Composition of the cuticular wax of Anabrus simplex Hald
by Graeme Levo Baker

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University


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# I ABSTRACT

The lipid solvent extractable material from the cuticle of Anabrus simplex Hald. (Mormon cricket) has been examined in an effort to elucidate its chemical composition. Hydrocarbons, free fatty acids, esters, free cholesterol and acidic resins are present in measurable amounts. Infrared spectrophotometry, column chromatography, and gas chromatography are utilized for the separation and characterization of these various components.

## II INTRODUTION

It has been well established that the exoskeletons of all insects are covered by a waxy, lipoidal layer. The properties of this layer appear to differ somewhat between species. This difference may be related to the moisture levels of the habitats occupied by the insects. The lipoidal component of the exoskeleton seems worthy of study since it has been shown to possess the mechanisms for the maintenance of the correct water balance between the insect and its environment [16]. In addition, it represents the barrier through which all contact poisons must pass before they can become effective as control agents. The Mormon cricket, Anabrus simplex Hald., was chosen for study because of its economic importance, availability, large size, and the presence of a good waxy protective layer. The semi-arid habitat of this insect suggests relatively large amounts of a lipid layer for moisture control.

Much has been written on the physical and chemical nature of lipids obtained from various insects. Most of this work has been dome by foreign investigators on insects that are of little or no economic importance in the United States. The major portion of the work has been done on waxes produced by the insects for purposes other than as an exoskeletal covering (i.e. beeswax, chinese insect wax, etc.) because these waxes are available in appreciable quantities. Publications of more specific studies relating to the chemical structure of the lipids of the exoskeleton itself have been infrequent. A few people have studied the cuticular lipids with an eye to elucidation of the chemical nature of the individual components [1,3,5,6,7,8].

## II INTRODUCTION

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Gilby and Alexander [5,6,7] have conducted the most exhaustive studies to-date. Their studies of the cuticular lipids of Ceroplastes destructor indicate that the wax consists of n-paraffin chain acids and alcohols that average about twenty-seven carbons. Significant amounts of short chain acids and alcohols (about $C_{12}$) were also reported. This work verifies the essentials of a paper published by Hackman in 1951 [8]. Beament [2] and Donnell [3] have obtained evidence on the exoskeletal wax of the cockroach. This wax has been shown to contain n-paraffins and alcohols. The presence of appreciable quantities of n-paraffins in an insect wax was also reported by Teng-Han Tang in 1939 [15]. The wax was presumably similar to chinese insect wax. Tang reported that the wax was composed of 21.80% hentriacontane, with the possibility of 4.72% cholestene. Ceryl alcohol ($C_{26}$) (7.45%), cerotic acid (35.72%) and unsaturated acids (unspecified) (29.89%) were also reported. This report was overlooked by the present investigator until the time of writing this paper. This was unfortunate, for it would have tempered a pre-conceived notion that insect waxes should consist chiefly of esters, free fatty acids, and alcohols. It is equally apparent that most of the other investigators in the field have also overlooked Tang's work.

In retrospect then, several generalizations on the reported composition of insect cuticular waxes can be made. Hydrocarbons, esters, free fatty acids and free alcohols have been reported. The hydrocarbons reported have all been saturated; the esters as such, have not been studied;

the acids and alcohols have been either saturated or unsaturated; and resinous materials are evinced by the lac producers. This knowledge of the composition of cuticular waxes is meager. The work reported herein amplifies such knowledge through a partial elucidation of the cuticular wax of Anabrus simplex Hald., the Mormon cricket.

## III PROCEDURE

### A. Collection of wax

Crickets were collected from various bands located in south-central Montana. No selection as to sex or age was made, but the crickets were predominantly in the adult stage. They were brought to the laboratory in cages and kept in a cool room for from three to five days. This action was prompted by the fact that, although the crickets are inactive at this temperature, digestion proceeds and thereby permits a relatively complete evacuation of the digestive tract. It was hoped that this procedure would minimize contamination of waxes extracted from the whole cricket. The enforced inactivity was found necessary in order to prevent cannibalism. Following this treatment the crickets were killed by subjecting them to chloroform and ammonia vapors in a closed container. The presence of the ammonia in the killing medium appeared to reduce the regurgitation of fluids from the crop. The crickets were then washed with water by spraying on large screens, air dried, and refrigerated until the extractions could be completed.

The cuticular "wax" was extracted by wrapping the crickets in cheese-cloth and extracting with a lipid solvent using a soxhlet continuous extraction apparatus. Since the solubility of the "wax" was not known several lipid solvents were tried; chloroform, benzene, diethyl ether, constant boiling chloroform-methyl alcohol, and skellysolve-B (industrial n-hexane). Variations in the amounts of wax extracted were insignificant. The "wax" extracted varied from 0.14% to 0.19% of total

cricket weight, with deviations between succeeding batches of the same solvent being as large as deviations between different solvents. Three different "wax" samples were collected, using three different solvents i.e. skellysolve-B, chloroform, and the constant boiling chloroform-methyl alcohol. The entire yield of "wax" was approximately 3 gms.

The "wax" extracts were washed with water in a saperatory funnel. Each liter of extract was washed successively with 250 ml., 250 ml. and finally 100 ml. of water. This procedure was used in an attempt to remove any water soluble materials that may have been extracted from the crickets. A characteristic "cricket odor" which had been apparent in the "wax" extract was noticeably transferred to the aqueous phase. This washing procedure was markedly hampered by the formation of stable emulsions, and losses of "wax" were unavoidable. The formation of emulsions was least troublesome with the skellysolve-B extracts. The washed extracts were dried over "drierite" and the solvent was then allowed to evaporate at room temperature. The evaporation was accelerated by blowing clean air over the surface of the solution.

The waxes obtained in this way were designated "wax X", "Y", and "Z" for the constant boiling chloroform-methyl alcohol, skellysolve-B, and chloroform extracts respectively. Throughout this paper X, Y, and Z will carry this connotation.

In the latter stages of this project verification of results was made by examination of the wax obtained from excised cuticles. The collection and treatment of the crickets was identical to previous collections. How-

ever as soon as the crickets had been washed, and air dried, they were frozen to await further handling. As time allowed the abdoninal sclerites were removed from the crickets, and all extraneous and cellular material were removed by careful brushing under water. The cleaned abdominal sclerites were then dried and refrozen for later extractions with an appropriate solvent.

B. Separations Based on Solubility:

Numerous attempts at solubility fractionations were made in the early stages of the current work. It became apparent that a degree of fractionation could be accomplished by the use of various combinations of skellysolve-B, diethyl ether and methyl alcohol. Attempts at fractionation involved both hot and cold solvent systems. Although this appraoch proved impractical for clean separations it did provide a clue to the general character of the wax.

The precipitation of a yellow wax by addition of methyl alcohol to either solutions of "wax Y" produced a mixture which was pure enough to elicit subsequent identification as a hydrocarbon. The soluble portion was shown to contain a ninhydrin positive component which to-date has been neither isolated nor identified.

Additional information was obtained from the methyl alcohol-soluble portion by removal of the solvent and treatment of the residue with n-hexane. The residue was only partially soluble in n-hexane and left a resinous residue. This resinous residue had solubility characteristics reminiscent of shellac, i.e. soluble in methyl alcohol, and basic aqueous

solutions (when heated) and insoluble in the non-polar organic solvents.

The solubility studies suggested, therefore, that hydrocarbons, a lac, and a nitrogenous material were components of the wax.

C. Saponification:

Saponification proved to be of value when infrared spectra indicated the presence of the suspected ester structures. Attempts to saponify the original "wax" were signally unsuccessful. This is, in retrospect, understandable since the hydrocarbons present would markedly complicate the handling of the saponification system. However, saponification and the following separation of the saponifiables from the non-saponifiables was accomplished by use of the following procedure:

1) ca. 0.5 g. of sample was heated at 150° C. with 5 ml. of 5% KOH in ethylene glycol for 2 hrs.

2) The mixture was then diluted to ca. 50ml. and acidified to congo red by the dropwise addition of 3 N $H_2SO_4$.

3) The acidified system was extracted with 3 successive volumes of diethyl ether (50, 25 and 25 ml.).

4) The ether extracts were combined and back-scrubbed with 2-25 ml. portions of water. The washed ether extracts were then dried by allowing them to stand over anhydrous sodium sulfate (drierite could not be used because it apparently absorbed some of the products).

5) After drying, the ether was removed by evaporation to yield the products of saponification.

6) This mixture was then taken up in ca. 500 ml. of n-hexane and

passed through a 3-stage Kies'[9] counter-current extraction assembly (Figure 1.). The stages of the Kies' apparatus were filled with 100 ml. of 30% aqueous ethyl alcohol solutions. In addition, tubes 1 and 2 contained 1 meq. of KOH and 0.5 meq. of KOH respectively.

7) The aqueous fractions were combined, acidified to congo red with $H_2SO_4$ and extracted three times with diethyl either (250 ml.; 250 ml.; and 100 ml.).

8) The ether extracts were combined, dried over $Na_2SO_4$, and the solvent was removed to obtain the free acids.

9) The n-hexane fraction that had passed through the Kies' stages was dried over $Na_2SO_4$ and the solvent removed to yield the non-saponifiables.

D. Preparation of methyl esters:

Various procedures for the qualitative identification of long chain acids were investigated and tried. Of the numerous methods reported in the literature, gas chromatography of the corresponding methyl esters proved to be the best. The methyl esters, both standards, and unknowns were prepared by the following scheme.

1) The sample size taken for esterification usually was of the order of 0.02 g., and this sample was refluxed with about 5 ml. of 10% (by wt.) $H_2SO_4$ in methyl alcohol for about 2 hours. The reflux proceeded nicely when the bath temperature was held at about 80° C.

2) The solution was then diluted to about 30 ml. with distilled water, and extracted three times with diethyl ether (25, 25 and 10 ml. portions). The combined ether extracts were back-scrubbed with 20, 20, and 10 ml.

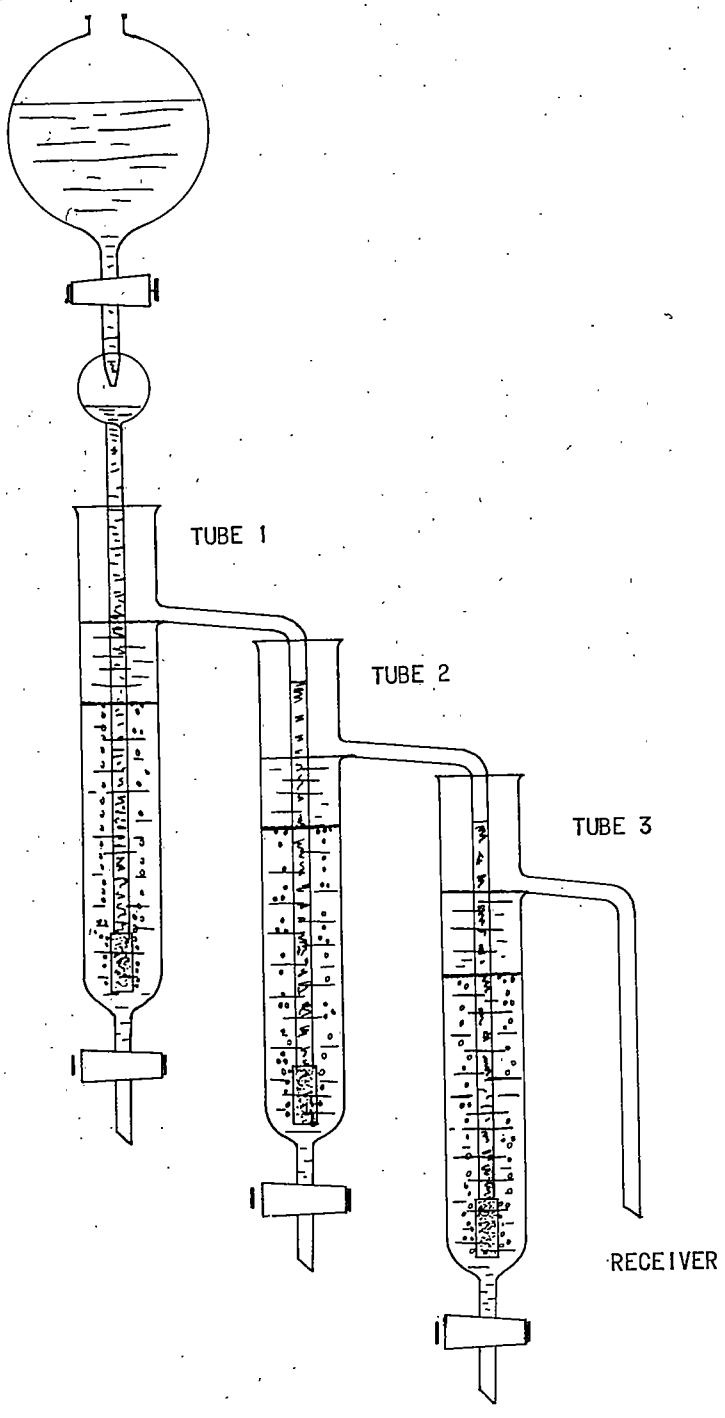



FIG. 1 - KIES APPARATUS

portions of water to remove any residual acids (including the sulfuric).

3) The washed ether extract was dried over $Na_2SO_4$ and the solvent removed by evaporation to yield the methyl esters.

E. Bromination of Esters:

The presence of unsaturated methyl esters can be shown by gas chromatography. Verification is made by brominating a system presumed to contain unsaturated esters, passing through the column and comparing the resulting chromatogram with that of the same system prior to bromination. The disappearance of a peak indicates an ester susceptible to bromination (i.e. unsaturated esters). Bromination was presumed to occur only on unsaturated systems and was carried out as follows:

1) The esters were dissolved in dry diethyl either to give approximately a 10% solution. This was cooled to the temperature of an ice-salt bath and bromine was added by means of a capillary until the color of bromine persisted.

2) The mixture was allowed to stand in the dark for about ten minutes and the bromine color was then discharged by the capillary addition of β-amylene.

3) The solvent was removed by evaporation and the reaction products were taken up in a minimum of benzene. This solution was suitable for gas chromatography.

F. Column Chromatography:

In the final analyses of the waxes, column chromatography using an alumina column showed the greatest promise for separations of the various

types of compounds in the wax. Numerous schemes were tried, but only silicic acid columns and alumina columns seemed advantageous. The alumina column seemed to give the best results and was, therefore, used for the separations reported here. This system provided for elution of the hydrocarbon components completely free of all other materials and allowed for some resolution of the individual esters in the wax. Other materials were eluted, though not identified, and acidic materials were retained by the column and could not be recovered.

Several column sizes, types of alumina and elution solvent systems were tested. The following system seemed the most satisfactory.

1) Column---- 10mm. or 16 mm. diameter x 30 cm. in length, fitted with a teflon stopcock.

2) Alumina--- adsorption alumina (Fisher Scientific Co. A-540); about 3 g. alumina were used for each 0.1 g. of material introduced on the column.

3) Eluants--- approximately 50 ml. of each of the following solvents were passed through the column in order: petroleum ether, 1:1 petroleum ether - benzene, benzene, 1:1 benzene - diethyl ether, diethyl ether, 1:1 diethyl ether - acetone, acetone, 1:1 acetone - methanol, and methanol.

4) Fractions- fractions were cut at ten minute intervals with an automatic fraction collector, and averaged about 4 ml. per fraction.

