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CHEMICAL ANALYSIS

Volume I: *The Analytical Chemistry of Industrial Poisons Hazards and Solvents*
By Morris B. Jacobs

Volume II: *Chromatographic Adsorption Analysis*
By Harold H. Strain

CHEMICAL ANALYSIS

A SERIES OF MONOGRAPHS ON
ANALYTICAL CHEMISTRY AND ITS APPLICATIONS

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Volume II

Chromatographic Adsorption Analysis

By HAROLD H. STRAIN

INTERSCIENCE PUBLISHERS, INC.
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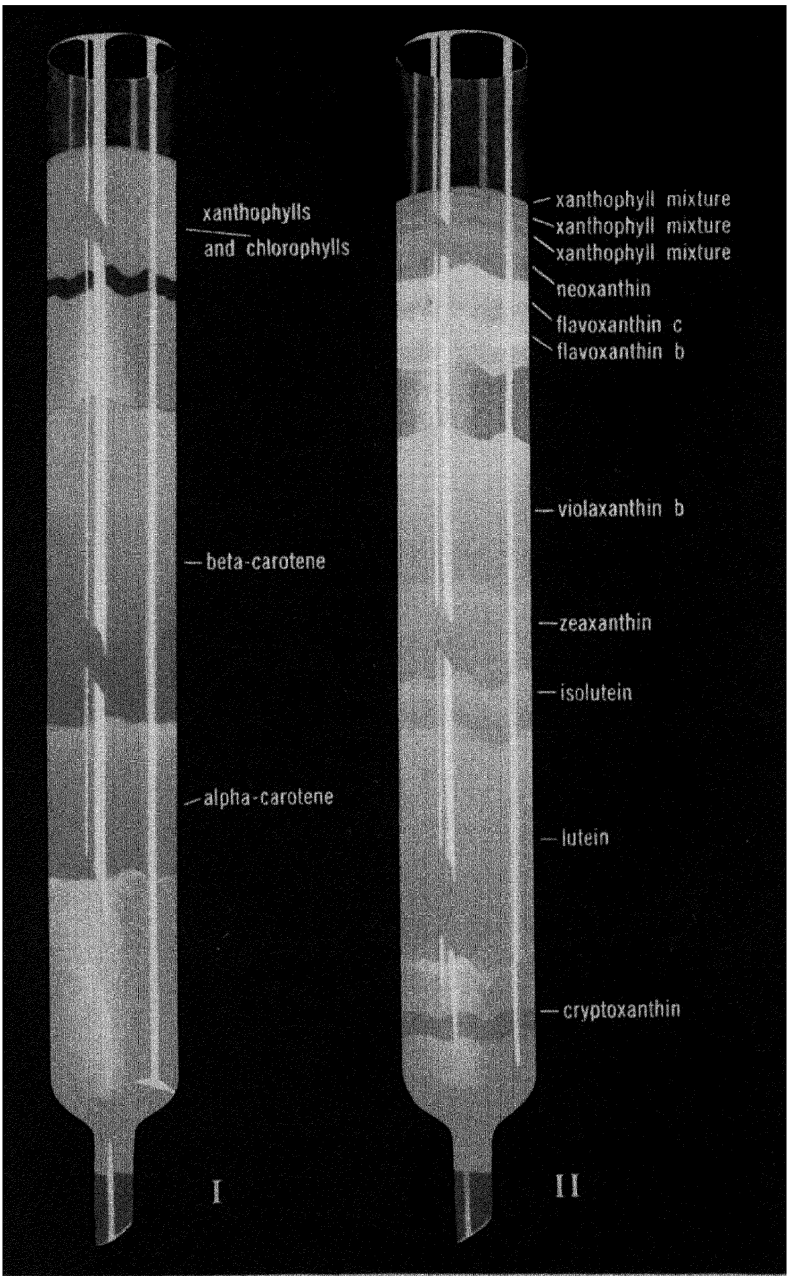

SEPARATION OF CAROTENES AND OF XANTHOPHYLLS BY ADSORPTION

I. Separation of carotenes by adsorption of petroleum ether extracts of leaves on a magnesia column

II. Separation of leaf xanthophylls by adsorption of a dichloroethane solution of these pigments on a magnesia column

Chromatographic Adsorption Analysis

BY

HAROLD H. STRAIN, PH.D.

