cules polar enough and small enough to penetrate these essentially porous, adsorbed films.

The present data prove that tristearin soil at the monolayer level is held by adsorption forces while cohesive forces are active at multimolecular soil thicknesses. The adsorption appears to be operative through the soil polar groups, and adsorbed soil is released only by more polar materials. Access of solvent molecules to the glass substrate surface presumably occurs through voids in the packed soil monolayer or at soil patch edges. Soil removal in these solvent systems takes place by dissolution of coherent soil and by stripping or preferential sorption mechanism at adsorbed soil patch edges. Nonpolar or weakly polar solvents act by dissolution of coherent soil, and polar solvents through preferential sorption and stripping and detaching at adsorbed soil sites. Combinations of polar and nonpolar solvents prove more effective in some instances than polar solvent alone.

REFERENCES

1. Adam, N. K., "Physics and Chemistry of Surfaces," Oxford University Press (1941).
2. Aickin, R. G., Australian Chem. Inst. J. and Proc., *6*, 68 (1939).
3. Anderson, S., and Kimpton, D. D., J. Am. Ceram. Soc., *36*, 175 (1953).
4. Baker, H. R., and Zisman, W. A., Ind. Eng. Chem., *40*, 2338 (1948).
5. Bikerman, J. J., "Surface Chemistry," Academic Press Inc., pp. 181, 272, 403, New York (1958).
6. Brunauer, S., "The Adsorption of Gases and Vapors," Vol. I, Princeton University Press, Princeton, N. J. (1943).
7. Epstein, H. T., J. Phys. and Colloid Chem., *54*, 1053 (1950).
8. Frazer, J. C. W., Patrick, W. A., and Smith, H. E., J. Phys. Chem., *31*, 897 (1927).
9. Hazel, J. F., and Schnable, G. L., J. Phys. Chem., *58*, 812 (1954).
10. Hensley, J. W., Long, A. O., and Willard, J. E., J. Am. Chem. Soc., *70*, 3146 (1948).
11. Hensley, J. W., Long, A. O., and Willard, J. E., Ind. Eng. Chem., *41*, 1415 (1949).
12. Johnson, C. E., Div. Colloid Chem., Am. Chem. Soc., April 7 (1957).
13. Jones, G. O., "Glass," Methuen and Company Ltd., p. 93 (1956).
14. Kahan, G. J., J. Colloid Sci., 6, 571 (1951).
15. Karle, J., J. Chem. Phys., *17*, 500 (1949).
16. Krasil'nikov, K. G., Kesilev, V. F., and Sysoev, E. A., Doklady Akad. Nauk S.S.S.R., *116*, 990 (1957).
17. Kühne, K. Silikattech., *6*, 191 (1955).
18. Langmuir, I., J. Am. Chem. Soc., *38*, 2221 (1916).
19. Langmuir, I., J. Am. Chem. Soc., *40*, 1361 (1918).
20. McBain, J. W., and Henniker, J. C., Colloid Chem., *7*, 67 (1950).
21. McHaffie, I.R., and Lenher, S., J. Chem. Soc., *127*, 1559 (1925); 1785 (1926).
22. Paneth, F., and Radu, A., Ber., *57B*, 1221 (1924).
23. Shepard, J. W., and Ryan, J. P., J. Phys. Chem., *60*, 127 (1956).
24 Tilton, L. W., Chem. and Eng. News, *36*, No. 45, 56 (1958).
25. Weyl, W. A., Research, *1*, 50 (1947).
26. Weyl, W. A., J. Soc. Glass Technol., *33*, T220 (1949).
27. Willard, J. E., J. Phys. Chem., *57*, 129 (1953).

[Received March 19, 1959]

# Separation and Chemical Assay of Lipide Classes[1]

**E. VAN HANDEL,[2] Division of Physiology, University of Tennessee, Memphis, and The Public Health Research Laboratories, Stuart, Florida**

THE INTEREST in human diseases which may be accompanied by abnormalities in lipide metabolism has greatly stimulated the development of methods for separation and micro-analysis of lipide classes. It therefore should be worthwhile to consider methods for micro-determination of cholesterol and its esters, triglycerides, and phospholipide constituents. The material has been restricted mainly to include those methods with which the author has had experience.