G. Gas Chromatography:

This technique progressed through many developmental stages during the course of this work, and therefore different instrumentation was used. The developments were of two types; instrument improvement itself, and screening of column substrates for efficiency in resolving specific mixtures. It gradually became apparent that high temperature gas chromatography (i.e., of the order of 300° C.) would be necessary for effective resolution of the hydrocarbons present in the wax. The column substrate that proved most useful at this temperature was a silicone grease (Dow-corning Hi-Vac Stopcock Grease) placed on Chromosorb-30/60 mesh (Johns-Manville Co.). "Apiezon" columns operating to about 250° C. also proved useful for hydrocarbon analysis. The methyl esters were run with silicone, reoplex, Lac-466 (Wilkin's Instrument Co.) and butanediol-succinate polyester columns. The butanediol-succinate polyester column seemed to be the most effective.

The most useful instrumentation was eventually afforded by an Aerograph Chromatograph (Wilken's Instrument Co.) using a hot-wire thermal conductivity cell, with reference and detector in a bridge circuit. However, at temperatures above 300° C., an instrument utilizing a single thermistor as detector and a specially designed proportional controller for temperature control was preferable. This instrument was designed by Dr. James Cason at the University of California.

H. Infrared Spectrophotometry:

All of the infrared data were taken in the rock salt region (5000 $cm^{-1}$

to 680 $cm^{-1}$). Spectra were obtained from materials in various physical states; liquids (as capillary films), solids (KBr disks and Nujol mulls), and in solution with various solvents. A Baird Associates instrument was used for the majority of the early studies. A Beckman IR-4 was used in the later phases of the work. The infrared interpretation has been made on the basis of personal observation and/or numerous articles discussing band assignments. The most inclusive of these articles is a review paper by O'Connor on "Application of Infrared Spectrophotometry to Fatty Acid Derivatives"[11].

# IV RESULTS

The "waxes X", "Y", and "Z", as extracted by the procedures outlined in the previous section, are essentially identical. Differences do exist, as evidenced by differences in color, but such differences may, or may not be due to the variations in the lipids of the respective waxes. This consideration is based on the absence of consistant color difference when the wax was extracted from excised cuticles by similar techniques. Moreover, no distinction, other than color was established by the investigation.

Therefore, although the terms "wax X", "wax Y", and "wax Z" will be used in describing the results, this does not imply any degree of difference. That is, data from one wax have been assumed to be applicable to another. The use of X, Y, and Z is retained only to firmly establish the basis on which the work was conducted. Where the term "wax" is used, a generality is implied, based on conclusions drawn from data obtained from one or more of the individual "waxes" "X", "Y", or "Z".

## A. General Character of Wax:

The extractable cuticular wax accounted for about 4.3% of air-dried weight of the excised abdominal sclerites. This figure agrees reasonably well with data obtained by Pepper and Hastings in 1947[12]. They estimated that the wax of cast exoskeletons amounted to about 5%. The moisture content variation in the two types of sample, although not measured, could account for the difference in measured wax percentages.

The wax is a light-yellow solid at room temperature (ca. 23° C.).

At this temperature it is soft, typically "waxy" and forms films. It has no sharp melting point, but gradually softens and becomes distinctly a liquid at temperatures above 50° C.

The infrared absorption pattern of wax obtained from excised cuticle is shown in Figure 2. Comparison of this spectrum with that of "Wax Y" (Figure 3) supports the contention that the two waxes are essentially identical. The broad band at 1550 $cm^{-1}$ in the spectrum of "Wax Y" is anomolous. It's appearance in the spectrum of "Wax Y" as well as "Waxes X" and "Z" is variable and inconsistent. It has been assumed, therefore, that the presence or absence of this band is dependent on the physical state of the sample. Thus the absence of this band in the spectrum of the excised cuticular wax does not imply a significant difference in the two waxes. Since all infrared data for the various waxes (including "X" and "Z" as well as "Y" and the excised cuticular wax) have indicated their essential identity it has been assumed that any information obtained from one is applicable to all. The strong band at 1713 $cm^{-1}$ is characteristic of the carbonyl stretch in fatty acids. The presence of an acid constituent in the wax is further indicated by the general appearance of the spectra, and particularly by the broad hydroxyl band extending across the entire carbon-hydrogen stretch region at 2851 $cm^{-1}$ and 2924 $cm^{-1}$. Esters are suggested by the band at 1740 $cm^{-1}$ (carbonyl stretch), and again by the smaller bands in the 1200 - 1100 $cm^{-1}$ region. Unsaturation is probable, as indicated by the shoulder at 3025 $cm^{-1}$. The band at 720 $cm^{-1}$ and

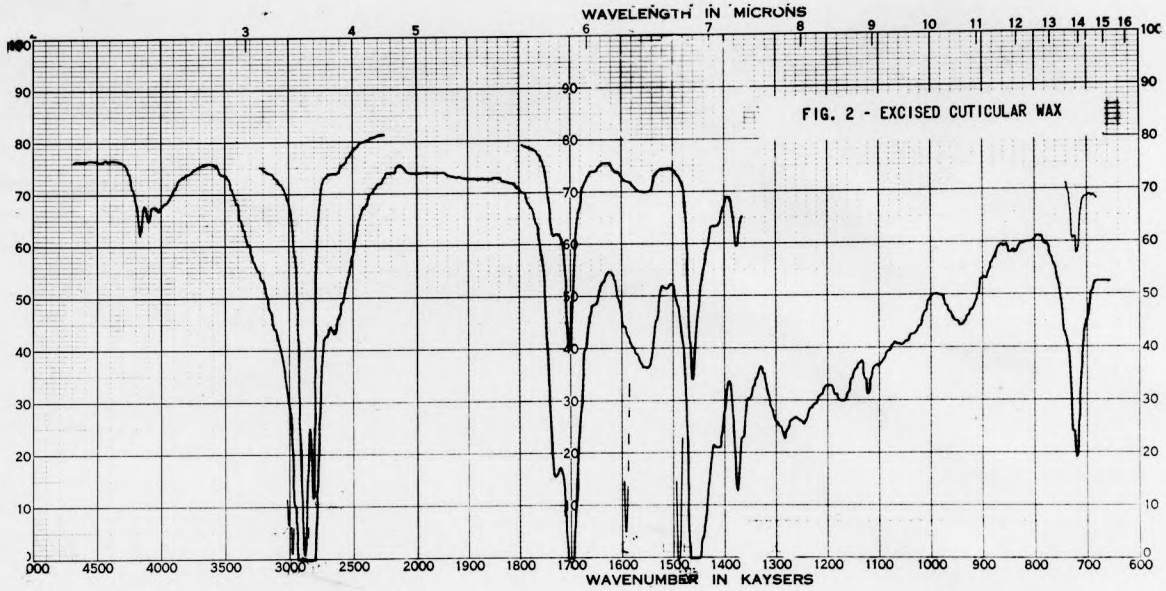



FIG. 2 - EXCISED CUTICULAR WAX

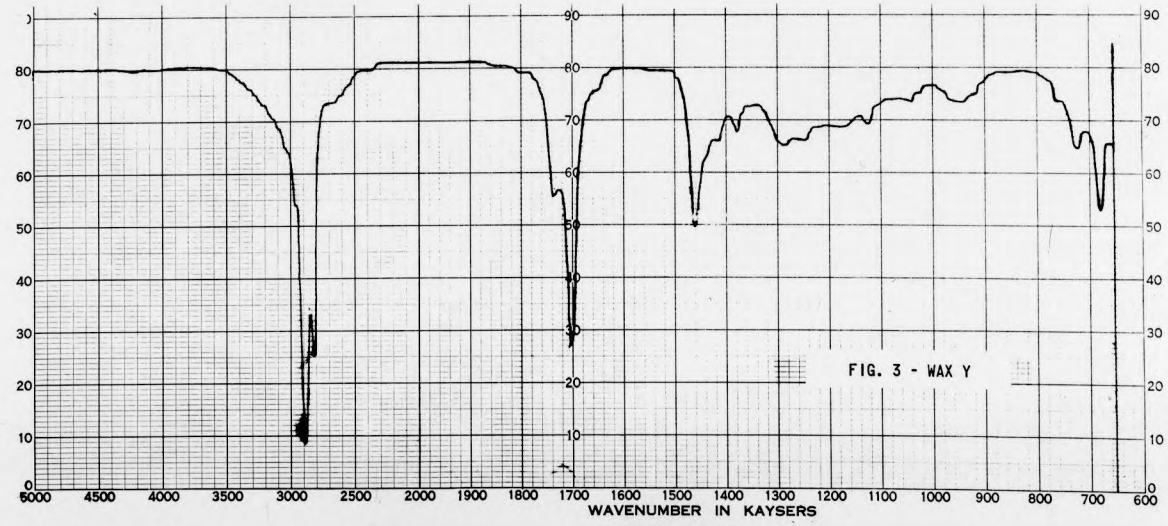



FIG. 3 - WAX Y

shoulder at 730 $cm^{-1}$ are characteristic of long-chain compounds and have been attributed to a rocking mode of multiple $-(CH_2)-$ groups [13,14]. These salient features of the spectrum of the wax will be reconsidered in the light of individual constituents separated from this mixture.

B. Constituents of Wax:

The preceeding spectra suggested the presence of appreciable quantities of free acids in the wax of the excised cuticle. This evidence was corroberated by passing 500 ml. of a skellysolve-B solution of 0.2114 g. of wax through a Kies' counter-current extraction apparatus. This resulted in the recovery of 0.0337 g. of acids which represented approximately 18% of the total material recovered from the separation (0.1778 g.). That the materials recovered were acids was established by means of the infrared spectrum of a capillary film (Figure 4). This spectrum has all of the band configurations associated with fatty acids and more important is free of extraneous absorption bands indicating a reasonably pure product. The band at 3020 $cm^{-1}$ is clearly resolved in this spectrum and firmly establishes unsaturation.

These acids were recovered from the salt plates used for infrared spectroscopy and were converted to the corresponding methyl esters. The conversion to methyl esters was reasonably successful although the recovery (0.0145 g.) was poor. The product was essentially the methyl ester as shown by the infrared spectrum of a capillary film (Figure 5). The carbonyl at 1740 $cm^{-1}$ and bands in the 1200 $cm^{-1}$ region clearly indicate the ester. Moreover, the absence of carboxylic-carbonyl stretch,

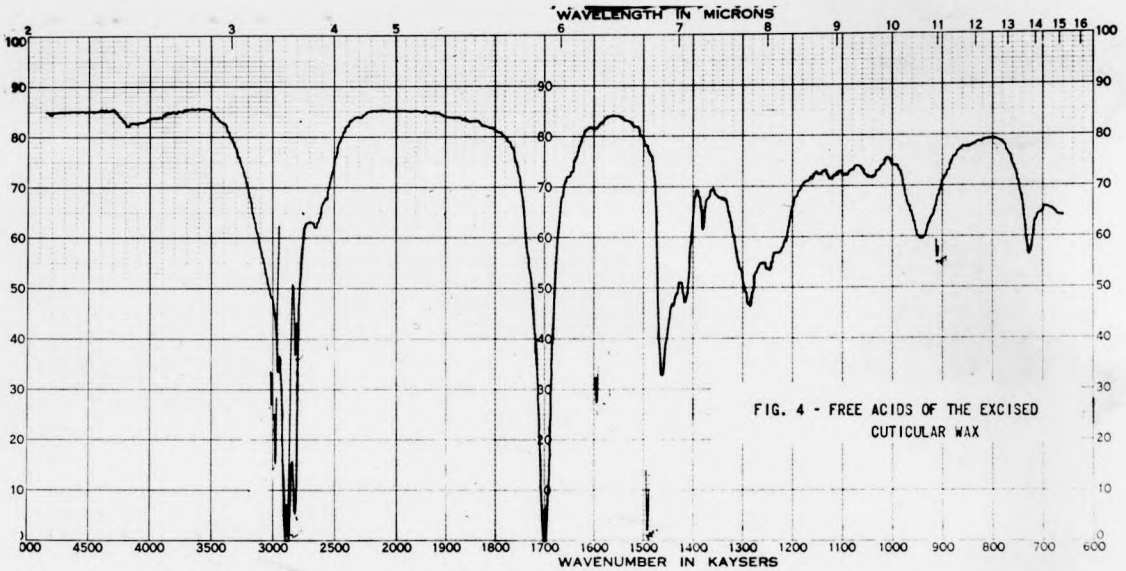



FIG. 4 - FREE ACIDS OF THE EXCISED CUTICULAR WAX

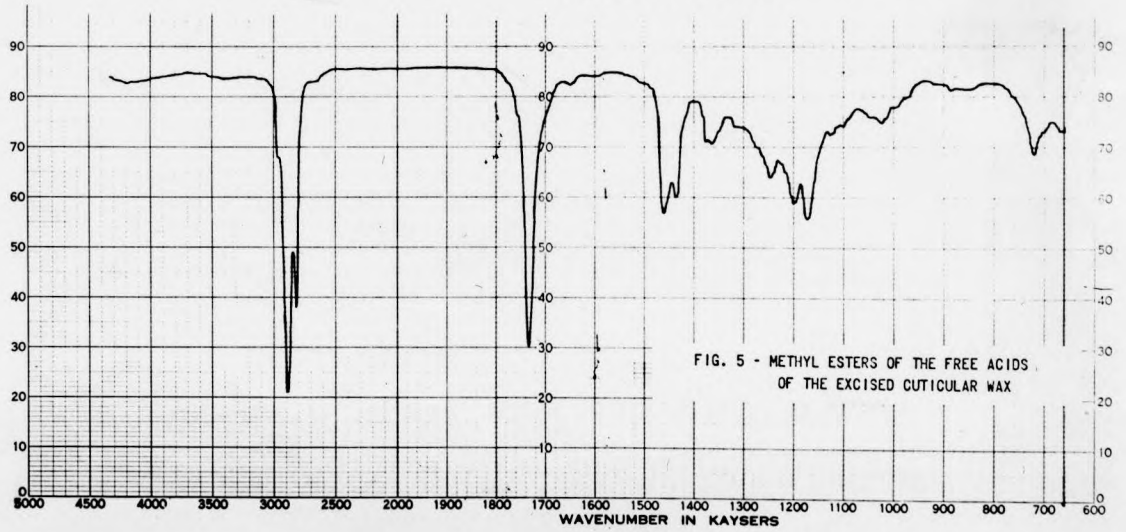



FIG. 5 - METHYL ESTERS OF THE FREE ACIDS OF THE EXCISED CUTICULAR WAX

and the carboxylic-hydroxyl band assures a reasonably pure product. Unsaturation is again predictable by the presence of the 3020 $cm^{-1}$ shoulder.

