1 from Z-2 by Digitonin

A 1.00 g sample of Z (2:1 Z-1 to Z-2) was submitted to the digitonin precipitation. The first crop of Z-1 digitonide weighed 2.00 g, gave a typical Z-type L.B. reaction (immediate blue turning slowly to green), and a silated sample showed only Z-1. Upon evaporation of the mother liquor to one-half volume, a precipitate weighing 0.38 g was filtered off. This was mostly Z-1 but had some Z-2 present. It was recrystallized from hot 80% ethanol to yield an additional 0.23 g of Z-1. The mother liquor from the recrystallization, when dried down, was mostly Z-2 with a small amount of Z-1. By the addition of more digitonin to the original filtrate and by a series of precipitations, recrystallizations and work up of Z-2 rich precipitates, 0.51 g of Z-2 digitonide was obtained.

Similarities of Z-1 and Z-2 to Peniocerol and Macdougallin

A search of the literature for dihydroxysterols revealed reports by Djerassi, Murray, and Villotti (28,29) on the isolation and characterization of peniocerol (cholest-8-ene-3 β , 6 α -diol) from the roots of the cactus, Peniocereus fosterianus. There were also reports by Djerassi, Knight, and Wilkinson (30) and Knight, Wilkinson, and Djerassi (31) on the isolation and characterization of macdougallin (14 α -methyl- Δ^8 -cholestene-3 β , 6 α -diol) from the roots of the cactus, Peniocereus macdougallii. Peniocerol and macdougallin were both found in different ratios in the roots of these two cacti. Peniocerol was also found in roots of the cactus, Wilcoxia viperina, by Djerassi,

Knight, and Brockmann (32), along with three unusual sterols having keto groups at C6.

A very small sample of very crude peniocerol and a small sample of impure macedougallin diacetate were kindly supplied by Dr. Carl Djerassi.

Thin-layer chromatography on silica gel G in 95:5 (v/v) chloroform:methanol of the crude peniocerol and macedougallin diacetate samples showed the main spot given by macedougallin diacetate corresponded in R_f to Z-1 and Z-2 diacetates but its red-purple color resembled that of Z-2 diacetate. The crude peniocerol sample was too crude: it gave only a brownish smear on TLC. Figure 19 shows thin-layer chromatography in two systems of the previously mentioned materials and others.

The GLC of the crude peniocerol showed two peaks. The predominant peak had the same retention time as Z, and the minor peak at a retention time similar to cholesterol. Silylated crude peniocerol showed one peak at the same retention time as silylated Z-1. The GLC of the macedougallin diacetate showed three strong peaks. The major one had a retention time the same as that of Z diacetate. The other two peaks were shorter retention times. A Liebermann-Burchardt reaction on the macedougallin diacetate sample gave a yellow-brown reaction.

Z-1 Free Sterol by Cleavage of Z-1 Digitonide

The 2.00 g sample of Z-1 digitonide was cleaved by the pyridine-ether procedure, and after acetone recrystallization, 0.09 g of free Z-1 sterol was obtained. It had a melting range of 183-198° (vac), and

Thurberol (Z-1b)	+		(Bl-Pu)
Peniocerol (Z-1a)	+		(Bl-Pu)
8(14) isomer	+		(Bl-Pu)
Macdougallin (Z-2)	+	(dashed)	(Rd-Pu)
Macdougallin (Djerassi)	+	(dashed)	(Rd-Pu)

Silica gel G; chloroform

Thurberol (Z-1b)	+	(Br)	
Peniocerol (Z-1a)	+		(Br-Bl)
8(14) isomer	+		(Br-Bl)
Macdougallin (Z-2)	+	(Br)	(Rd-Pu)
Macdougallin (Djerassi)	+	(Br)	(Rd-Pu)

Silver nitrate-silica gel G; chloroform

Figure 19. Thin-layer chromatography of diacetates.