## Determination of Total Cholesterol in Serum

The desire for convenient analysis of small quantities of cholesterol and its esters in serum and some tissues is evidenced by the great number of modifications of the basic chemical procedures. In material where sterols other than cholesterol can be expected, such as adrenals or intestinal contents, the determination is very tedious. For cholesterol assay in serum, four different colorimetric methods are in use, including, in order of increasing specificity: direct addition of the color-forming reagents to a sample of serum (1, 2); addition of the reagents to the lipides isolated from serum (or tissue) by solvent extraction (3); application of the reagents for color development to the unsaponifiable fraction (4); and precipitation with digitonin of nonesterified 3 *beta* hydroxy steroids from a saponified lipide extract and subsequent addition of the reagents to the sterol digitonide (5).

*Development of a Colored End-Product.* Three different color producing reactions are suitable: the reaction with acetic anhydride and sulfuric acid to produce a blue to blue-green color (Liebermann-Burchard reaction), reaction with ferric chloride which forms a purple complex (2), and reaction with zinc chloride-acetyl chloride for cherry red (Tschugaeff reaction) (6). The Liebermann-Burchard (L.B.) reaction is the most widely used. It actually yields two colored components, one having an absorption maximum at 625 $m\mu$ and a more stable product which can be conveniently determined at 430 $m\mu$ (7). The latter wavelength can be used only if no related steroids and bile acids are present to give interfering green-yellow to yellow reaction products.

In the L.B. reaction, chloroform may be used as the solvent (8) although acetic acid is preferred. Free and esterified cholesterol have different absorption characteristics, but this difference is much less when the reaction is carried out in acetic acid than when chloroform is used as the solvent (Table I). For that reason alone it is best to use acetic acid rather than chloroform when the esters have not been saponified. Another disadvantage of chloroform as the solvent is the instability of the reaction product at 625 $m\mu$ (7, 9). At 25°C. the maximum develops within 6–8 min. for esterified cholesterol and within 10–12 min. for free cholesterol, then declines at a rate of 2½–

[1] Presented at the 49th annual meeting, American Oil Chemists' Society, Memphis, Tenn., April 22, 1958.
This investigation was supported in part by a grant from the National Heart Institute, National Institutes of Health, United States Public Health Service (H-2181).
[2] Present address: Public Health Research Laboratories, Box 595, Stuart, Fla.

TABLE I

Optical Density of Cholesterol[3] and Some Cholesteryl Esters[3] Produced in the Liebermann-Burchard Reaction, Measured at Maximum Color Development in a Coleman Model 6D Spectrophotometer with 19-mm. Round Cuvettes

| Reagent | Wavelength | Cholesterol 0.4 mg. | Cholesteryl acetate 1.11×0.4 mg. | Cholesteryl myristate 1.54×0.4 mg. | Cholesteryl oleate 1.68×0.4 mg. |
|---|---|---|---|---|---|
| a | 625 | 0.594 ±1% | 0.814 ±1% | 0.840 ±1% | 0.880 ±½% |
| a | 430 | 0.495 ±2% | 0.605 ±1% | 0.630 ±½% | 0.650 ±2% |
| b | 625 | 0.468 ±1% | 0.495 ±½% | 0.483 ±1% | 0.522 ±½% |
| b | 550 | 0.176 ±2% | 0.190 ±2% | 0.176 ±2% | 0.188 ±2% |
| c | 625 | 0.452 ±½% | 0.472 ±2% | 0.457 ±2% | 0.503 ±1% |
| c | 550 | 0.205 ±½% | 0.214 ±2% | 0.196 ±2% | 0.214 ±2% |

a Six ml. of a mixture: 50 ml. of chloroform, 10 ml. of acetic anhydride, 1 ml. of sulfuric acid (7).
b Four ml. of a mixture: 20 ml. of acetic anhydride and 1 ml. of sulfuric acid, added to the sterols dissolved in 2 ml. of acetic acid (4).
c Six ml. of a mixture: 2 ml. of water, 15 ml. of 12% *para* toluenesulfonic acid in acetic acid, 37.5 ml. of acetic anhydride, and 5 ml. of sulfuric acid (1), using the technique described in text.
Optical densities represent the average of not less than 4 and not more than 8 replicates (±% standard error), run on different days.