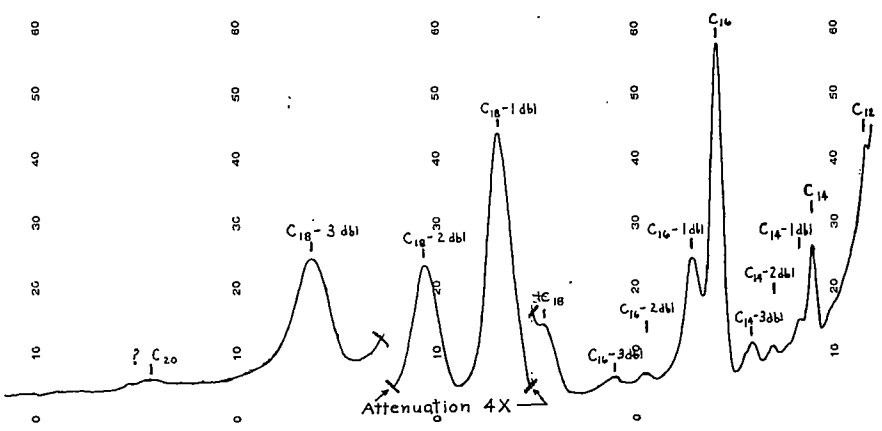
The methyl esters were recovered from the salt plates and dissolved in benzene to yield a solution that approximated 0.14 g. of ester in 0.25 ml. of benzene. This system was separated by means of a gas chromatograph operating at 210° C. The column substrate was a polyester of butanediol and succinic acid ("Craig" column - Wilken's Instrument Co.) The results of this separation are portrayed in Figure 6. The peak assignments were based on the data obtained by chromatographing known esters under identical conditions and plotting log retention time vs. the number of carbons. This is illustrated by Figure 7 and adequately shows the straight line function of members of an homologous series. This plot was used to establish the nature and approximate percentage of individual esters in the injected sample. The percentage composition was based on the relative areas under the respective peaks of the chromatogram. An emperical formula to account for deviations from linearity wherein molecules exhibiting large differences in molecular weight are concerned was published by Eastman[4] in 1957. This relationship

$$\left(\frac{M_i}{W}\right) = \frac{A_i \sqrt{M_i}}{\sum_i A_i \sqrt{M_i}}$$

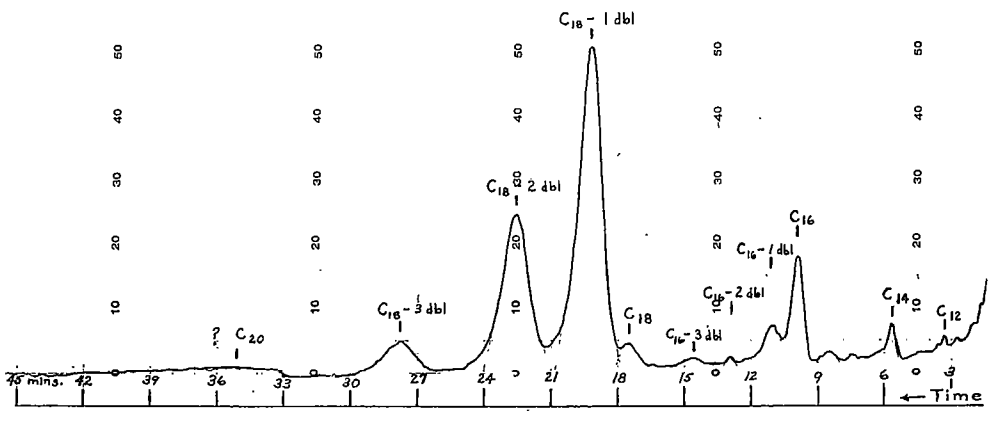
was used to estimate the relative amounts of the methyl esters present. In turn, it was assumed that the conversion efficiency for all acids to esters was constant and therefore this relationship could be

FIG. 6 - METHYL ESTERS OF THE FREE ACIDS OF THE EXCISED CUTICULAR WAX

5' POLYESTER COLUMN
210°C
40 λ INJECTION




SAME - 10 λ INJECTION



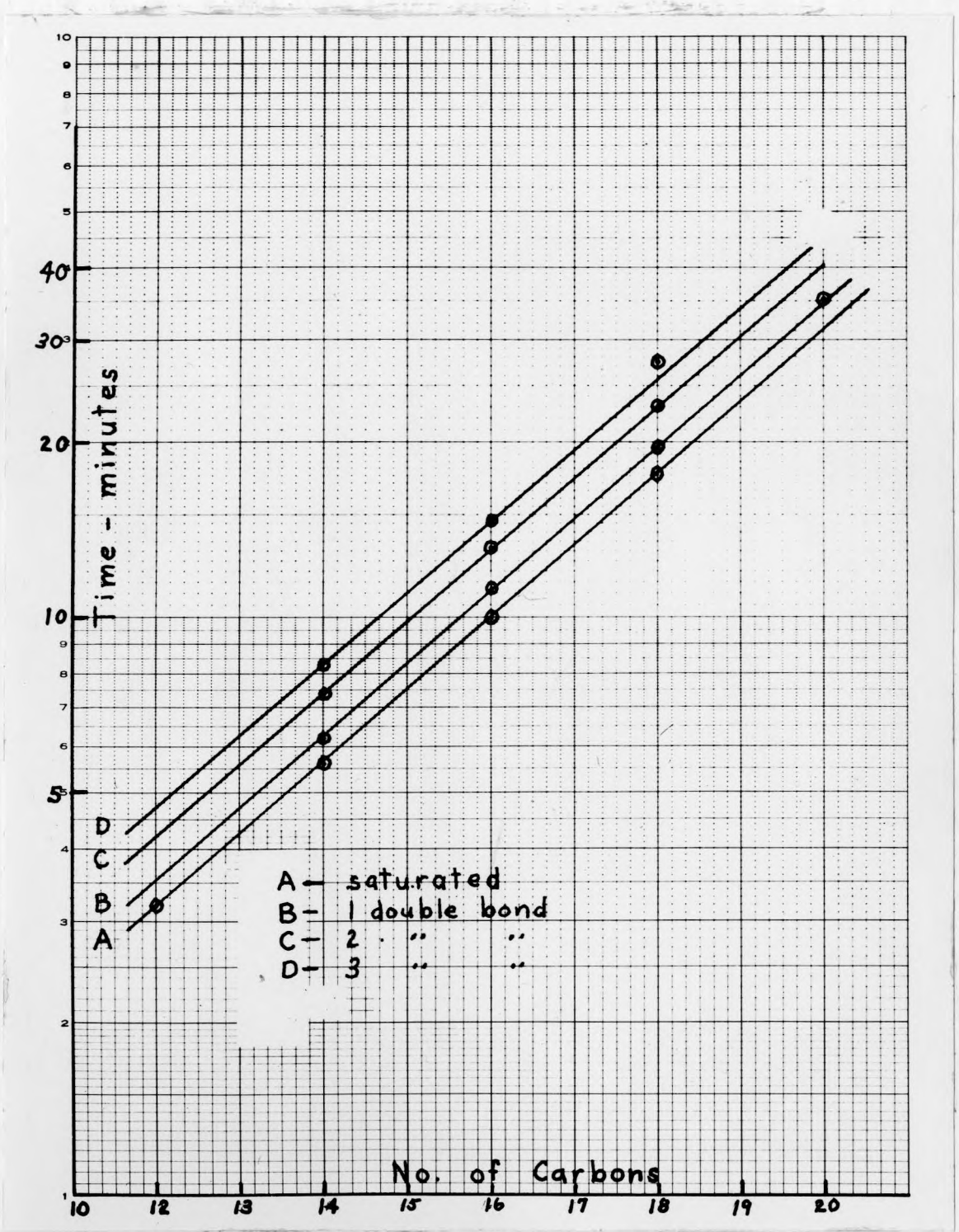



Fig. 7 - Retention Time of Methyl Esters of Free Acids of the Excised Cuticular Wax

used as an expression of the precentages of free acids. The types and relative amounts of free acids to be found in cricket wax are tabulated in Table I.

TABLE I

FREE ACIDS IN CRICKET WAX

| TYPE | % OF TOTAL FREE ACIDS | % OF WAX |
|---|---|---|
| $C_{12}$ | 0.4 | 0.1 |
| $C_{14}$ | 1.7 | 0.3 |
| $C_{14}$ - 1 double bond | 0.5 | 0.1 |
| $C_{14}$ - 2 double bonds | 0.3 | 0.1 |
| $C_{14}$ - 3 double bonds | 0.6 | 0.1 |
| $C_{16}$ | 7.9 | 1.4 |
| $C_{16}$ - 1 double bond | 4.0 | 0.7 |
| $C_{16}$ - 2 double bonds | 0.3 | 0.5 |
| $C_{16}$ - 3 double bonds | 0.7 | 0.1 |
| $C_{18}$ | 2.4 | 0.4 |
| $C_{18}$ - 1 double bond | 48.4 | 8.7 |
| $C_{18}$ - 2 double bonds | 26.8 | 4.8 |
| $C_{18}$ - 3 double bonds | 6.0 | 1.1 |
| $C_{20}$ | Trace | |

The presence of unsaturation in the acids as reported in Table I was verified by bromination of some of the methyl ester mixture used for the chromatogram of Figure 6. The bromination was carried out in a manner that would bring about addition and minimize substution.

The brominated mixture was then passed through the same column under identical conditions. In every case where unsaturation is reported, the peak for that particular ester was either markedly diminished or absent.

The "neutral" materials that passed through the Kies' system with the skellysolve-B amounted to 0.1441 g. or about 82% of the total material recovered. The infrared spectrum of this material is shown in Figure 8. The marked decrease in acid content is clearly apparent by comparison of Figure 8 with Figure 2. However, the acid band at 1710 $cm^{-1}$ is present and persisted at approximately the same intensity even after the material had been recycled through the Kies' set-up. This must be interpreted as indicating carboxyl groups which are involved in molecular configurations more complex than that of the free acids and whose solubility preference remains with the skellysolve-B. The ester band at 1736 $cm^{-1}$ is still evident as expected. The carbon-hydrogen stretch and deformation bands are extremely strong as compared to the carbonyl intensities. This implies either few carbonyl structures in huge molecules or, as the evidence accumulated, more likely a mixture containing considerable quantities of free hydrocarbons.

At this point it was decided to sacrifice the acidic component of this mixture for the possibility of further elucidating the hydrocarbon and ester portions. Previous studies had indicated that hydrocarbons could be freed from such a mixture by passing through an alumina

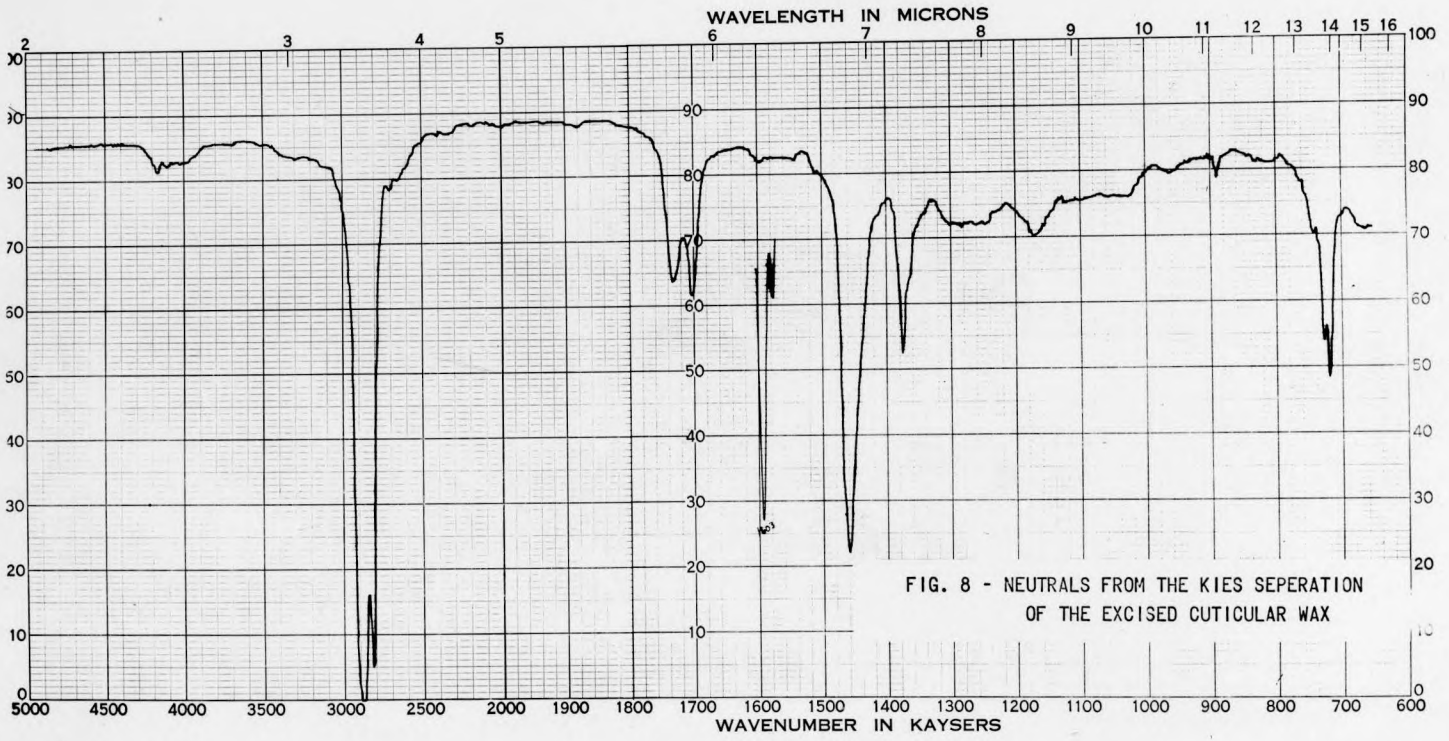



FIG. 8 - NEUTRALS FROM THE KIES SEPERATION OF THE EXCISED CUTICULAR WAX

column. This was done by eluting the mixture from the column with increasingly polar solvents (described in the preceeding section). The acidic materials were lost because they would not elute from the column. The results of this attempt at chromatographic separation are illustrated in Figure 9. The 28 fractions were recombined into the 5 fractions indicated for further consideration.

Fraction number 1, which accounts for 57.6% of total recovery (47.2% based on total wax), gave the infrared spectrum shown in Figure 10. This spectrum indicates paraffinic hydrocarbons with very long chains (note bands at both 730 $cm^{-1}$ and 720 $cm^{-1}$). A carbon-hydrogen analysis on material obtained in this same fashion gave carbon 85.00%, hydrogen 14.96%

Fraction number 2 is probably a mixture of esters plus hydrocarbons. This is based on its infrared spectrum (Figure 11) in which the bands characteristic of esters are present. They appear weaker than is usual for most esters. It is assumed, therefore, that rather large amounts of hydrocarbons are also present in this cut. The shoulder in the 3000 $cm^{-1}$ region of this spectrum suggests unsaturation. It should be noted that this band did not appear in the spectrum of the hydrocarbon cut. It is likely then that the unsaturation is present in the ester moiety of this number 2 fraction.