Bl = blue
Pu = purple

Rd = red
Br = brown

it appeared to sublime partially at a lower temperature than the melting range. In air it appeared to decompose at 164°C . The GLC showed only Z-1. The UV spectrum showed the usual absorption with the maximum shifted to 250 $\text{m}\mu$ and end absorption at 209 $\text{m}\mu$. Because of the limited solubility of this preparation in carbon disulfide, a KBr disk was prepared and showed the strong 3300 cm^{-1} OH absorption and some absorption at 800 cm^{-1} . The mass spectrum showed molecular ion peaks only at 402 and 400 m/e at a ratio of about 4:3. This indicated that the 416 and 401 m/e peaks in the spectrum of the Z sterol material were due to Z-2 molecular ion and the $(\text{Z-2})^{+}\text{-CH}_3$, as proposed. This spectrum also showed that Z-1 was definitely composed of two substances differing by 2 in their molecular weight and closely related in structure. This was seen from the presence of the following pairs of ion peaks: 402, 400; 387, 385; 384, 382; 369, 367; 351, 349; 289, 287; 271, 269; 253, 251 m/e . These represent: M^{+} , $\text{M}^{+}\text{-CH}_3$, $\text{M}^{+}\text{-H}_2\text{O}$, $\text{M}^{+}\text{-CH}_3\text{-H}_2\text{O}$, $\text{M}^{+}\text{-CH}_3\text{-2 H}_2\text{O}$, $\text{M}^{+}\text{-C}_8\text{H}_{17}$, $\text{M}^{+}\text{-C}_8\text{H}_{17}\text{-H}_2\text{O}$, and $\text{M}^{+}\text{-C}_8\text{H}_{17}\text{-2H}_2\text{O}$ for each of the two compounds. The $\text{M}^{+}\text{-H}_2\text{O}$ and $\text{M}^{+}\text{-2 H}_2\text{O}$ ions showed the two OH groups present in both compounds.

Hydrogenation of Z-1 Sterols

A 15.3 mg sample of the preceding Z-1 sterol was dissolved in 5 ml of 95% ethanol and hydrogenated for 4.5 hr over 5% Pd/C. The catalyst was filtered off, and the solution was evaporated to dryness. The hydrogenated Z-1 showed no UV absorption at 247-248 $\text{m}\mu$, and the end absorption was strong and shifted to 213 $\text{m}\mu$. The TLC showed essentially one spot at the same mobility as the Z-1. The spot reacted with both

sulfuric acid and with a potassium permanganate spray, showing at least one double bond was still present. The GLC showed a single peak at slightly less retention time than Z-1 free sterol. This material showed no immediate blue L.B. reaction but slowly turned green, then pink.

Acetylated Z-1

Direct acetylation of 1.85 g of Z-1 digitonide by acetylation method 4 gave 0.47 g steryl diacetates. Three crystallizations from acetone yielded 128.4 mg of white Z-1 diacetate which gave a single peak on GLC at a much longer retention time than the free sterol. The melting range was $54-70^{\circ}$ (air) and $56-70^{\circ}$ (vac). The UV spectrum showed absorption at 247-248 μ and an end absorption at 210 μ , at the same intensity. The IR spectrum showed absorption at 807 cm^{-1} . The TLC on silver nitrate-silica gel G with chloroform gave two spots, the more mobile, designated Z-1a, reacted blue to sulfuric acid, and the less mobile, designated as Z-1b, reacted brown immediately with the spray and turned darker upon heating and appeared to be the diene. The mass spectrum of the mixture showed ion pairs at m/e values of 426, 424 ($M^+ - \text{CH}_3\text{COOH}$); 366, 364 ($M^+ - 2\text{ CH}_3\text{COOH}$); 351, 349 ($M^+ - 2\text{ CH}_3\text{COOH} - \text{CH}_3$); 313, 311 ($M^+ - \text{CH}_3\text{COOH} - \text{C}_8\text{H}_{17}$); and 253, 251 ($M^+ - 2\text{ CH}_3\text{COOH} - \text{C}_8\text{H}_{17}$). When the spectrum was run at a much greater sensitivity, molecular ion peaks at 486 and 484 m/e were observed. (This sensitivity also showed traces of Z-2 present, an example of the extreme sensitivity of mass spectroscopy.)