3% of the optical density per min. When acetic acid is used, the maximum is reached in about 25 min. and stays constant for at least 15 min. (9). If sulfuric acid is previously mixed with acetic anhydride, there is no change in temperature on mixing with acetic acid. The blue color with maximum absorption at 625 $m\mu$ is not light-sensitive (9). For these reasons it is neither necessary to use a shielded, constant-temperature bath nor to time the reaction with a stop-watch.

Pearson *et al.* modified the L.B. reagent by adding *para* toluenesulfonic acid [2] and found that, mole for mole, cholesteryl acetate and cholesterol have the same absorption characteristics (1); but, for naturally occurring esters, small differences in molecular absorption may still exist (Table I). Pearson *et al.* chose a wavelength of 550 $m\mu$ because at that wavelength the color is stable for 15 min. With the technique described below the color is also stable for 15 min. at the absorption peak. Measuring at 625 $m\mu$ more than doubles the optical density, and in this manner one stays farther away from interfering yellow-green and yellow reaction-products of serum. The blank used by Pearson *et al.* (12% *para* toluenesulfonic acid in acetic acid added to serum) reduces net absorption about 10–15% more than the reagent blank at 550 $m\mu$ and 5–10% at 625 $m\mu$.

The original procedure calls for adding reagents without mixing until the last step, which is the addition of sulfuric acid. This has given rise to violent reactions (10). These are avoided and the determination simplified if the following technique is followed.

Four ml. of a mixture, containing 3% *para* toluenesulfonic acid, 25.5% acetic acid, and 71.5% acetic anhydride, are added from a pipette to 0.2 ml. of plasma in a test tube while being agitated by a cork placed eccentrically on the shaft of a small electric motor. The standard contains 0.4 mg. of cholesterol[3] in 4 ml. of the same mixture and 0.2 ml. of water. After cooling, 0.4 ml. of sulfuric acid is rapidly added, again with gentle agitation, and tubes are immediately placed in cold water so that the reaction does not proceed above room temperature. The absorption is measured at 625 $m\mu$ (or 550 $m\mu$) after 40–60 min. The serum blank is prepared by adding 4.4 ml. of 12% *para* toluenesulfonic acid in acetic acid to 0.2 ml. of serum.

Several normal human sera and normal rabbit plasmas have been analyzed (Table II) by this method (column b) and by the method of Abell (4) (column a).

Direct methods may lead to over-estimation because of the relative lack of specificity of the reagents for cholesterol. In the Pearson method there is wide divergency in parts of the absorption spectrum between the green reaction-product of serum and the blue-green reaction-product of cholesterol (1). Greater specificity is achieved by the method of Abell *et al.* (4), where plasma is saponified and the unsaponifiable sterols extracted with petroleum ether. Abell showed by countercurrent distribution that the unsaponifiable product extracted from plasma behaves like authentic cholesterol. Consequently the absorption spectrum of the reaction product of the petroleum ether extract closely resembles that of pure cholesterol in the L.B. reaction.