Fraction number 3 may include esters and alcohols, esters involving hydroxy acids, or various combinations of all three. Evidence for this stems from the infrared spectrum of a capillary film of this

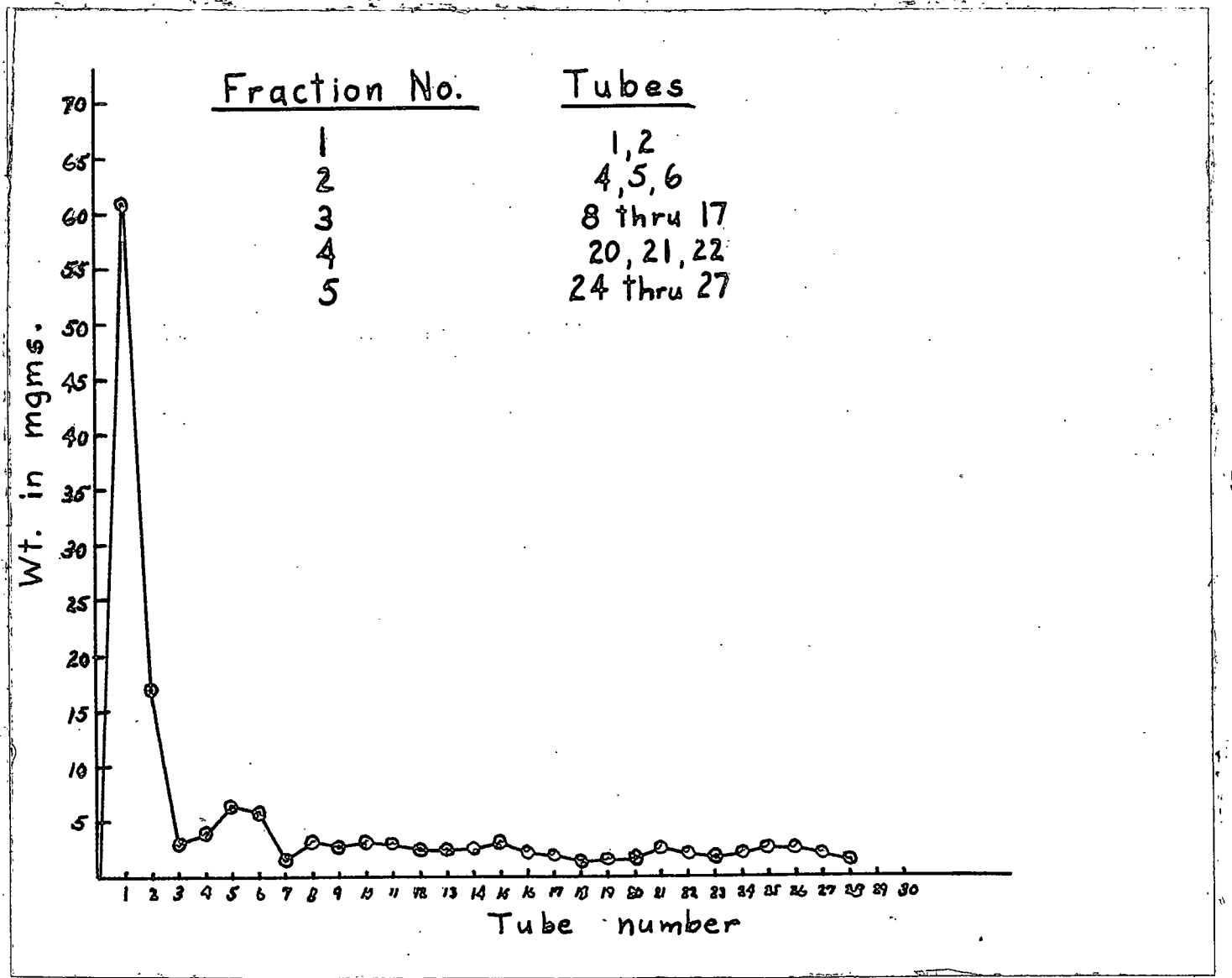



Fig. 9 - Alumina Chromatography of Neutrals of Excised Cuticular Wax

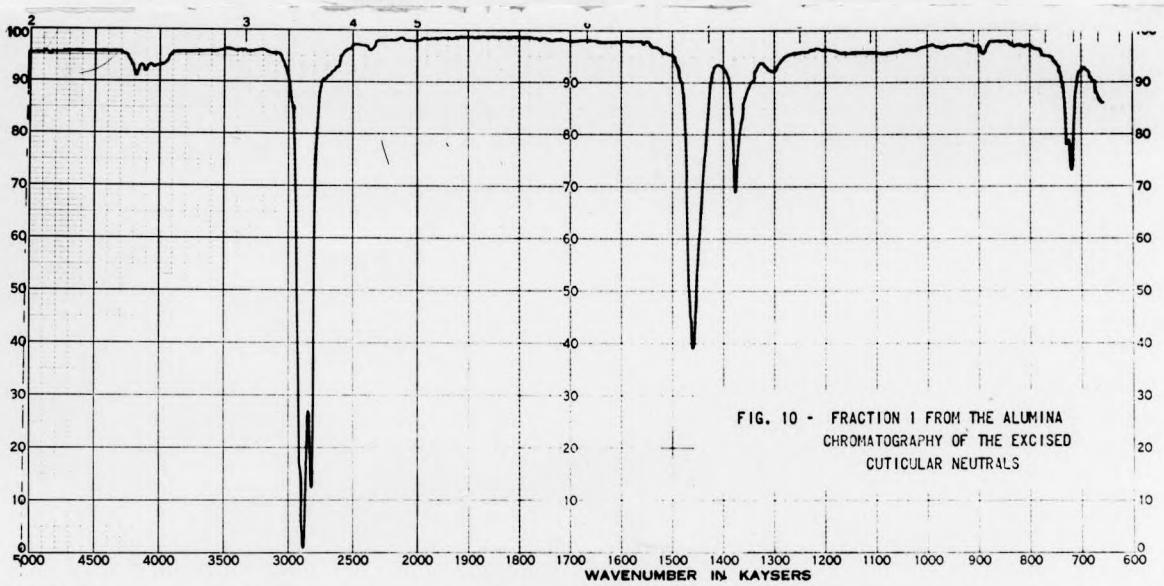



FIG. 10 - FRACTION 1 FROM THE ALUMINA CHROMATOGRAPHY OF THE EXCISED CUTICULAR NEUTRALS

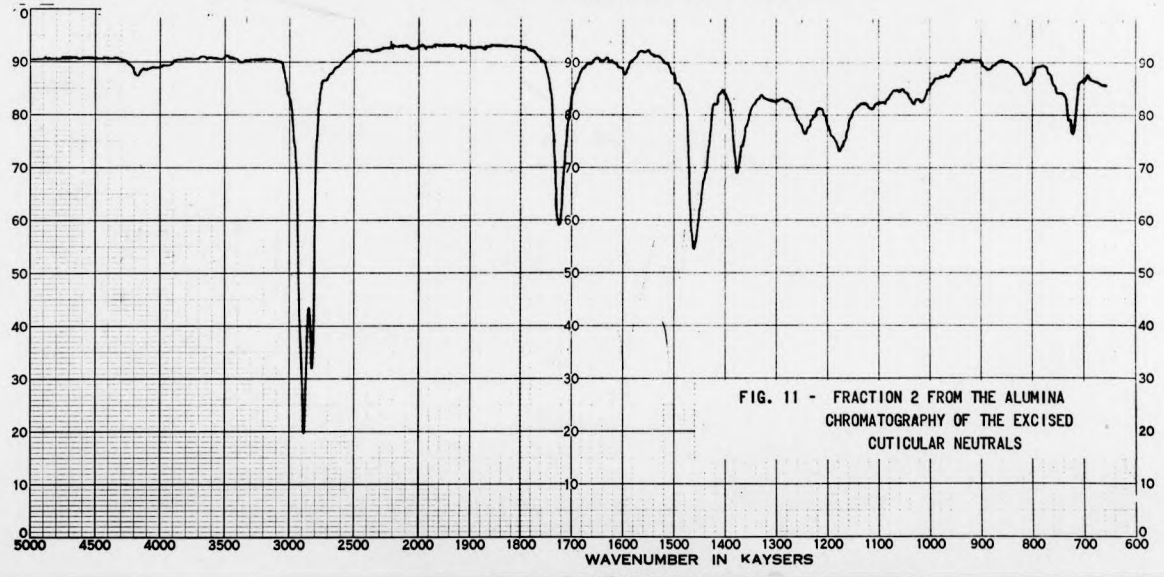



FIG. 11 - FRACTION 2 FROM THE ALUMINA CHROMATOGRAPHY OF THE EXCISED CUTICULAR NEUTRALS

material (Figure 12) in which the ester bands have become quite strong and a strong hydroxyl band is also apparent at 3400 $cm^{-1}$.

Fraction number 4 and number 5 give essentially the same spectra as number 3 so that no additional information can be inferred.

This particular series of tests utilized virtually all of the material obtained from the excised cuticles. However, since all of the evidence gathered for the wax from the excised cuticle had duplicated similar evidence for waxes "X", "Y" and "Z", it was decided to repeat the alumina chromatography studies with the remainder of wax "Y". The chromatographic separation of 1.4143 g. of this wax, which included all material, not just the neutrals as in the previous case, is illustrated by the plot of weight vs. tube number (Figure 13). The 55 cuts were recombined into the 10 fractions illustrated.

Fraction number 1 was a white solid. The material melted gradually at about 30° C. Its infrared spectrum (Figure 14) clearly established it as a hydrocarbon and is virtually identical to the spectrum for a similar cut from the wax of the excised cuticle (Figure 10). The failure of the band at 720 $cm^{-1}$ to exhibit two band heads should have been suggestion enough that this material might still contain some of the petroleum ether that was used as an eluant. However, at the time it was not construed in this way and it was not until later that it became obvious that these lower hydrocarbons could be retained in the form of solid solutions. Although this did not lead

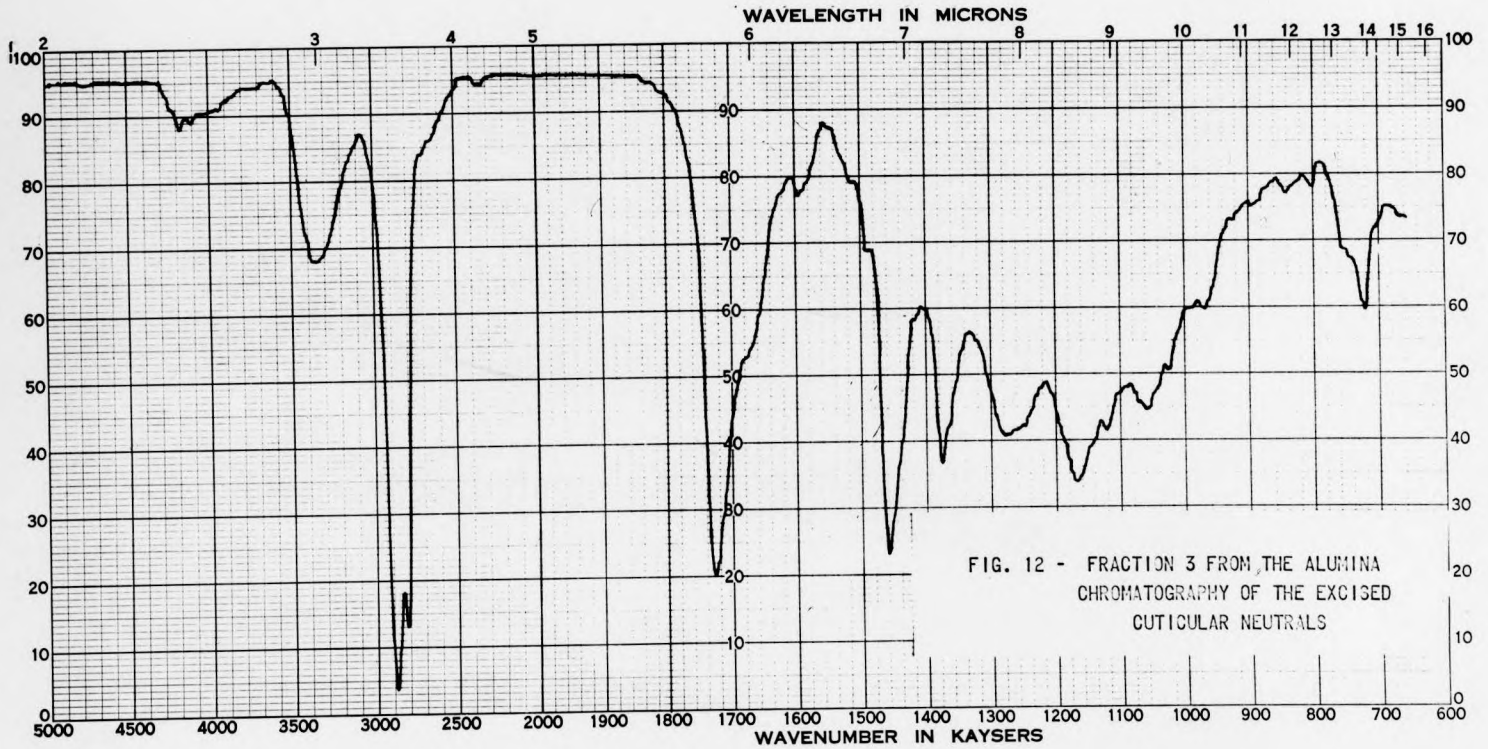



FIG. 12 - FRACTION 3 FROM THE ALUMINA CHROMATOGRAPHY OF THE EXCISED CUTICULAR NEUTRALS

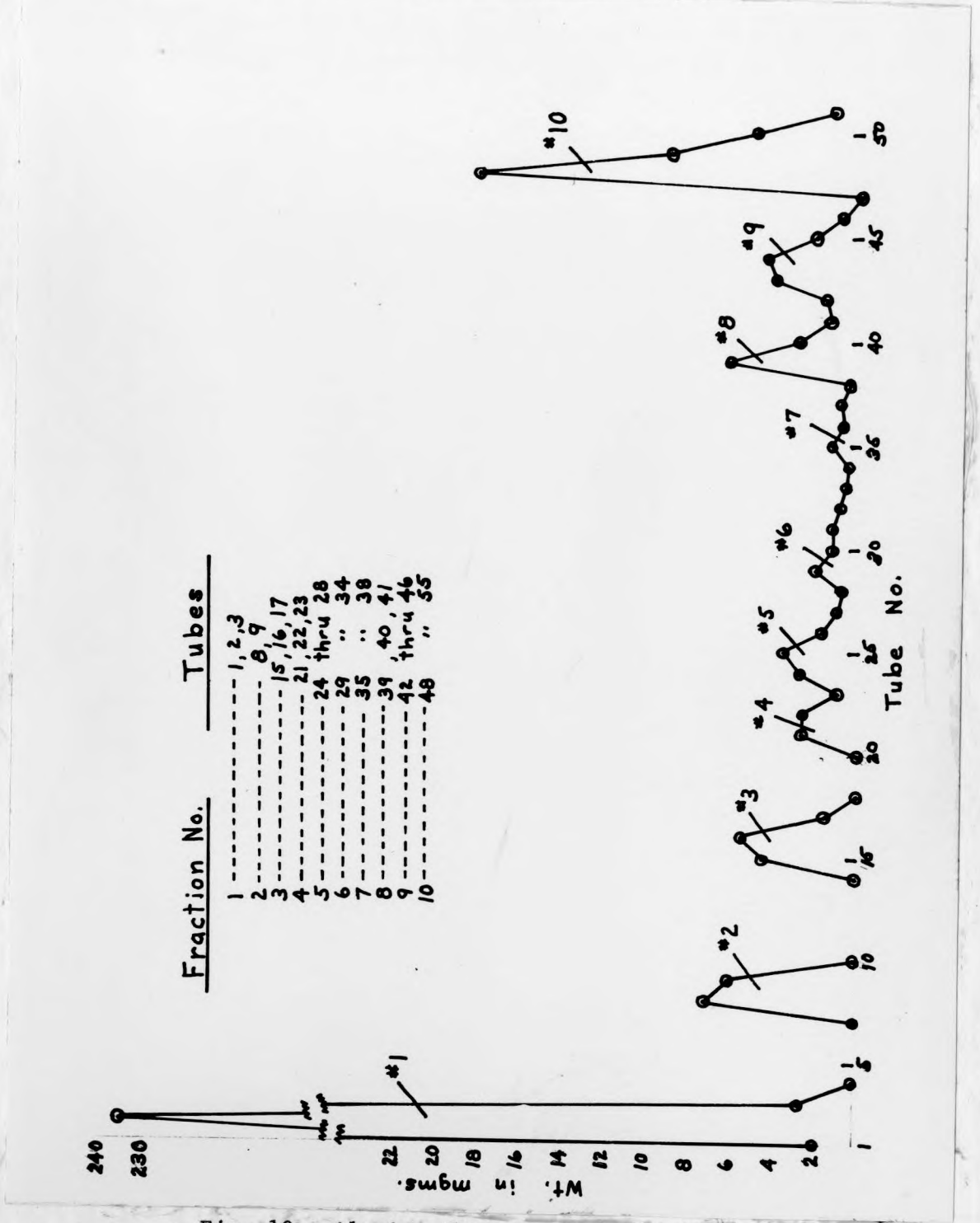



Fig. 13 - Alumina Chromatography of "Wax Y"

to erroneous conclusions as to the type of hydrocarbons present, it undoubtedly affected the weight percentage calculated for hydrocarbons. The hydrocarbon content calculated from these particular separation data was 57.8%.