Hydrogenation of Z-1 Diacetate

A 350 mg sample of monoene-diene diacetate mixture was hydrogenated overnight in ethyl acetate with 5% Pd/C and several drops of acetic acid. The catalyst was removed and solvent evaporated. The residue was crystallized from methanol to get 209 mg of plates, m.p. 138-140.5° (air) and 140-141° (vac) (lit., m.p. 141-142°) (33). The L.B. reaction gave an immediate blue color, remained blue for at least one hour, then turned purple. The TLC with chloroform on silver nitrate-silica gel B showed one blue reacting spot (see Figure 19). The GLC showed a single peak at slightly less retention time than the starting material but not separable from it. Its UV spectrum showed only strong end absorption at 212.5 m μ , ϵ 6,715. The IR spectrum showed no 805 cm⁻¹ band. A mass spectrum showed ions at 426, 411, 366, 351, 313, 253, and 211 m/e representing M⁺-CH₃COOH, M⁺-CH₃COOH-CH₃, M⁺-2CH₃COOH, M⁺-2CH₃COOH-CH₃, M⁺-CH₃COOH-C₈H₁₇, M⁺-2CH₃COOH-C₈H₁₇ S.C. and M⁺-2CH₃COOH-155, respectively. The entire spectrum matched exactly with that of a sample of 8(14)-cholesten-3 β , 6 α -diol diacetate kindly supplied by C. Djerassi. An NMR spectrum at 100 Mc in CDCl₃ showed shift values of .78 (C19), .82 (C18), .89, .95, and 2.00 ppm and in CCl₄ at .78 (C19), .82 (C18), .88, .94, 1.92, and 1.96 ppm. Calculated values for C19 and C18 were .75 and .84, respectively. No vinyl proton peak was present. The NMR spectrum matched exactly a xeroxed NMR spectrum of 8(14)-cholesten-3 β , 6 α -diol diacetate kindly supplied by C. Djerassi. The identity of this material was unquestionable. Both Z-1a monoene diacetate and Z-1b diene diacetate produced 8(14)-cholesten-3 β , 6 α -diol

diacetate upon hydrogenation under isomerizing conditions. This was also seen by hydrogenation of a mixture of the free sterols.

Hydrogenation of 8,14-cholestadien-3 β -ol acetate under the same conditions gave plates that melted at 75-76.5 $^{\circ}$ (air and vacuum). The literature values for the melting point of 8(14)-cholesten-3 β -ol acetate were 76-77 $^{\circ}$ (34) and 77-78 $^{\circ}$ (35).

A small sample of a mixture of the Z-1a diacetate and Z-1b diacetate was hydrogenated overnight with Raney nickel at room temperature. The product before crystallization from methanol showed no conjugated diene in its UV spectrum and the end absorption was the type typical of a $\Delta^{8(9)}$ rather than a $\Delta^{8(14)}$ double bond (a gradual slope rather than sharp). The GLC showed one main peak at the retention time of Z-1a and Z-1b diacetate and just a trace of impurity. After crystallization from methanol the product melted at 48-50 $^{\circ}$, the mp of Z-1a diacetate (see below). The mixture of Z-1a and Z-1b diacetate apparently hydrogenated to Z-1a diacetate over nickel.

Hydrogenation of 8,14-cholestadien-3 β -ol acetate under the same conditions with Raney nickel also produced a product that appeared to be 8(9)-cholesten-3 β -ol acetate. The UV spectrum showed no conjugated diene and gave a typical end absorption curve of a $\Delta^{8(9)}$ rather than $\Delta^{8(14)}$ double bond. The melting range of the product was 91.5-98 $^{\circ}$ rather than the 76-78 $^{\circ}$ range of the 8(14)-cholesten-3 β -ol acetate. The literature value for 8(9)-cholesten-3 β -ol acetate is 107-108 $^{\circ}$ (36). With some impurities it would be expected to be lower so the 91.5-98 $^{\circ}$ is reasonable for the $\Delta^{8(9)}$ with impurities. It appeared then that the

14(15) double bond had been hydrogenated under these conditions without any isomerization from the 8(9) to the 8(14) position.

Reaction with Maleic Anhydride

To help decide the positions of the conjugated double bonds, a 49.8 mg sample of the monoene-diene diacetate mixture and 20.6 mg of maleic anhydride were mixed in 5 ml of benzene and refluxed for 4 hours. The benzene was evaporated and the residue was crystallized from acetone. The UV spectrum, TLC, and melting range indicated that no adduct was formed. This ruled out a 7,14 diene.