TABLE II

Total Serum Cholesterol (mg. per 100 ml.) as Determined by the Methods of Abell *et al.* (a) and Pearson *et al.* (b)

| a Abell | b Pearson | |
|---|---|---|
| 625 $m\mu$ | 625 $m\mu$ | 550 $m\mu$ |
| Human serum | | |
| 222 | 231 | 228 |
| 190 | 193 | 193 |
| 128 | 118 | 135 |
| 196 | 210 | 214 |
| 144 | 142 | 156 |
| 237 | 240 | 274 |
| 203 | 210 | 216 |
| Rabbit plasma | | |
| 45 | 56 | 54 |
| 70 | 81 | 85 |
| 55 | 50 | 52 |
| 34 | 35 | 39 |
| 53 | 75 | 87 |
| 37 | 40 | 50 |
| 54 | 61 | 62 |
| 25 | 26 | 25 |

For the ferric chloride method (2) Herrmann has demonstrated that there may be as much as 25% difference between the direct reaction with plasma and that of the petroleum ether extract (11).

## Separation of Cholesterol from Cholesteryl Esters

About 65–75% of total cholesterol in plasma is esterified with fatty acids. Methods available for separation of cholesterol from its fatty esters depend upon precipitation of free cholesterol with digitonin (5), or upon elution from aluminum oxide (12), or silicic acid (13, 14, 15). If a solution of lipides in petroleum ether is adsorbed on silicic acid, cholesteryl esters may be eluted with small amounts of chloroform-petroleum ether. All other lipides, including free cholesterol and triglycerides, remain adsorbed (13, 14). Chloroform will elute cholesterol and triglycerides quantitatively while the phospholipides remain and can be eluted with methanol.

Separation of cholesteryl esters from cholesterol and triglycerides is achieved as follows:

A mixture of ½ g. of Super-Cel and ½ g. of nonactivated silicic acid[4] is slurried with chloroform in a constricted glass

[2] The reason for addition of this reagent is unexplained, and very similar results are obtained if 3% acetic acid is substituted for the 3% *para* toluenesulfonic acid.
[3] All cholesteryl esters were purified by elution from large silicic acid columns with 10% chloroform in petroleum ether. Cholesterol was purified by elution with chloroform-petroleum ether 3:1 (v/v). The appearance of only one spot on a chromatogram was used as the criterion for purity.
One mg. of cholesterol is equivalent to 1.11 mg. of cholesteryl acetate, 1.25 mg. of caproate, 1.54 mg. of myristate, and 1.68 mg. of oleate.
[4] Silicic acid (Mallinckrodt). 100-mesh A.R. Hyflo Super-Cel (Johns Manville).

tube (1.0 cm. in inner diameter and 40 cm. in length), in which a small plug of glass wool has been inserted. After drainage of the chloroform about 10 ml. of petroleum ether are passed through. The mixture of lipides (max. 20 mg.) in petroleum ether (b.p. 60°–70°C.) is placed on the column and eluted with 20 ml. of 1:9 (v/v) chloroform-petroleum ether. The eluate contains cholesteryl esters. Subsequently the column is eluted with 20 ml. of chloroform. This fraction contains all of the triglycerides and free cholesterol.

The separation by the above procedure of cholesteryl oleate and caproate from cholesterol in molecular ratios from 1:10 to 10:1 is shown in Table III. Results were in good agreement with the Sperry-Webb method for free cholesterol even with biological specimens of widely different free/total cholesterol ratios

TABLE III

Determination of Cholesterol Esters,[a] Free Cholesterol,[a] and Triglycerides, Following Elution from Silicic Acid

| Adsorbed on silicic acid (mg.) | | a Chol. ester* | a Trigl.** | b Cholesterol* | b Trigl.** |
|---|---|---|---|---|---|
| Chol. oleate | 0.84 | 0.84 | | | |
| Cholesterol | 5.0 | | | 5.0 | |
| Corn oil | 4.0 | | <0.04 | | 3.8 |
| Chol. oleate | 1.68 | 1.71 | | | |
| Cholesterol | 10.0 | | | 10.0 | |
| Corn oil | 4.0 | | <0.04 | | 3.7 |
| Chol. oleate | 8.4 | 8.2 | | | |
| Cholesterol | 5.0 | | | 5.1 | |
| Corn oil | 4.0 | | <0.04 | | 3.8 |
| Chol. oleate | 16.8 | 16.7 | | | |
| Cholesterol | 1.0 | | | 1.1 | |
| Corn oil | 4.0 | | <0.04 | | 3.9 |
| Chol. caproate | 1.25 | 1.30 | | | |
| Cholesterol | 10.0 | | | 10.0 | |
| Chol caproate | 12.5 | 12.8 | | | |
| Cholesterol | 1.0 | | | 1.1 | |
| Chol. caproate | 0.62 | 0.62 | | | |
| Cholesterol | 5.0 | | | 4.9 | |
| Chol. caproate | 6.25 | 6.35 | | | |
| Cholesterol | 0.50 | | | 0.53 | |