Previous work on the hydrocarbons obtainable from cricket wax had indicated that a wide range of hydrocarbons was probably present. With this in mind a sample of this material was separated by gas chromatography utilizing a 2½ foot, ¼ inch silicone column operated at 255° C. The chromatogram (Figure 15) fails to give complete resolution although it is satisfactory for an estimation of molecular size and relative amounts, The log plot of retention time vs. number of carbons places the major peaks in the $C_{27}$ to $C_{32}$ region (Figure 16). The relative amounts were calculated on the basis of molecular weight and area under the curve using the same emperical relationship as was used for the methyl esters. The results are tabulated in Table II.

TABLE II

HYDROCARBONS IN CRICKET WAX

| Molecular Size | % of Total Hydrocarbons | % of Wax |
|---|---|---|
| $C_{20}$ group ($C_{12}$ to $C_{25}$) | 12.5 | 7.2 |
| $C_{27}$ to $C_{28}$ | 20.6 | 11.9 |
| $C_{30}$ to $C_{31}$ | 55.3 | 31.9 |
| $C_{32}$ | 11.4 | 6.6 |

An effort was made to resolve these same hydrocarbons using a 5 foot "Apiezon" column. This column cannot be operated at as high a

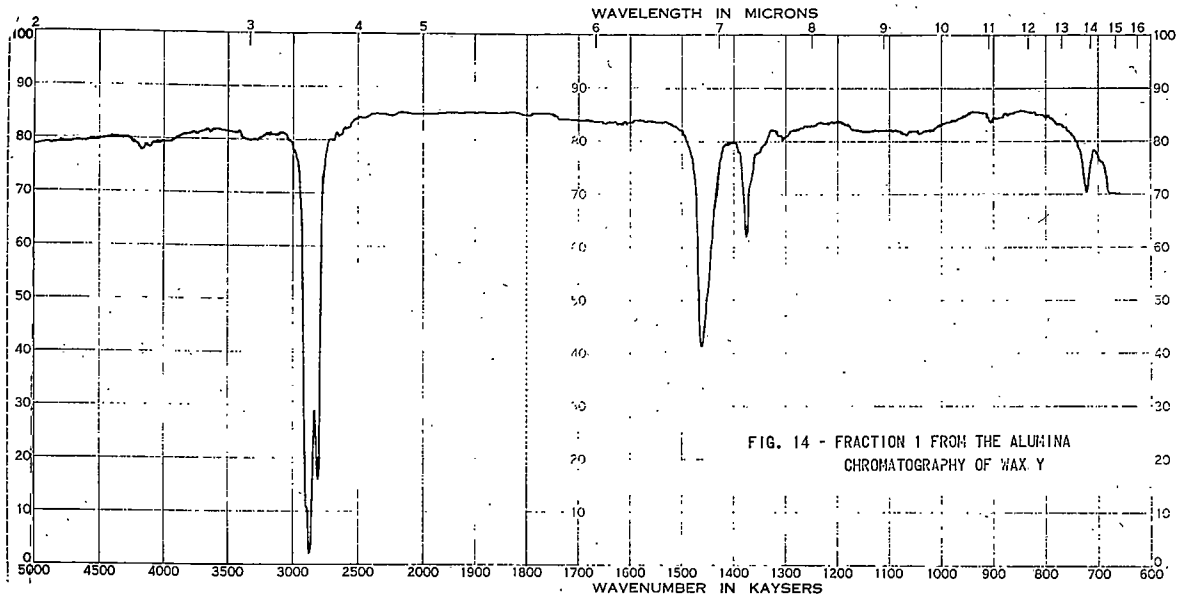



FIG. 14 - FRACTION 1 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

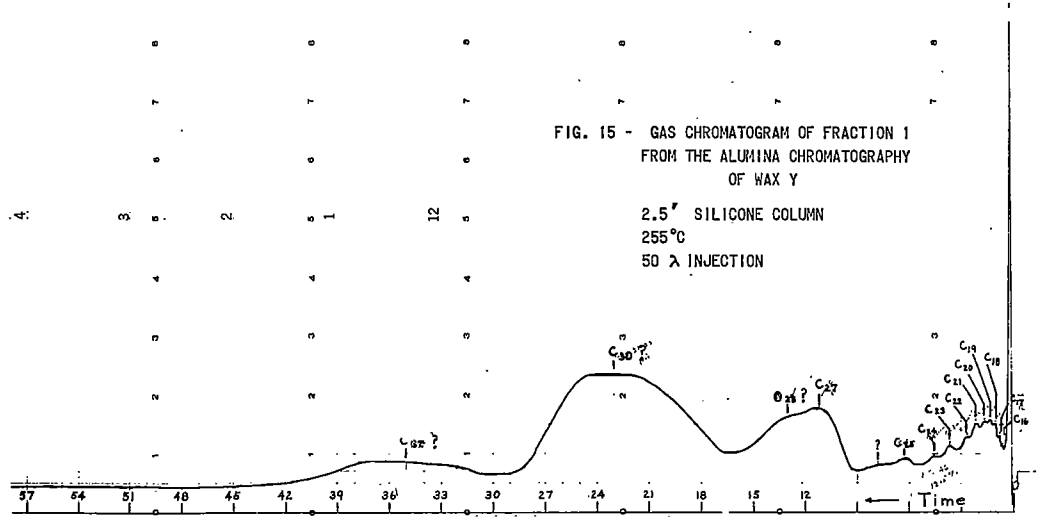



FIG. 15 - GAS CHROMATOGRAM OF FRACTION 1 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

2.5' SILICONE COLUMN
255°C
50 λ INJECTION

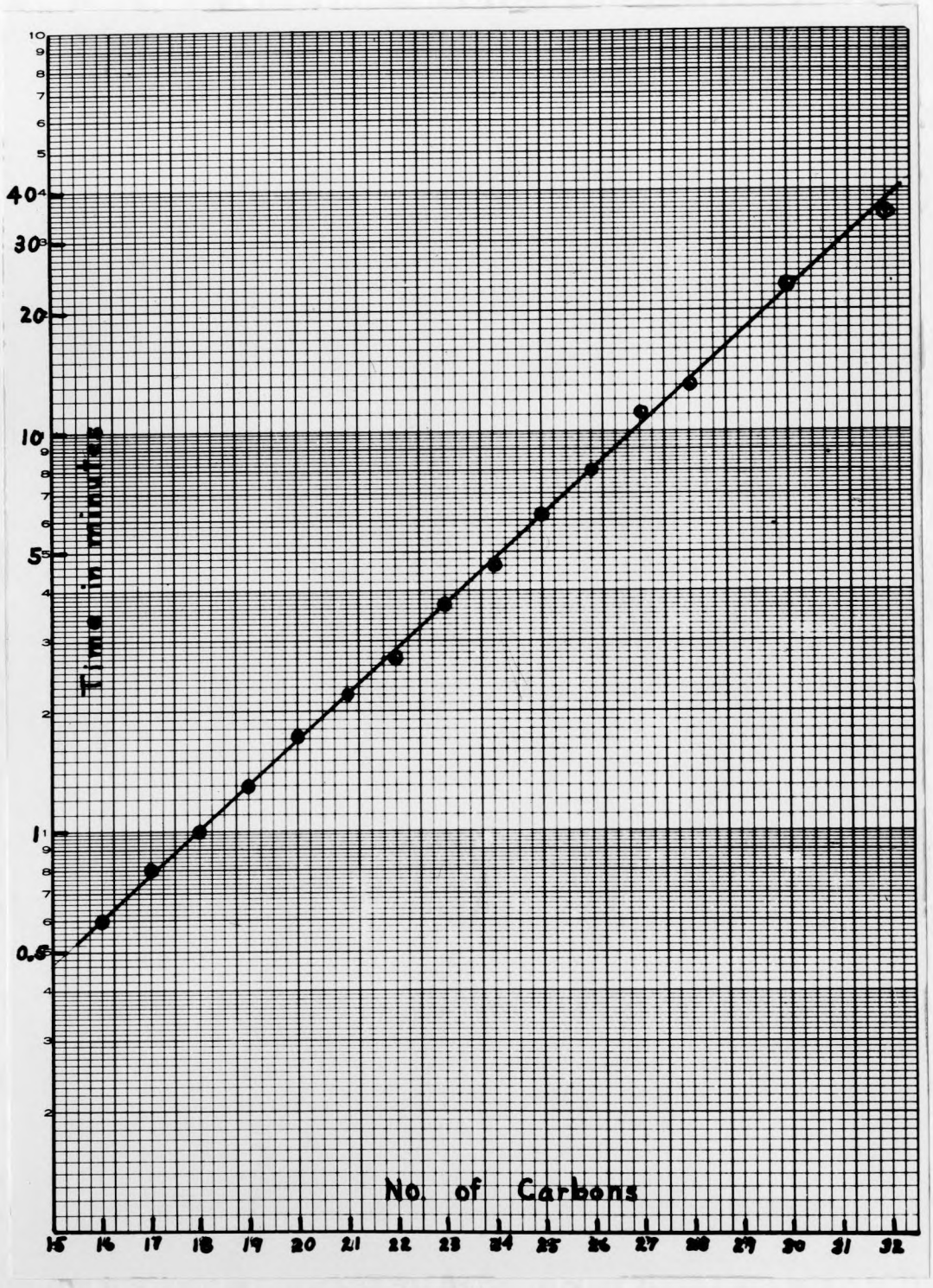



Fig. 16 - Retention Time of Hydrocarbons of Fraction Number 1 on Silicone Column

temperature as is possible with silicone and as a result resolution beyond $C_{27}$ failed. Up to this point, however, 19 different peaks were obtained (Figure 17). These data applied to the log plot (Figure 18) clearly establishes the presence of hydrocarbons with 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and 27 carbons as components of the wax. The instability of the base line above $C_{25}$ (Figure 17) is thought to be due to thermal cracking resulting from the long residence time at this temperature. This could obiliterate a $C_{26}$ peak if $C_{26}$ were a minor constituent. This is mentioned since $C_{26}$ is conspicuously absent and because the chromatogram does suggest the presence of a peak at a time compatible with the log plot for $C_{26}$ elution.

Fraction number 2 from the alumina chromatography of "Wax Y" is a dark-yellow oil that may be esters of long chain acids and alcohols. This is indicated by the infrared spectrum of a capillary film (Figure 19) which shows rather strong carbon-hydrogen bands as compared to the ester carbonyl. This fraction represents 3.2% of the original wax.

Fraction number 3 represents 2.7% of the total and is characterized by its infrared spectrum shown in Figure 20. This spectrum is typical of esters. The single notable feature here is the unsaturation indicated in the 3020 $cm^{-1}$ region. This fraction, too, was a yellow oil.

The yellow, waxy-solid of Fraction number 4 represents about 1.4% of the total. The salient features of its infrared spectrum (Figure 21) are the absence of unsaturated carbon-hydrogen stretch and the