Carnegie Institution of Washington
Stanford University, California

WITH 37 ILLUSTRATIONS

1942

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PREFACE

Many important advances in the natural sciences have depended upon the detection and isolation of specific, homogeneous chemical compounds. Discovery of methods for the preparation of both natural and synthetic substances in a high state of purity has often stimulated development in varied fields of research. These preparatory methods have played fundamental roles in such diverse studies as inorganic, organic and biological chemistry, in physiology, biology and medicine and in physics. Preparation of the elements, separation of isotopes, and purification of hormones, enzymes, proteins and other products of vital activity has involved utilization of powerful and refined methods for the resolution of mixtures

In the past ten years, extensive use has been made of a unique columnar adsorption method for the detection, isolation and purification of numerous compounds not preparable by other methods. Substances so similar in chemical structure and reaction that they are inseparable by the most selective methods have been prepared quickly, efficiently and conveniently by means of this new technique. Known as *chromatographic adsorption* because of its original use with pigments, this adsorption procedure has permitted the writing of new chapters in our knowledge of chemical compounds. It is a tool that has broken the turf in many fresh fields, especially in chemistry and biology. An indication of its widespread use may be gained by perusal of the bibliography appended to this publication.

In this summary of our knowledge regarding the chromatographic adsorption method, major emphasis has been placed upon experimental procedure. Undoubtedly, the greatest and probably the most important interest concerns application of the method to new problems, to the detection and preparation of new compounds. These applications depend, to a large extent, upon knowledge of the previous investigations, particularly upon those in related fields. As a consequence, reference has been made to most of the published investigations.

Because of the war in Europe, many of the leading foreign, scientific journals have not been available since 1939. For this reason,

some recent important papers may have been omitted from the bibliography. Appearance of reports on the chromatographic adsorption method in many different languages has made it desirable to follow the example of Chemical Abstracts and to translate the titles.

Apparatus used by various investigators has been redrawn, often with considerable modification. The aim has been to simplify the drawings and to emphasize the novel features introduced by the original author.

In the course of the preparation of this publication, many helpful suggestions were made by Dr. H. A. Spoehr and Dr. J. H. C. Smith. Dr. J. W. McBain called attention to several important papers. This constructive assistance turned out to be of great value. It is gratefully acknowledged.

HAROLD H. STRAIN
Stanford University, California
April, 1941

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I. HISTORICAL INTRODUCTION

A good technique sometimes renders more service to science than the elaboration of highly theoretical speculations

CLAUDE BERNARD.

In 1906 at the then Russian city of Warsaw, there was devised a new and ingenious adsorption method of chemical analysis that was destined to influence the life of man and beast the world over. It was a strikingly original and simple invention, yet it projected the light of discovery into the forbidding mazes of chemical substances that constitute all things—mineral, vegetable and animal.

In effect, this unique adsorption technique provided scientists with a particularly efficient procedure for preparation of chemical compounds in a high state of purity. Isolation and identification of chemical substances, prerequisites to investigations of composition and molecular structure, were thus brought to a new state of perfection. A plateau in the domain of chemical exploration was attained.

Through the extension of his perception resulting from use of the adsorption analysis, the chemist beheld familiar compounds in new roles. Because it became possible to separate many substances from other similar compounds, the course of many reactions could be followed with greater precision. The adsorption method eventually made feasible the isolation of numerous ephemeral substances such as vitamins, drugs, and pigments. It contributed to a better understanding of obscure natural phenomena, as for example, the nutrition of plants and animals, the effects of hormones upon the form and character of man and animals, and the occurrence and functions of vitamins in plants and animals. Through its use, undreamed of reactions were found as parts of the complex machinery of living cells. The achievements attributable in whole or in part to this new chromatographic adsorption method of analysis transcend by far the consequences of those stirring political events that have occurred in the city of its origin.