a—mg.eluted with 20 ml. of chloroform:petroleum-ether 1:9.
b—mg. eluted with 20 ml. of chloroform.
*Determined by the L.B. reaction, using cholesteryl oleate, caproate, and cholesterol as standards.
**According to Van Handel and Zilversmit (18).

(Table IV). The efficiency of the separations was further checked chromatographically on silicic acid impregnated glass paper (16), using petroleum ether as the mobile phase and 8% alcoholic phosphomolybdic acid (17) as a staining agent.

*Separation of Fatty Acids from Sterols.* The glycerides from the chloroform fraction can be saponified; cholesterol can then be extracted with petroleum ether. After acidification of the soaps, fatty acids are extractable with petroleum ether. Similarly the fatty acids and cholesterol from the cholesteryl ester fraction can be separated by saponification. In this way cholesterol from free and from esterified cholesterol becomes separately available for further analysis, as do fatty acids from sterol esters and glycerides. Therefore no further attempts have been made to separate triglycerides and cholesterol from each other by silicic acid chromatography.

## Determination of Triglycerides

Few investigators have attempted to survey the variations of blood and tissue triglycerides in health and disease. This is probably on account of the inadequacy of current analytical methods. In many biological samples, triglycerides constitute a minor

TABLE IV

Determination of Cholesterol from Liver of Normal and Cholesterol-Fed Rabbits, Chromatographically, and According to the Method of Sperry and Webb

| Esterified cholesterol mg./g. of tissue | Free cholesterol mg./g. of issue | | Ratio free/total cholesterol |
|---|---|---|---|
| As 10% chloroform fraction from column* | As chloroform fraction from column | As digitonide | |
| 0.31 | 1.12 | 1.17 | 0.79 |
| 0.3 | 0.9 | 1.0 | 0.75 |
| 0.60 | 1.80 | 1.90 | 0.75 |
| 1.15 | 2.3 | 2.1 | 0.67 |
| 1.25 | 1.75 | 1.85 | 0.58 |
| 1.00 | 1.17 | 1.12 | 0.54 |
| 0.98 | 1.00 | 0.95 | 0.50 |
| 11.8 | 4.8 | 3.5 | 0.29 |
| 25.9 | 5.1 | 4.6 | 0.16 |
| 31.3 | 5.7 | 4.7 | 0.15 |
| 18.0 | 3.3 | 2.4 | 0.15 |
| 26.3 | 3.7 | 3.5 | 0.12 |
| 25.7 | 2.8 | 2.8 | 0.10 |

* Compared with a cholesteryl oleate standard.

fraction of the total lipides, and methods which depend on the difference between total lipides and the sum of the determined cholesterol, cholesteryl esters, and total phospholipides are liable to serious error. A much more specific method for triglycerides depends on the determination of esterified glycerol after removal of the interfering phospholipides. The separations of phospholipides from nonphosphorus containing triglycerides is accomplished by placing plasma directly on zeolite and extracting the triglycerides with chloroform. Alternatively the separation can be brought about by passing a chloroform extract of total lipides through a small silicic acid column. The glycerides are saponified, liberated glycerol is oxidized by periodic acid, and the formed formaldehyde is determined colorimetrically after reaction with chromotropic acid. As little as 0.02 mg. of triglycerides can be determined (18).