Separation of Z-1a (Monoene) from Z-1b (Diene) as Diacetates

Several small silver nitrate-silica gel columns were tried for the separation of Z-1a diacetate from Z-1b diacetate using chloroform as the developing solvent since that system had been able to separate the two diacetates in TLC. The columns were only slightly successful and no pure Z-1a diacetate nor Z-1b diacetate was obtained from those columns. Because of this, TLC on preparative plates became the method of choice to obtain a small amount of Z-1a diacetate and Z-1b diacetate for analytical data needed for their characterization. The separation of the two diacetates was accomplished on preparative thin-layer plates of silver nitrate-silica gel G with chloroform as the developing solvent. As much as 54 mg of the diacetate mixture was spotted in a band on each plate. Elution of the scraped bands was accomplished with ethyl acetate, according to the method given in Chapter 3. Eluted material was purified on a small column of silica gel-celite with 8:2

(v/v) chloroform:acetone, followed by evaporation of the solvent and crystallization of the diacetate from methanol.

Properties of Z-1a Diacetate and Its
Characterization as Peniocerol Diacetate

The Z-1a diacetate crystallized from methanol in long, fine needles, m.p. 48.5-50° (vac). Djerassi et al. (29) reported peniocerol diacetate as needles, m.p. 48-50°. The L.B. reaction gave an immediate long-lasting blue turning eventually to green and then to pink. The GLC showed essentially a single peak and the TLC is shown in Figure 19.

The spectral data, melting point, and hydrogenation to 8(14)-cholesten-3 β , 6 α -diol diacetate leaves no doubt that the Z-1a from the organ pipe cactus is peniocerol, 8(9)-cholesten-3 β , 6 α -diol (Figure 20). A summary of the comparative spectral data is shown in Table 5.

Properties of Z-1b Diacetate

The Z-1b diacetate crystallized from methanol as small, fine needles, m.p. 103-104°. The TLC on OV-101 showed essentially a single peak (very slight traces of contaminant) at the same retention time as peniocerol diacetate (Z-1a diacetate). The TLC with chloroform on plain silica gel G shows a blue reacting spot at the same R_f as peniocerol diacetate (Z-1a diacetate) but with chloroform on silver nitrate-silica gel G it shows a brown reacting spot of smaller R_f than peniocerol diacetate (Z-1a diacetate) (see Figure 19). Analysis (Schwartzkopf Microanalytical Laboratory)--Found: C, 76.88; H, 10.22. Calculated for C₃₁H₄₈O₄: C, 76.78; H, 10.01. All the spectral data for Z-1b diacetate are shown in Table 6.

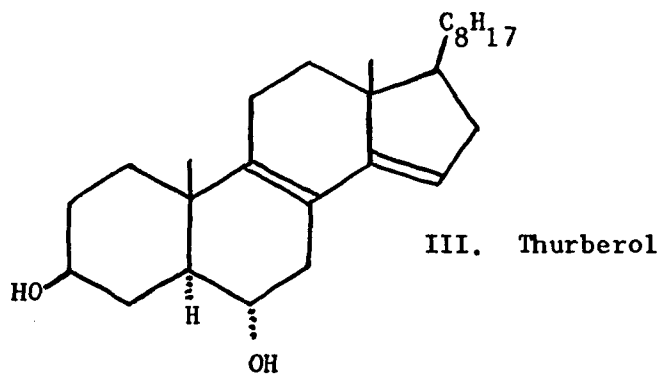
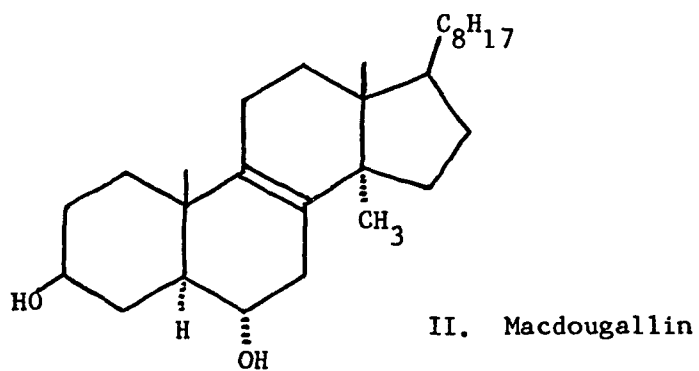
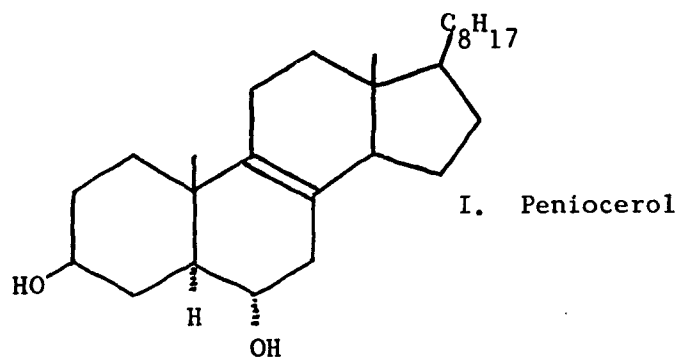