M. Tswett, the inventor of this excellent analytical method that has contributed so much to chemical progress, was himself a botanist,

not a chemist. In the course of his investigations of the pigments in plants, he performed a simple experiment that was to form the basis for the chromatographic adsorption method. Into the constricted portion of a glass tube like that shown in figure 1, Tswett placed a plug of cotton, and above this he tamped small portions of finely divided adsorptive material, such as precipitated chalk. This porous filter or adsorption column as it is now called was attached to a suction flask and a green, petroleum ether extract of dried leaf material was drawn through it. Under these conditions, two green pigments, the chlorophylls, were held by the adsorptive solid near the top of the column, one yellow pigment, identical with the carotene of carrot roots, passed rapidly through the tube with the solvent, and two or three other yellow pigments, known as xanthophylls, formed yellow bands or zones on the adsorbent below the green bands near the top. In this way the pigments were gradually separated from one another. According to Tswett, the components of the pigment mixture were resolved into a regular pattern akin to the light rays in the spectrum.

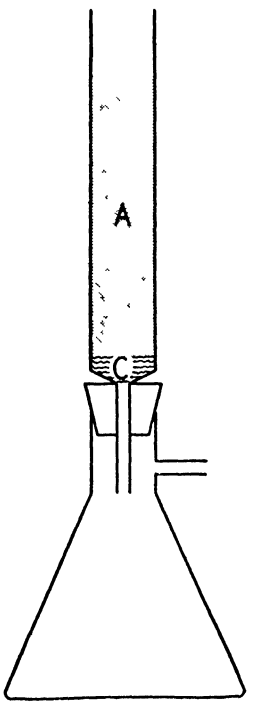


Fig 1 Simplest adsorption apparatus A, adsorbent, C, cotton or glass wool

Formation of the bands on the adsorbent was traced to the fact that weakly adsorbed pigments moved through the column faster than the strongly adsorbed ones. Completeness of the separation of the several bands from one another was improved by washing the column with fresh portions of the solvent. Subsequent work has demonstrated this to be an essential step in the preparation of pure compounds by Tswett's method.

After the leaf pigment mixture was separated into bands on the column and after these bands had been completely separated from one another by washing the column with fresh solvent, Tswett pressed the moist adsorbent from the glass tube. He thus obtained a cylinder of cohesive adsorbent containing the bands of pigments. When this cylinder was divided between the bands with a knife, the

separation of the pigment mixture into its constituents was compelted.

Only one more step remained in the analytical procedure; namely, the liberation of the pigments from the adsorbent. This was accomplished by extraction or elution of the pigments from the solid material with alcohol. It caused immediate dissolution of the adsorbed compounds, and it thus provided solutions of the pure pigments. By this technique, one of the greatest desiderata of the chemist, the resolution of complex mixtures into their constituents, was realized.

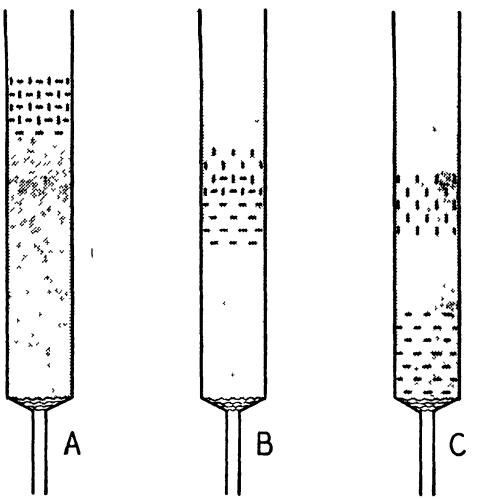


Fig. 2. Development of the chromatogram A, solution of two compounds, ı and –, passed into upper portion of the column B, column washed with fresh solvent that carries ı and – along at different rates C, complete separation of ı from – with slight widening of bands.

For the description of the various stages in Tswett's adsorption technique, a complex terminology has been developed. The series of colored bands upon the adsorption column is referred to in German as a "chromatogramm." This word has been transposed to English with omission of the final *m*. Percolation of fresh solvent through the column in order to obtain further separation of the bands is as shown in figure 2 described as "development of the chromatogram." Resolution of mixtures into their several constituents with adsorption columns is known as "chromatographic analysis" or "chromatographic adsorption analysis." Utilization of chromatographic

methods is called "chromatography." "Chromatograph," a verb denoting the resolution of mixtures by adsorption on Tswett columns, represents a new usage that would not be suspected from the Greek roots of the word itself but that is now widely accepted in this highly specialized field.