## Analysis of Phospholipides

Determination of lipide phosphorus by dry or wet ashing and subsequent application of the Fiske and Subbarow reagent has not been the subject of much controversy. Some phospholipides, *e.g.,* from plasma (19), bile (20), or egg (21a) have a relatively simple composition. Others, *e.g.,* from brain, liver, and oil seeds, are much more complex and show a great variety of nitrogenous and sugar-containing components. Quantitative determination of the several components depends on assay of the products of hydrolysis: fatty acids, sphingosine, glycerophosphoric acid, choline, ethanolamine, amino acids, inositol, aldehydes, and sugars. A study of the analysis of the nitrogenous phosphatides, with emphasis on the composition of egg phosphatides and commercially available "lecithins," has been published as a monograph (21).

In the animal kingdom, two choline-containing phospholipides occur: lecithin and sphingomyelin. The latter can be differentiated from lecithin by its resistance to mild alkaline hydrolysis (22). The occurrence in soybean of an alkaline-resistant phosphatide, not containing choline, led the author to the detection and isolation of a new class of phosphatides, showing a nitrogen to phosphorus molar ratio of 2:1 and a molecular weight of about 1,600 (21b, 23). They contain a sphingosine-like base, glucosamine, inositol, and reducing sugars (24).

Carter proposed the name "phytoglycolipides" for this class of sphingolipides.

The determination of ethanolamine and serine in hydrolysates usually has been done by assay of ammonia, which is liberated upon oxidation by periodate (25, 26). The assumption however that periodate liberates ammonia only from compounds having the amino group and a hydroxyl group on adjacent carbon atoms (*e.g.*, ethanolamine and serine) does not hold. Choline also develops ammonia on oxidation with periodate. Therefore choline has to be removed from the hydrolysate prior to de-amination with periodate (21c).

The isolation of individual, intact phospholipides is a prerequisite for the elucidation of chemical structures and for work in intermediary metabolism that involves tracer techniques. Combinations of countercurrent distribution and chromatography undoubtedly will lead to a more comprehensive knowledge of the structure and more accurate analysis of this interesting and important class of lipides.

## Summary

A survey is given of the micro determination of three major lipide classes: cholesterol, triglycerides, and phospholipides. The merits of a popular direct method for serum cholesterol and a system for rapid chromatographic separation of esterified cholesterol from other lipides have been discussed.