Figure 20. Structures of peniocerol, macdougallin, and thurberol.

Table 5. Physical properties of Z-1a and peniocerol.

	Z-1a	Peniocerol
	<u>Free sterol</u>	<u>Peniocerol Lit. (29)</u>
Melting range	163-180°	168-171°
IR spectrum	3300 cm ⁻¹	3330 cm ⁻¹
Mass spectrum	402 m/e	402 m/e
	<u>Diacetate</u>	<u>Peniocerol Lit. (29,31)</u>
Melting range (ppm)	48.5-50° needles	48-50° needles
UV spectrum	end abs. at 208 mμ typical 8(9) type	typical 8(9) type end abs. at 210 mμ
IR spectrum	1725 and 1240 cm ⁻¹	1735 and 1243 cm ⁻¹
NMR spectrum (CDCl ₃ , 100 Mc)	.58 (C-18)(calc: .58) .82 .88 .94 1.03 (C-19)(calc: .99) 2.00 2.02	.60 (C-18) .85 .90 1.05 (C-19) 2.06 2.10
Mass. spectrum	426 m/e (M ⁺ -CH ₃ COOH) 366 (M ⁺ -2 CH ₃ COOH) 351 (M ⁺ -2 CH ₃ COOH-CH ₃) 313 (M ⁺ -CH ₃ COOH-C ₈ H ₁₇) 253 (M ⁺ -2 CH ₃ COOH-C ₈ H ₁₇)	426 366 351 313 253
(traces of diene)		

Table 6. Physical properties of thurberol (Z-1b) diacetate.

Spectrum	
UV spectrum	247.5 m μ , ϵ 18,000 (in 95% ethanol)
IR spectrum	808, 895, 915, 937, 980, 1032, 1070, 1130, 1165, 1240, 1365, 1730, and 2920 cm ⁻¹ (in CS ₂)
NMR spectrum (ppm)	.79 (C-18) (calc: .81) (CDCl ₃ , 100 Mc) .83 .89 .96 1.07 (C-19) (calc: 1.06) 2.01 acetate proton 2.05 " " 5.34 vinyl proton
Mass spectrum	484 m/e (M ⁺) 424 (M ⁺ -CH ₃ COOH) 364 (M ⁺ -2 CH ₃ COOH) 349 (M ⁺ -2 CH ₃ COOH - CH ₃) 311 (M ⁺ -CH ₃ COOH - C ₈ H ₁₇) 251 (M ⁺ -2 CH ₃ COOH - C ₈ H ₁₇) 236 (M ⁺ -2 CH ₃ COOH - C ₈ H ₁₇ - CH ₃) 209 (M ⁺ -2 CH ₃ COOH - 155)

The data clearly show that the Z-1b from organ pipe was 8,14-cholestadien-3 β ,6 α -diol (Figure 20). This is a new compound not previously described in the literature, and the proposed trivial name for this compound is Thurberol.