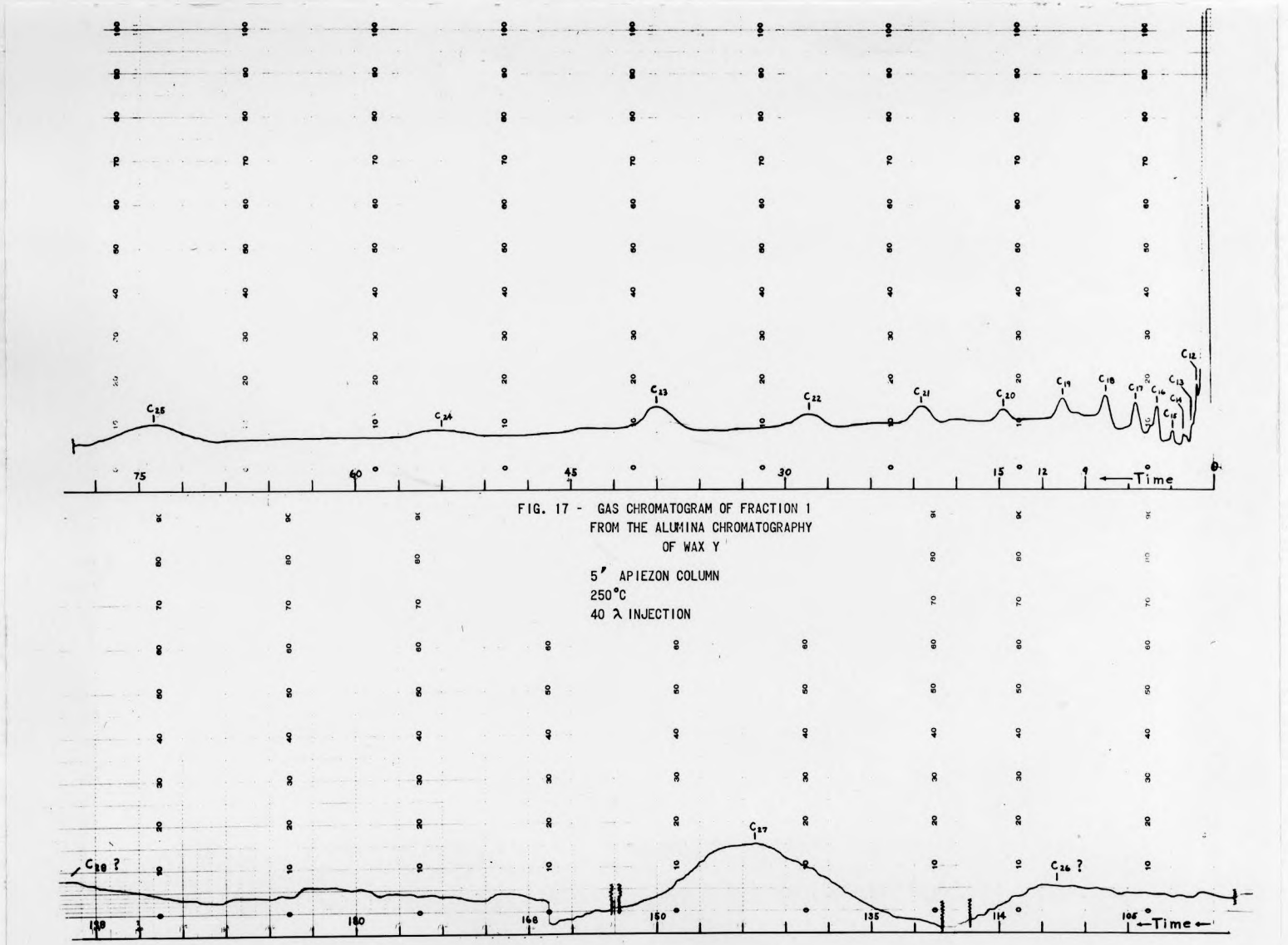



FIG. 17 - GAS CHROMATOGRAM OF FRACTION 1
FROM THE ALUMINA CHROMATOGRAPHY
OF WAX Y

5' APIEZON COLUMN
250°C
40 λ INJECTION

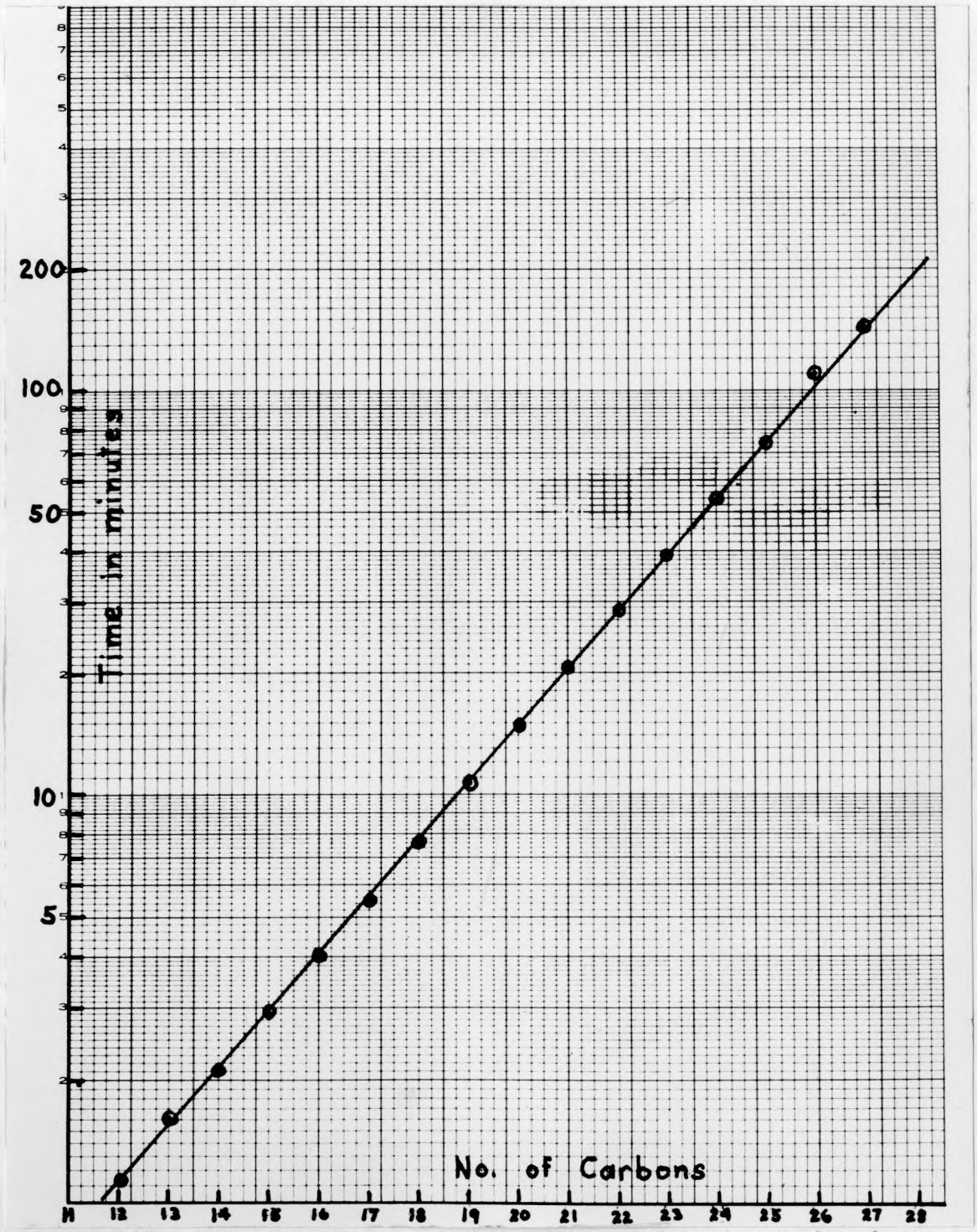



Fig. 18 - Retention Time of Hydrocarbons of Fraction Number 1 on Apiezon Column

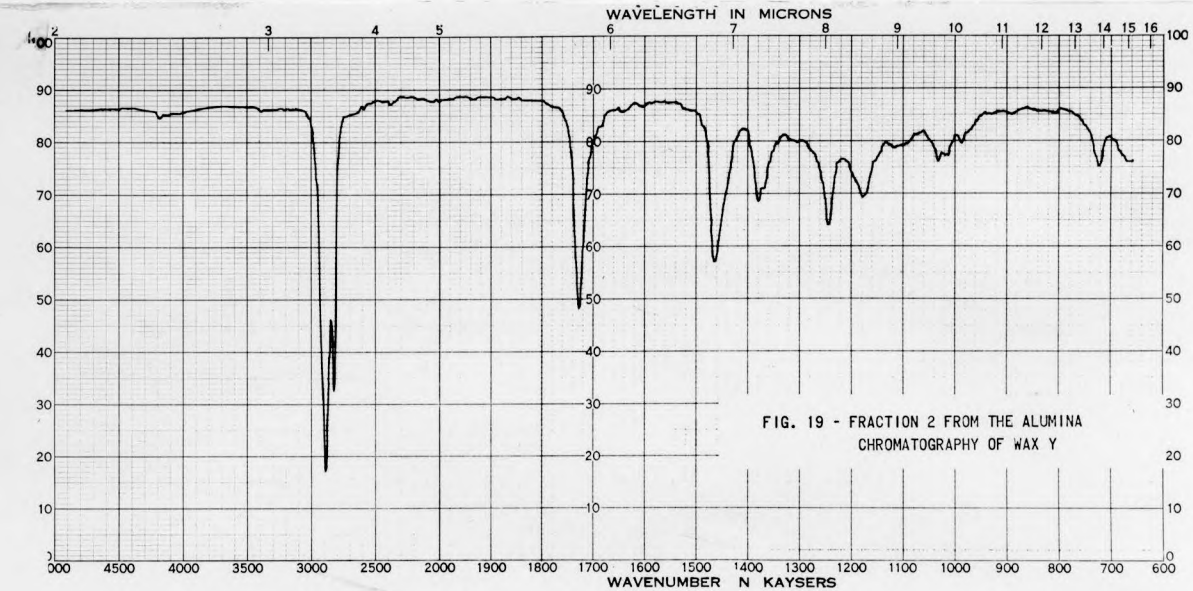



FIG. 19 - FRACTION 2 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

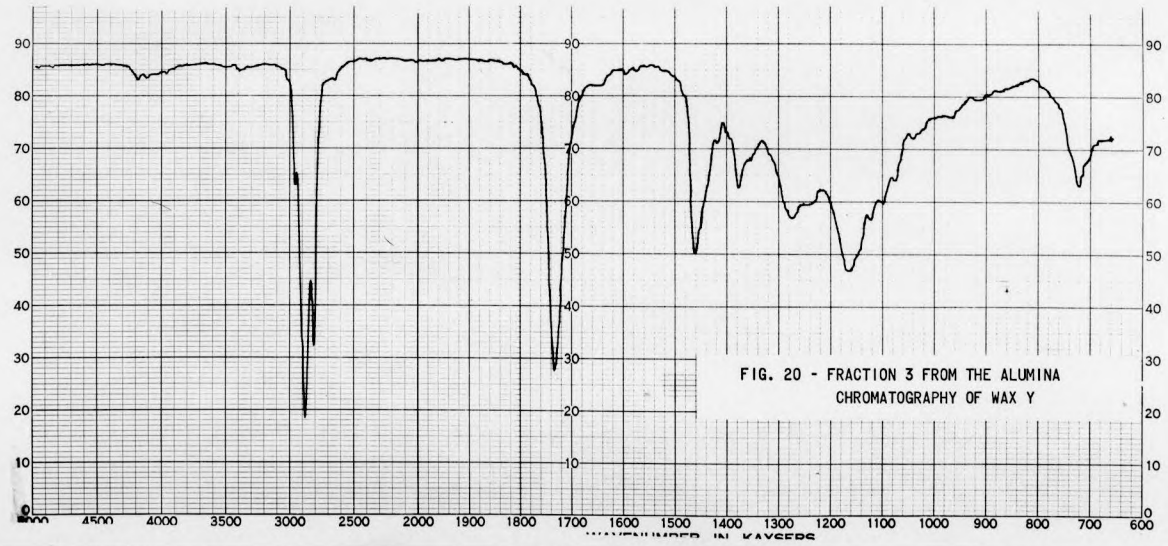



FIG. 20 - FRACTION 3 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

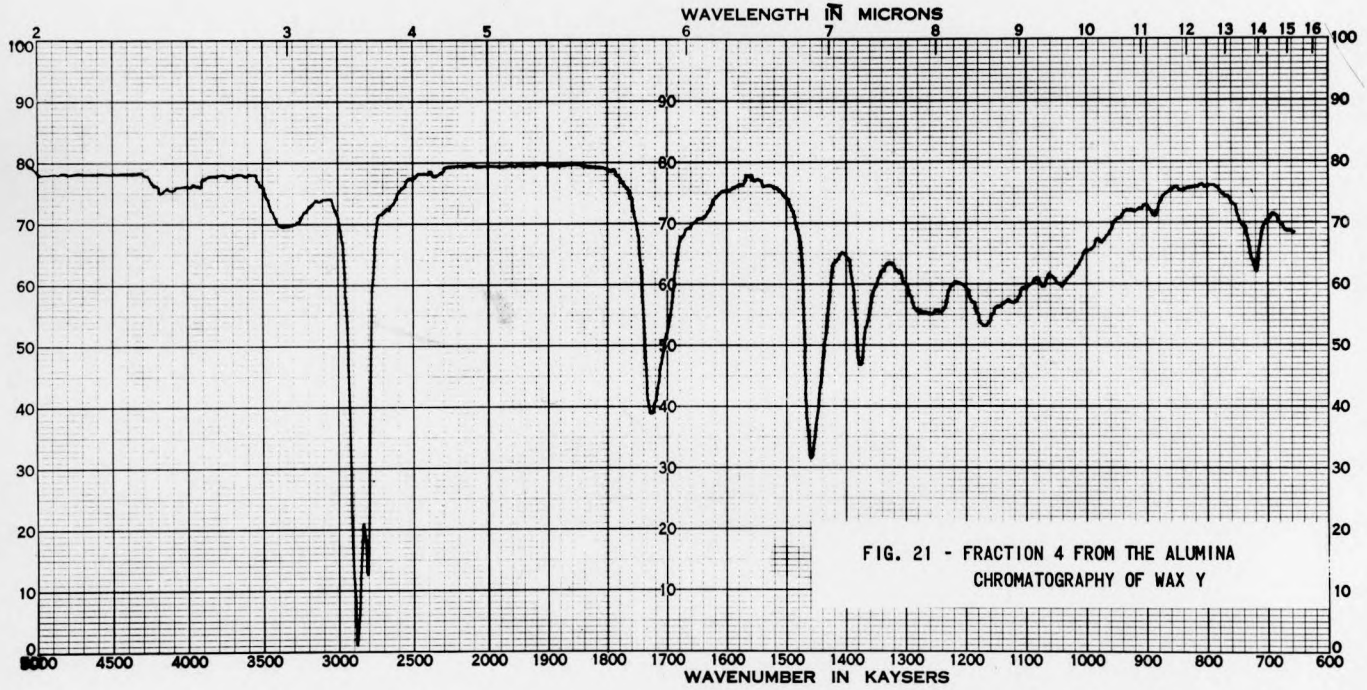



FIG. 21 - FRACTION 4 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

appearance of hydroxyl stretch. In other respects an ester is indicated. This fraction may be the result of esters of hydroxy acids. Equally possible is that the fraction contains free alcohols in an admixture with esters. This probability is enhanced by the relatively small carbonyl band.

Fraction number 5 is a white solid (some yellow occluded) that will not form films for infrared spectroscopy as have the previous fractions. It was necessary to examine this cut in carbon disulfide solution. The markedly enhanced band at 1050 $cm^{-1}$ in Figure 22 suggests that this cut may contain a secondary alcohol. The hydroxy stretch, as it appears in solutions, is evident in the 3500 $cm^{-1}$ region. Unsaturation is indicated though not pronounced in this spectrum. This fraction, accounting for 2.3% of the total, may well be an alcohol with a trace of esters. The spectrum of this material is similar in many respects to various published spectra for sterols [15,16]. No degree of certainty of identification is possible here since this fraction is very likely a mixture. However, if the absorbancies at 1736 $cm^{-1}$ and 1170 $cm^{-1}$ are discounted as due to a trace of extraneous ester, the remaining structure is indeed comparable to the spectrum of cholesterol (Figure 23).