## REFERENCES

1. Pearson, S., Stern, S., and McGavack, T. H., Anal. Chem. *25*, 813–814 (1953).
2. Zlatkis, A., Zak, B., and Boyle, A. J., J. Lab. Clin. Med., *41*, 486–492 (1953).
3. Bloor, W. R., J. Biol. Chem., *77*, 53–73 (1928).
4. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., J. Biol. Chem., *195*, 357–366 (1952).
5. Sperry, W. M., and Webb, M., J. Biol. Chem., *187*, 97–110 (1950).
6. Hanel, H. K., and Dam, H., Acta. Chem. Scand., *9*, 677–682 (1955).
7. Kenny, A. P., Biochem. J., *52*, 611–619 (1952).
8. Forbes, J. C., J. Lab. Clin. Med., *16*, 520–521 (1930).
9. Kabara, J., J. Lab. Clin. Med., *44*, 246–249 (1954).
10. Jones, B. J., and Moreland, F. B., Clin. Chem. *1*, 345 (1955).
11. Herrmann, R. G., Proc. Soc. Exper, Biol. Med., *94*, 503–506 (1957).
12. Schon, H., Hoppe-Seyler's Z. Physiol. Chem., *303*, 81–90 (1956).
13. Freeman, N. K., Lindgren, F. T., Yook, C. NG., and Nichols, A. V., J. Biol. Chem., *227*, 449-464 (1957).
14. Wycoff, H. D., and Parsons, J., Science, *125*, 347–348 (1957).
15. Michaels, G. D., Fukayama, G., Chin, H. P., Wheeler, P., Proc. Soc. Exper. Biol. Med., *98*, 826–829 (1958).
16. Dieckert, J. W., Carney, W. B., Ory, R. L., and Morris, N. J., Anal. Chem., *30*, 1442 (1958).
17. Martin, R. P., Biochem et Biophys Acta, *25*, 408–416 (1957).
18. Van Handel, E., Zilversmit, D. B., J. Lab. & Clin. Med., *50*, 152–157 (1957).
19. Axelrod, J., Reichenthal, J., and Brodie, B. B., J. Biol. Chem., *204*, 903–911 (1953).
20. Polonovsky, M., Bourillon, R., Bull. Soc. Chim. Biol., *34*, 712–719 (1952).
21. Van Handel, E., "The Chemistry of Phosphoaminolipids," D. B. Centen's Publishing Company Ltd., Amsterdam (1954): a) Chapter V; b) Chapter IX; and c) Chapter II.
22. Schmidt, G., Benotti, J., and Thannhauser, S. J., J. Biol. Chem., *166*, 505–511 (1946).
23. Van Handel, E., Rec. Trav. Chim., *72*, 763 (1953).
24. Carter, H. E., Celmer, W. D., Galanos, D. S., Gigg, R. H., Lands, E. M., Law, J. H., Mueller, K. L., Nakayama, T., Tomizawa, H. H., and Weber, E., J. Am. Oil Chemists' Soc., *35*, 335–343 (1958).
25. Artom, C., J. Biol. Chem., *157*, 585–595 (1945).
26. Burmaster, C. F., J. Biol. Chem., *165*, 1–6 (1946).

[Received April 22, 1958]

# Analysis of Plant Waxes by Means of Chromatography and X-Ray Diffraction

**N. WIEDENHOF, Laboratory for General and Technical Biology, Technical University, Delft, the Netherlands**

COLUMN CHROMATOGRAPHY of a number of plant waxes by means of $Al_2O_3$ led to the isolation of fractions containing paraffins, esters, alcohols, or acids, as main constituents. In the fractions these substances could be identified by means of x-ray analysis. The waxes that were studied were derived from Candelilla, carnauba palm, sugar cane, and kapok.

## Introduction

Cole (6) applied chromatography on columns of $Al_2O_3$ Woelm, anionotropic pH 4[1] to various plant waxes and succeeded in separating them into functionally homogeneous groups. However the activity of the alumina is not described entirely in the customary way (4), and the percentage of water which it contained is not mentioned. Preliminary experiments with a mixture of known composition were carried out on $Al_2O_3$ (Fisher).

Schuette and Baldinus (15) have analyzed the paraffins occurring in Candelilla wax, qualitatively as well as quantitatively, by chromatography on $Al_2O_3$ grade $F_{20}$ (Al Ore Company). They too failed to record the activity of the $Al_2O_3$ in the customary way.

[1] Supplied by M. Woelm, Eschwege.

It seemed desirable to continue Cole's attempts to separate the plant waxes in functionally homogeneous groups and to use the same separation, *viz,* $Al_2O_3$ Woelm, anionotropic pH 4[1] but to standardize it according to Brockmann and Schodder (4).

For identifying the main components in the waxes themselves, as well as in the fractions that had been obtained chromatographically, x-ray analysis seemed to be the most suitable method.

In crystallized normal long-chain aliphatic products the chain molecules are arranged into piles of uni-molecular layers of parallel chains in which the chain direction is perpendicular or at an oblique angle to the basal planes of the layers. Consequently the thickness of the layers depends on the chain length of the compound in question and on the angle of the chains with the basal plane. This thickness can be measured by x-ray diffraction as the so-called long spacing. Therefore, provided a reference standard is availalbe, the x-ray long spacing affords a means of determining the chain length of an unknown member of a homologous series of n-aliphatic long-chain compounds.

It is known that in this way the normal aliphatic wax components may be identified, not only after isolation (5, 12, 13, 14, 17) but occasionally in the