Characterization of Z-2 as Macdougallin

The only preparation of Z-2 in an amount sufficient for some analytical data was obtained by reworking of mother liquors from a Z-1 digitonide precipitation, and accumulation of digitonides rich in Z-2. Further enrichment of Z-2 was achieved by recrystallizations of this material in 80% ethanol. The Z-2 was enriched in the mother liquors and later precipitates. Such a Z-2 enriched (approx. 70% Z-2:30% Z-1) digitonide precipitate (3.8 g) was recrystallized from 1:2 (v/v) methanol:acetone. This actually cleaved the digitonide; the digitonin precipitated and was removed, and the filtrate evaporated to dryness. This residue was extracted with boiling acetone several times. The acetone was evaporated to dryness, and the residue recrystallized from acetone to get 0.38 g of chloroform soluble Z-2. This material reacted to L.B. reagent with an immediate purple, changing to blue, then changing to green. The GLC showed only possible traces of Z-1. Other physical properties of Z-2 are shown in Table 7.

A portion of Z-2 (0.17 g) was acetylated in a minimum volume of dry pyridine with 0.3 ml acetic anhydride overnight. After work-up, the Z-2 diacetate was crystallized from methanol to get 82.7 mg of small, colorless needles, m.p. 122-122.5°. The TLC showed essentially one spot with a small amount of a tan reacting impurity suspected of

Table 7. Physical properties of Z-2 and macdougallin.

	Z-2	Macdougallin
	<u>Free sterol</u>	
Melting range	157-158° (vac) (impure sample)	173-174° Lit. (31)
Mass spectrum	416 m/e	416 m/e
	<u>Diacetate</u>	
Melting range	122-122.5° (air and vac)	124-127° Lit. (31)
IR spectrum	1730 and 1240 cm ⁻¹	1735 and 1250 cm ⁻¹ Lit. (31)
NMR spectrum (CDCl ₃ , 100 Mc)	.69 ppm (C-18) .83 .89 .95 1.04 (C-19) 2.01 2.03	.70 ppm Lit. (31) .84 1.04 2.02 2.04
(CCl ₄ , 100 Mc)	.72 ppm (C-18) .84 .90 1.06 (C-19) 1.94 1.98	.72 ppm (Djerassi sample) .84 .90 1.06 1.93 1.98
Mass spectrum	500 m/e (M ⁺) 440 (M ⁺ -CH ₃ COOH) 425 (M ⁺ -CH ₃ COOH-CH ₃) 380 (M ⁺ -2CH ₃ COOH) 365 (M ⁺ -2CH ₃ COOH-CH ₃) 267 (M ⁺ -2CH ₃ COOH-C ₈ H ₁₇)	
	(Z-2 and Djerassi sample same)	

being a diene. Djerassi's sample of macdougallin diacetate on the same plate gave the same two spots; the main spot in each had the very distinctive red-purple color not seen in any other compound chromatographed. The GLC showed a single peak with a shoulder of higher retention time exactly as shown by the macdougallin diacetate. The UV spectrum showed a trace of absorption in the 230-260 $m\mu$ region, too broad and shallow to see the maxima accurately. The end absorption was at 206 $m\mu$, the same as the end absorption of the macdougallin diacetate. Other physical properties of Z-2 diacetate are shown in Table 7.

A 25.8 mg sample of Z-2 diacetate was hydrogenated overnight in 10 ml of ethyl acetate and 1 ml of glacial acetic acid over 5% Pd/C. The catalyst was filtered off and the solvent evaporated. The TLC and GLC showed no change in the Z-2 diacetate from starting material, as was the case reported for macdougallin (31).

The comparison of data for Z-2, its diacetate, and macdougallin and its diacetate is summarized in Table 7 and shows that Z-2 is macdougallin (Figure 20).

CHAPTER 7

SUMMARY AND BIOCHEMICAL IMPLICATIONS

Among the *Cereus* cacti of the Sonoran Desert, Organ pipe cactus has been shown to be unique in its lipid chemistry. Within the limited scope of this research concerning the fatty acids and sterols, it has been demonstrated that this cactus has a much greater content of extractable lipid material than all the other *Cereus* cacti that have been examined. Senita would appear to have the next greatest amount. It is certainly interesting to note that saguaro and cardon, host plants for *D. nigrospiracula*, have extremely low amounts of extractable lipids. The younger organ pipe tissue showed an even greater amount of extractable lipids and fatty acids than mature tissue.