The spectra of fractions number 6 and number 7 are nearly identical. These combined fractions would therefore account for 2.2% of the total. The infrared spectrum of fraction number 6 (Figure 24) is again strongly reminiscent of esters and once again shows unsaturation. The hydroxy

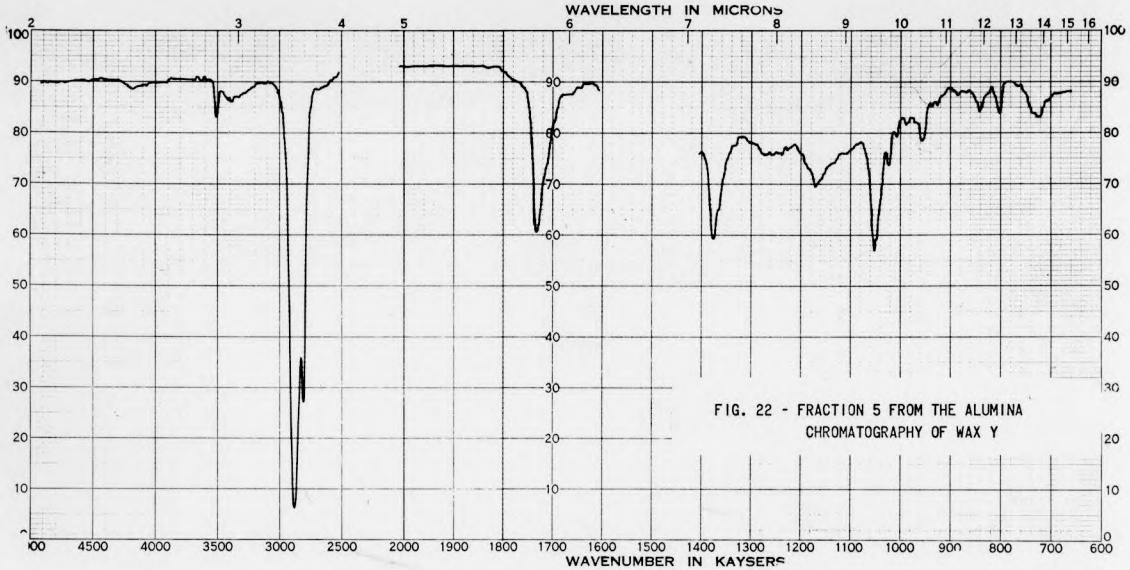



FIG. 22 - FRACTION 5 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

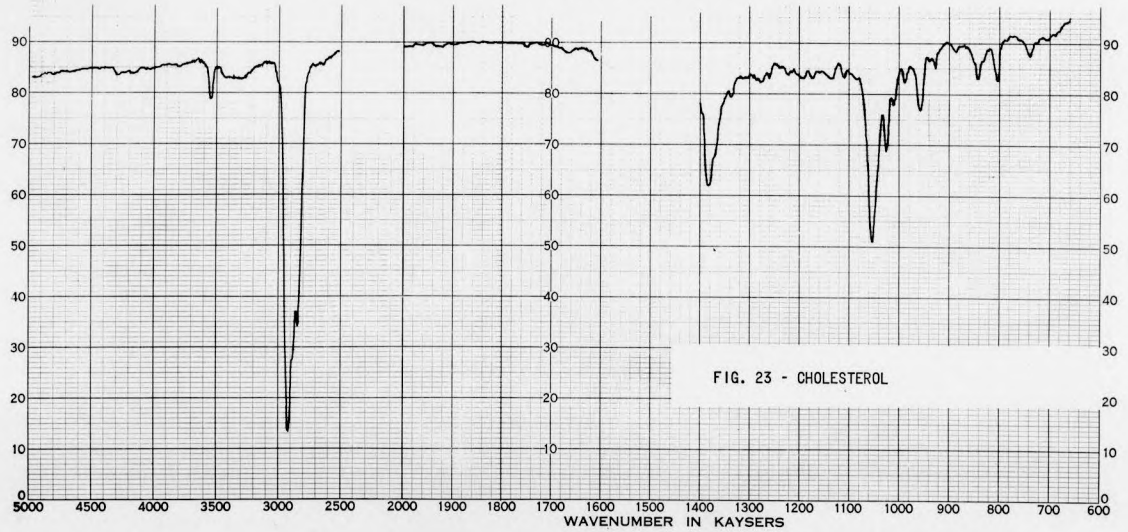



FIG. 23 - CHOLESTEROL

stretch is quite evident and the suggestion is for an unsaturated hydroxy ester.

The light-yellow solid of fraction number 8 that amounts to 2.4% of the total is anomolous. Although the general ester and hydroxy bands do appear in this spectrum (Figure 25) the very strong bands at 1640 $cm^{-1}$, and 1280 $cm^{-1}$ and 840 $cm^{-1}$ are dominant. These strong and broad bands might be the result of nitro groups but other groups or combinations of groups could also account for them. Inexperience in the interpretation of infrared data leaves this particular fraction essentially unidentified.

Fractions number 9 and number 10 give infrared spectra that are nearly identical. The combined fractions account for 12.9% of the total. The yellow-green oil may have a slight residual methanol impurity as suggested by the strong methyl band at 1380 $cm^{-1}$ and strong hydroxyl bands (Figure 26). All things considered, however, it seems more likely that components of the wax are responsble for these bands. The carbonyl band has broadened perceptibly which suggests more than one type of carbonyl structure. This fraction is very likely a mixture of esters and alcohols with the strong possibility of some polymerization. Carboxyl systems are quite likely and their elution from the alumina could be accounted for if they were not free but associated with a large polymer containing ester linkages, hydroxy groups, and probably aldehyde and ketone structures. Such is the system presumed for shellac. The spectrum

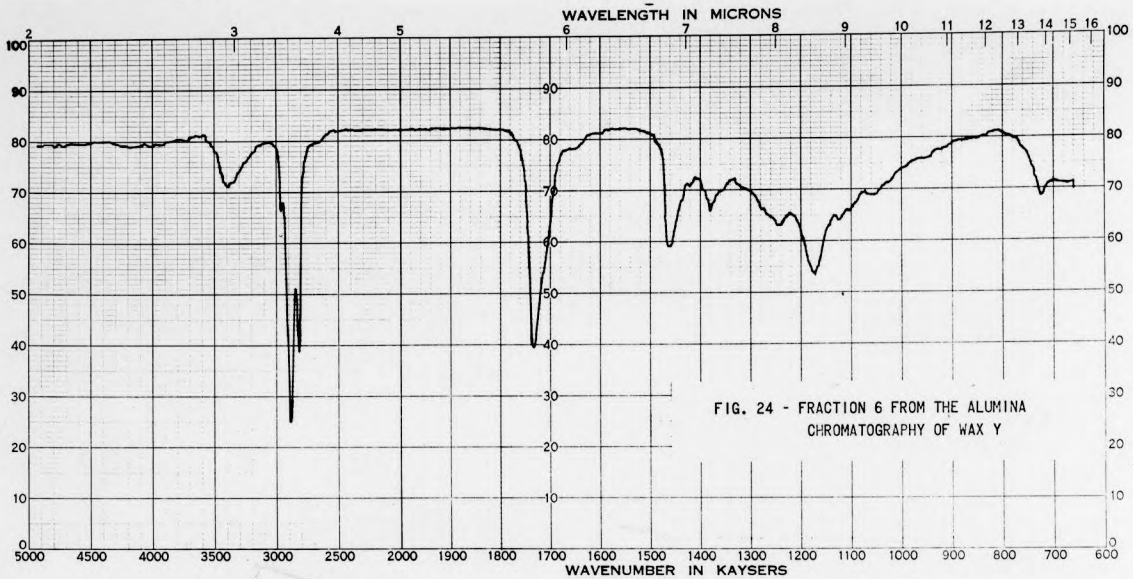



FIG. 24 - FRACTION 6 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

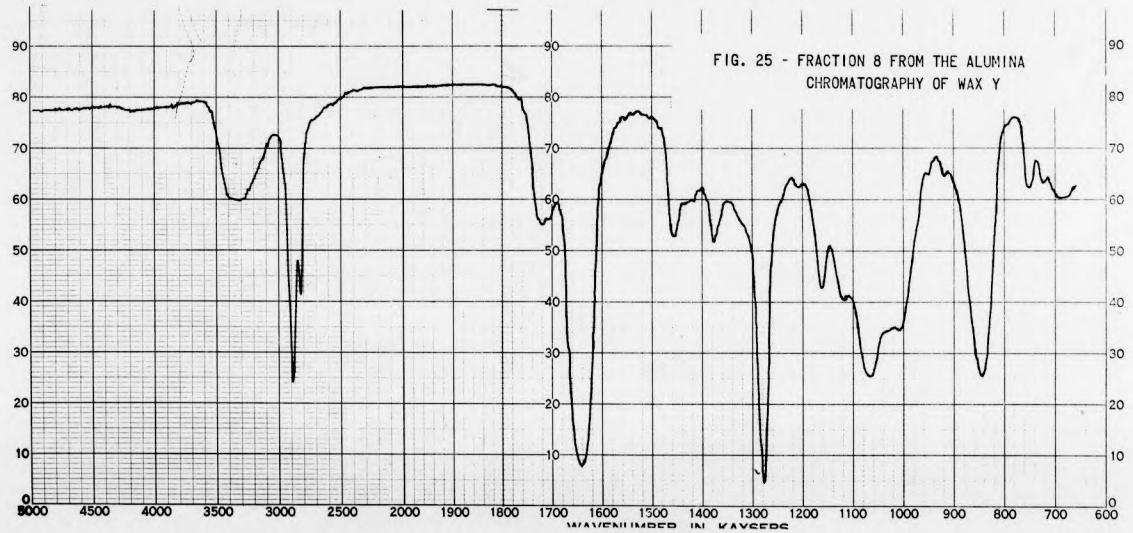



FIG. 25 - FRACTION 8 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

of a commercial shellac sample is presented for comparison (Figure 27). Mild support is given to the shellac suggestion by the infrared pattern shown in Figure 28. This is the spectrum of a material obtained from the original "Wax Y" by extraction with methanol, evaporating the solvent and finally washing with skellysolve-B. This material, therefore, resembles shellac in its solubility characteristics; soluble in methanol, insoluble in skellysolve-B.

A tabulation of the data from the alumina chromatography of "Wax Y" is presented in Table III.

TABLE III

SEPARATION OF "WAX Y" VIA ALUMINA CHROMATOGRAPHY

| Fraction No. | Probable Composition | % of Total |
|---|---|---|
| 1 | hydrocarbons; $C_{12}$ to $C_{32}$ | 57.8 |
| 2 | esters; probably saturated | 3.2 |
| 3 | esters; some unsaturation | 2.7 |
| 4 | esters and alcohols and/or hydroxy esters; saturated | 1.4 |
| 5 | cholesterol and trace of esters | 2.3 |
| 6 | esters and alcohols and/or hydroxy esters; unsaturated | 1.5 |
| 7 | same as 6 | 0.7 |
| 8 | unidentified | 2.4 |
| 9 | esters and polymerized products with carboxyl groups; similar to shellac | 3.1 |
| 10 | same as 9 | 9.8 |
| | acidic material remaining on column | 14.9 |