In contrast to other cacti investigated, organ pipe has essentially no long chain fatty acids, except in its younger stems, but is well supplied with short chain fatty acids (six carbons to twelve carbons in length). These fatty acids appear to occur in the plant in ester linkages with the hydroxy groups of the three dihydroxysterols, peniocerol, macdougallin, and thurberol. They are probably also esterified to the hydroxy groups of the triterpenes, calenduladiol and betulin, based upon the extractability of these with Skellysolve B from a dry tissue.

The lack of long chain fatty acids poses questions about the photosynthetic apparatus and processes in this cactus, since

α -linolenic acid is considered ubiquitous in normal photosynthetic systems. Does photosynthesis occur only in the young stems and meristematic tissue at the growing tips of mature stems, or are the chloroplast lipids different in this plant?

Perhaps the high lipid content, the large quantity of short chain fatty acids or the deficiency of long chain fatty acids provide an environment unable to support the growth of certain yeasts or D. nigrospiracula. It is interesting to note in this connection that, in the process of drying saguaro and organ pipe tissue in air, mold growth was a problem with saguaro tissue, but mold never grew on the wet organ pipe tissue.

Phytosterols and Drosophila

The common phytosterols, cholesterol, campesterol, stigmasterol, and sitosterol, probably esterified with short chain fatty acids, were found in extremely small quantities in organ pipe cactus tissue. This is in contrast to saguaro and cardon, host plants for D. nigrospiracula, where the phytosterols were found in ample quantities as free sterols. If D. nigrospiracula requires any or all of the normal phytosterols, perhaps they are not in sufficient quantity in organ pipe for this fly, which is large by Drosophila standards. It is also possible that D. nigrospiracula is unable to use phytosterol esters while D. mojavensis, being more polyphagous, has the ability to use esterified sterols. These possibilities are amenable to being tested.

Dihydroxysterols and Drosophila

The closely related three dihydroxysterols, peniocerol, macdougallin, and thurberol, were found in organ pipe cactus in greater quantities than the phytosterols. They too are esterified to the short chain fatty acids.

This is the first time that the peniocerol and macdougallin have been found in aerial portions of cacti. They were isolated from the large tuberous roots of cacti that have a much smaller aerial structure than organ pipe. In organ pipe, these sterols and thurberol, a diene, are exposed to *Drosophila*. Is it possible that sterols with an extra hydroxy in the 6α position could act as inhibitors to the normal use of sterols by *D. nigrospiracula* or as inhibitors of the normal sterol metabolism of yeasts? Slaytor and Bloch (37) showed that rat liver tissue metabolized peniocerol to cholesterol under aerobic conditions and to 7-cholesten- 3β -ol under anaerobic conditions. Macdougallin was metabolically inert; conversion to cholesterol or 7-cholesten- 3β -ol was insignificant due to the 14α methyl group. Perhaps *D. mojavensis* can use these sterols but *D. nigrospiracula* cannot.

Peniocerol, macdougallin, and thurberol are the only 6α -hydroxysterols ever found in plants. Their possible use by phytophagous insects as precursors for ecdysone, the molting hormone, which is oxygenated at C6, could open up a whole new area of research.

Thurberol and Sterol Biosynthesis

It is interesting to consider where the new compound may fit into cholesterol and phytosterol biosynthesis. Lutsky and Schroepfer (38) found easy conversion, in rat liver tissue, of 8,14-cholestadiene-3 β -ol to cholesterol. Johnston and Bloch (39) suggested that the 5(6) double bond of cholesterol arises by a two-step reaction involving a hydroxylation at C-6 followed by elimination of water. In thurberol the C-6 hydroxyl is already there, but in the alpha configuration, which is not the more favorable trans diaxial position (6 β) for elimination of water. Even Δ^7 cholesten-3 β ,6 α -diol was not an intermediate in conversion of Δ^7 cholestenol to 7-dehydrocholesterol.

Finding macdougallin, thurberol, and peniocerol in the same tissue points to thurberol, III, as an intermediate in the biosynthesis of peniocerol, I, from macdougallin, II (see Figure 20). This lends credence to the hypothesis that a Δ^{14} bond is formed during elimination of the 14 α -methyl group in sterol biosynthesis as outlined by Schroepfer et al. (40). Whether or not peniocerol is further metabolized is an open question. The point in the biosynthetic scheme at which the three sterols are esterified is also unknown.

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