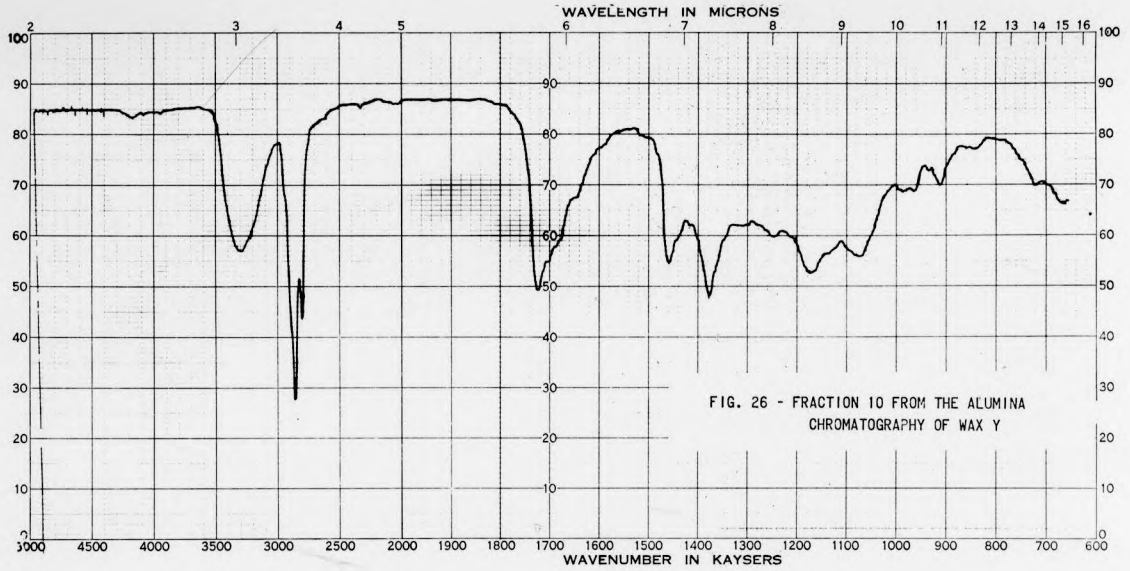



FIG. 26 - FRACTION 10 FROM THE ALUMINA CHROMATOGRAPHY OF WAX Y

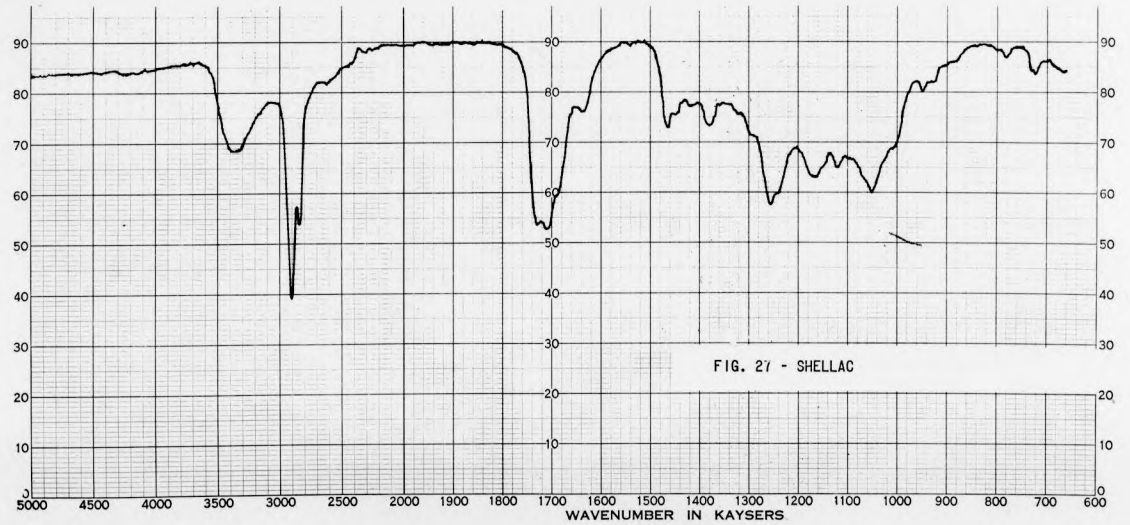



FIG. 27 - SHELLAC

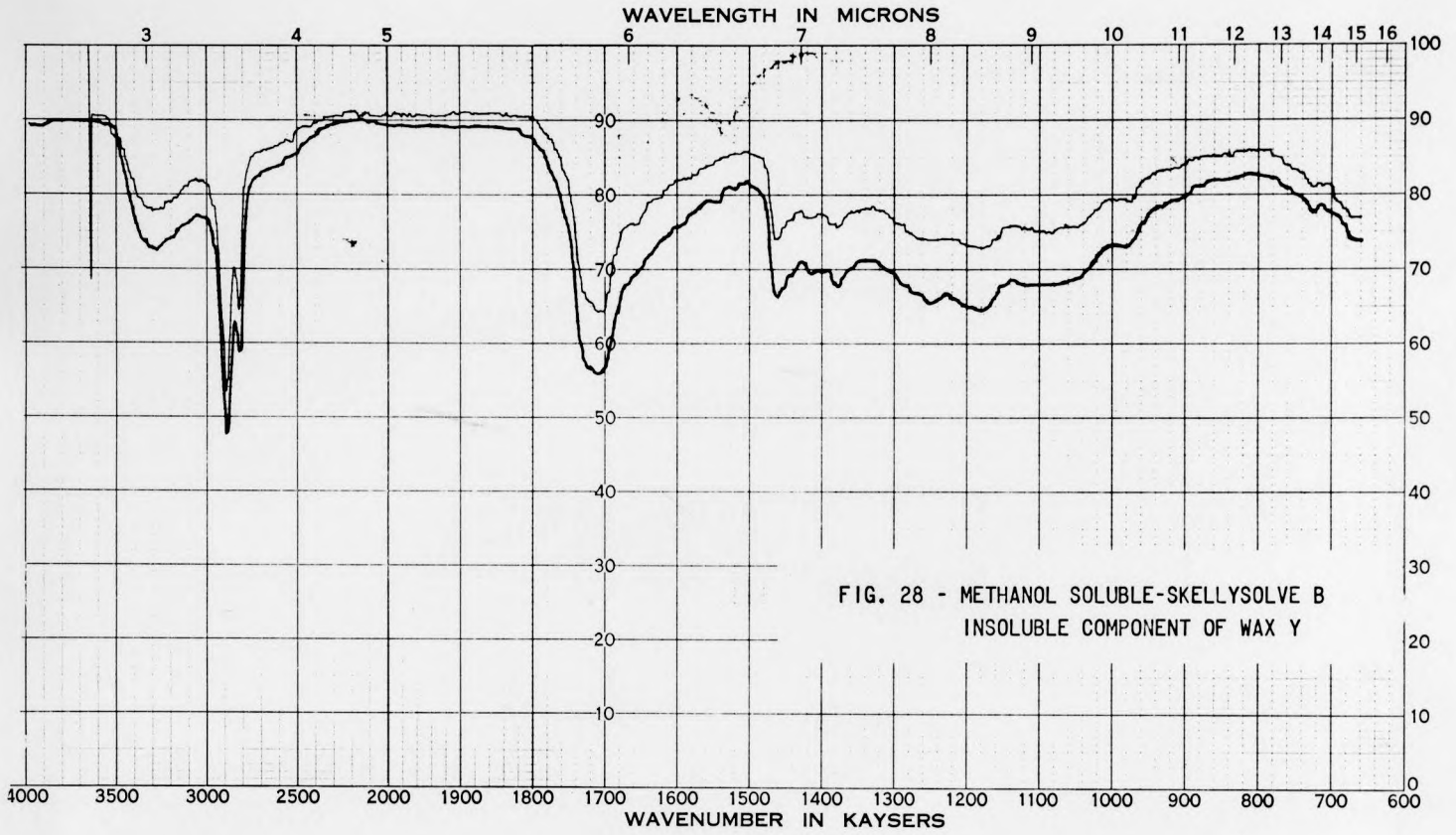



FIG. 28 - METHANOL SOLUBLE-SKELLYSOLVE B INSOLUBLE COMPONENT OF WAX Y

The obvious approach on completing this phase of the work was to examine the individual fractions via saponification, countercurrent separation, etc. However, not enough material was available at this time to conduct such an extensive investigation of the individual fractions. The results to be cited now are, therefore, based on correlations of this latter phase of the work involving separation with an earlier phase in which saponification studies were made on "Wax Z" (i.e. a chloroform extract). In this particular investigation attempts were being made to effect solvent separations and a fraction of "Wax Z" was obtained by extracting it with methanol. It is this methanol soluble system that is considered now. In the light of later information it seems that this methanol soluble system probably contains all of the wax components with the exception of the higher molecular weight hydrocarbons. This extract, designated $M_{s-z}$, was saponified and the acids and neutrals separated by the Kies' technique. The acids were in turn converted to the methyl esters and investigated via gas chromatography. The chromatography was carried out at 204° C. using a 5 foot LAC 446 column. Identification of peaks from the chromatogram (Figure 29) was made in the usual way. The esters involved here originated from the free acids present in $M_{s-z}$ as well as from acids freed as the result of saponification. Comparison of this chromatogram with that for the free acids (Figure 6) emphasizes the significantly larger peaks for the saturated acids $C_{16}$, $C_{18}$,

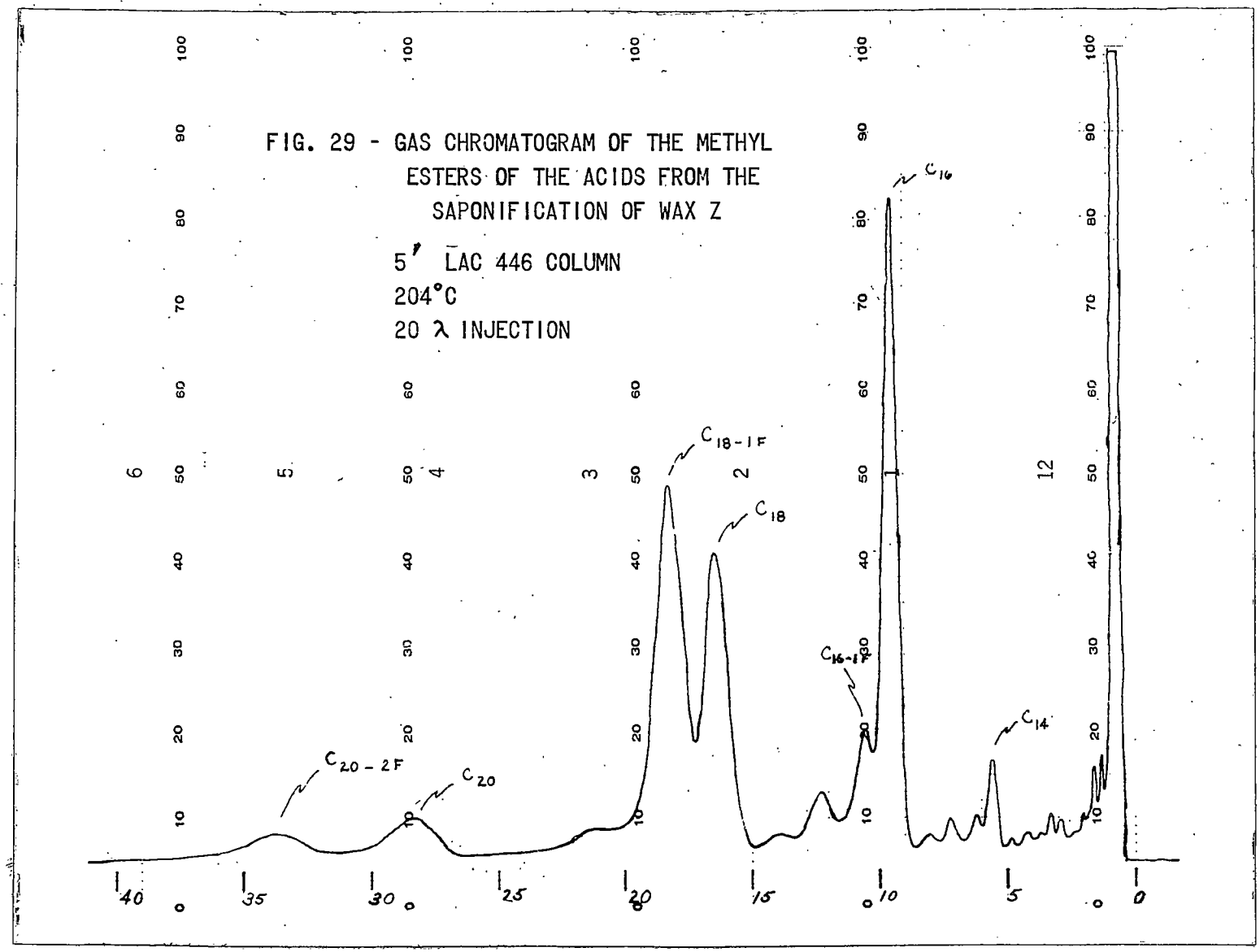



FIG. 29 - GAS CHROMATOGRAM OF THE METHYL ESTERS OF THE ACIDS FROM THE SAPONIFICATION OF WAX Z

5' LAC 446 COLUMN
204°C
20 λ INJECTION

$C_{20}$ and $C_{22}$. This implies, of course, that although the esters do contain unsaturated systems, as evinced by their infrared spectra, their fatty acids composition must exhibit saturated acids to a greater degree than do the free acids. This conjecture is supported by the fact that the ester fractions from alumina chromatography of "Wax Y" seem to be predominantly saturated. Fractions number 2, 4, 5, 9 and 10 show little, if any, unsaturation and account for 19.8% of the total whereas fractions 3, 6, and 7 which exhibit the unsaturation represent only 5.7%.

# V DISCUSSION: LIMITATION OF INTERPRETATION

The interpretation of data as set forth in the previous section can best be classified as a reasonable estimate of the constitution of the wax of the Mormon cricket. It is based on a summation of data gathered from many trials, many of which were unsuccessful, and necessitated a recovery of materials. This has led to obvious discrepencies in the data presented. A glaring example is in the estimated amounts of free acids in the wax. These acids obtained by means of a counter current extraction appear to account for approximately 18% of the total wax (page 20), whereas a calculation based on the amount of material retained on the alumina column indicates 14.9% (Table III).

The possibility of error in the hydrocarbon content has already been discussed (page 31) and is manifested by comparison of 47.2% for the hydrocarbon content of excised cuticular wax (page 28) with the 57.8% reported for "Wax Y" (page 34). It must be assumed, therefore, that all percentage relationships may be in error by the same order of magnitude since this known hydrocarbon error would reflect itself in all other calculations.

No attempt has been made to make a critical assignment of the various acids whose molecular size and type was indicated by gas chromatography. A $C_{18}$ acid with sufficient branching, for example, could be mistakenly assigned as a $C_{16}$ acid. The position of a double bond in an acid has not been indicated and, although it is tempting to speak of a $C_{18}$-1 double bond acid as oleic acid, no such evidence has been presented.

The assignment of molecular size to the hydrocarbons via gas chromato-

graphy has the same possibility for confusion that has already been cited for the acids.

In short, the evidence presented has rather clearly indicated the general chemical nature and probable molecular size for the lipids of the cuticular wax of the Mormon cricket but in not one instance has a specific structure been proven.

# VI CONJECTURE

Interpretation of the evidence pertinent to elucidation of Mormon cricket wax composition has been reported in the previous sections. A short resume' will be presented here which represents the author's ideas on the probable make-up and function of this wax. These ideas are obviously not based completely on facts or they would not be presented in this manner. Rather these thoughts stem from a summation of all things involved in this project - fact and fancy.

The wax produced by a cricket probably consists of hydrocarbons, free fatty acids, cholesterol, cholesteryl esters of fatty acids, an acidic resin similar to shellac and an amine emulsifier.

An aqueous emulsion of this system is probably exuded just prior to moult into the region between the newly formed cuticle and the exuvium about to be cast. In this region it can serve two functions: 1) It can aid in dissolution of materials bonding the old cuticle to the body of the cricket. 2) It can act as a lubricant in the actual release of the cast skin after all direct bonds have been destroyed. The newly exposed surface is indeed labile. The wax now responds to its new environment by "drying" just as commercial floor waxes do. The presence of the shellac in the wax provides a film with sufficient rigidity to give the insect a degree of protection until the "tanning" of the proteins within the matrix of the new cuticle is complete. A concurrent function of the dried film, of course, is the rather well established function of moisture control. This is not to suggest that the cricket ceases to produce

these waxes after this time, but that the waxes are exuded to the surface and perhaps produced at a much greater rate just prior to moult. Perhaps these same waxes are produced throughout the life of the cricket for repair of this surface coat so subject to physical abrasion.

The production of this wax is, of course, of interest. Although the mechanism for the production of fatty acids in biological systems of any type is not too clearly established, reasonable suggestions have been made and it may be assumed that similar processes produce these fatty acids. Certainly the food eaten could be in itself a more direct source of these same fatty acids. Cholesterol syntheses have likewise been proposed and similar mechanisms may account for its production in the cricket. A modification of plant sterols ingested with the food might be a short-cut. The creation of esters from existing alcohols and fatty acids creates no problems and since no structural identity has been given the amine it would be foolish to consider a mechanism for its production. The biochemical production of hydrocarbons is another matter. Presumably the hydrocarbons could result from the reduction of fatty acids (reductions are not uncommon in known biological processes). An alternative would be decarboxylation of fatty acids. This fits well for odd hydrocarbons resulting from even acids, but requires very large acids which have not been found (certainly not to be ruled out, however, since an acid of such size would not have been found by any of the techniques employed in this study). An intriguing thought is that these hydrocarbons might stem from cholesterol, or be the result of an alterna-

tive pathway to the final steps in the production of cholesterol. This thought is derived from the molecular size of the predominant hydrocarbons ($C_{27}$ to $C_{32}$ comparing favorably with the molecular size of sterols). It is conceivable that cholesterol might form the nucleus for such systems by a mechanism that opens the rings. This would produce long chain hydrocarbons with considerable branching. This idea has just a tinge of support from the infrared spectrum of the hydrocarbons in which the intensity of the methyl-methylene band is more intense than would be expected for heptacosane, indicating more methyl groups. This could be a result of shorter chain hydrocarbons mixed with the heptacosane as well as branching of a $C_{27}$ system. Current literature generally suggest mechanisms for the production of squalene via either mevalonic acid, or dimethylacrylic acid. Subsequent coupling with decarboxylation and loss of water presumably accounts for the build-up of isoprene units. It is suggested that the squalene is the precursor for cholesterol. Such a mechanism requires reduction and ring closure. It is conceivable that similar reductions could occur at stages prior to ring closure and in this way account for hydrocarbons of the types observed here.

Such thoughts are indeed conjecture and must remain so until the precise structures of these various compounds are known. It is to be hoped that this paper can provide a starting point for this more specific study.

## VII SUMMARY

A study of the cuticular wax of the Mormon cricket, Anabrus simplex Hald., has been made. An estimate of the chemical composition of the wax has been made by interpretation of this data. Table IV is a summary of the probable constitution of the wax.

TABLE IV

SUMMARY OF THE COMPOSITION OF MORMON CRICKET WAX

| Chemical Class | % of Wax | Individual Component | % of Class | % of Wax |
|---|---|---|---|---|
| Hydrocarbons | 48-58 | $C_{12}$, $C_{13}$, $C_{14}$, $C_{15}$, $C_{16}$ / $C_{17}$, $C_{18}$, $C_{19}$, $C_{20}$, $C_{21}$ / $C_{22}$, $C_{23}$, $C_{24}$, $C_{25}$, $C_{26}$ | 12.5 equally distributed | 6-7 |
| | | $C_{27}$ - $C_{28}$ | 20.6 | 10-12 |
| | | $C_{30}$ - $C_{31}$ | 55.3 | 27-32 |
| | | $C_{32}$ ? | 11.4 | 5-7 |
| Free Acids | 15-18 | $C_{12}$ | 0.4 | 1 |
| | | $C_{14}$ | 1.7 | 0.4 |
| | | $C_{14}$ - 1 double bond | 0.5 | 0.1 |
| | | $C_{14}$ - 2 " " | 0.3 | 0.1 |
| | | $C_{14}$ - 3 " " | 0.6 | 0.1 |
| | | $C_{16}$ | 7.9 | 1-1.4 |
| | | $C_{16}$ - 1 double bond | 4.0 | 0.6 |
| | | $C_{16}$ - 2 double bond | 0.3 | 0.1 |
| | | $C_{16}$ - 3 " " | 0.7 | 0.1 |

TABLE IV (continued)

| Chemical Class | % of Wax | Individual Component | % of Class | % of Wax |
|---|---|---|---|---|
| Free Acids | 15-18 | $C_{18}$ | 2.4 | 0.4 |
| | | $C_{18}$ - 1 double bond | 48.4 | 7-9 |
| | | $C_{18}$ - 2 " " | 26.8 | 4-5 |
| | | $C_{18}$ - 3 " " | 6.0 | 1 |
| | | $C_{20}$ | trace | trace |
| Esters | 9-11 | saturated | 34 | 3.2-3.9 |
| | | saturated & unsaturated | 28 | 2.7-3.3 |
| | | saturated and/or hydroxy | 15 | 1.4-1.7 |
| | | unsaturated and/or hydroxy | 23 | 2.2-2.7 |
| Free alcohols | 2-3 | cholesterol | | 2-3 |
| Polymers | 12-14 | acidic resins of varying molecular size | | 12-14 |
| Unidentified | 2-4 | may be nitro containing compounds | | 2-4 |
| Emulsifier | trace | probably an amine | | trace |

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