Although this terminology indicates that the adsorption method is limited to use with colored substances, it was discovered that colorless as well as colored compounds form a series of well-defined bands in the adsorption columns. Numerous modifications of the procedure, described in chapter VI, made it possible to locate these bands of colorless materials on the columns. In this way chromatographic methods became applicable to the resolution of mixtures of all types of chemical compounds.

In spite of the fact that Tswett resolved the pigments of leaves into more constituents than had previous investigators and even though he considered most of the factors that have since been shown to affect the use of adsorption columns, his new method did not find wide application to the preparation of chemical compounds until twenty five years after its discovery. There were three principal reasons for this. One was the failure to prepare sufficient quantities of materials for analysis by the macro chemical methods that were then available. Another was the belief, sponsored primarily by the ranking German chemists Willstatter and Stoll, that adsorption might alter or decompose labile compounds such as the leaf pigments. The third was the war of 1914–1918 which stopped the publications of Tswett.

A few other workers soon made use of the chromatographic adsorption method. As early as 1912, Rogowski in Dhéré's laboratory and later Dhéré and Vegezzi investigated the pigments of leaves and of snail livers. Palmer (1913) and Palmer and Eckles (1914) utilized adsorption columns for resolution of the pigments of butterfat. Attempts were also made to separate the yellow, fat-soluble pigments of various natural sources by Coward (1924) and by von Lipmaa (1926). In all these investigations as in those of Tswett, only minute quantities of pigments were recovered from the columns. (Palmer 1922).

When in 1931 Kuhn and Lederer resolved carrot root carotene into two similar, isomeric hydrocarbons by adsorption upon columns of fibrous alumina, a great impetus was given to the use of chromatographic adsorption methods. Crystalline carotene, regarded for more than a century as a homogeneous substance, was separated into

α- and β-carotene. Each of these was prepared in good yield and in sufficient quantities for ultimate analysis by the Pregl micro combustion procedures.

In the same year, Kuhn, Winterstein and Lederer extended the use of adsorption columns to preparation of crystalline leaf xanthophyll (lutein) and to the isolation of the xanthophylls of egg-yolks. Red carotenoid pigments of bacteria infecting salt fish were likewise prepared in a crystalline state by the use of the adsorption technique (Petter).

A full quarter of a century had elapsed between Tswett's discovery and the application of his technique to preparation of homogeneous compounds in sufficient quantity for further chemical examination. Then like a dormant plant, the latent method suddenly burst into flower attracting workers from the hives of virtually all industry and research.

Owing to the importance of the carotenes and some related carotenoids as natural precursors of vitamin A, a great deal of attention in many countries was quickly directed to the possible use of adsorption columns for preparation of other biologically significant compounds. The use of fibrous alumina by Kuhn and Lederer, to which reference has already been made, demonstrated the importance of the choice of adsorbent, particularly in respect to method of preparation and activation. Subsequent work along these lines resulted in development of highly active and selective adsorbents, a number of which are described in chapter IV. Extensive industrial developments made many of these adsorbents available on a large scale and at a reasonable cost. Similar advances made several new organic compounds available as solvents. Under certain conditions water was also used as a solvent for the formation and development of chromatograms.

With the development of active and selective adsorbents, Tswett's adsorption method was soon utilized for separation of isotopes, for resolution of mixtures of the elements, and for separation of mixtures of inorganic ions. In this latter respect chromatographic adsorption may yet displace or supplement the more conservative methods of qualitative analysis. In the field of organic chemistry virtually every class of substance (hydrocarbons, alcohols, ketones, aldehydes, acids, esters, sulfonic acids, heterocyclic compounds, dyes, drugs and their substitution products) has been investigated with chromatographic adsorption methods. Various natural products as toxins,

enzymes and proteins have been studied with the aid of adsorption columns. Tswett columns are finding rapid application in industrial operations as well as in experimental work. Preparation of still more active and selective adsorbents, will undoubtedly lead to wider use of the chromatographic adsorption method. Reference to the literature reveals that applications of the method are still expanding rapidly in many different fields.

Chemical compounds are always adsorbed in the same sequence on the columns providing the same solvents and adsorbents are employed under the same conditions. Only one or two unverified exceptions to this rule have been reported; namely, the adsorption of lutein and zeaxanthin (Strain 10). Changes in the solvents or adsorbents may reverse the relative positions of some adsorbed compounds. This was first reported by Tswett who found that one of the yellow xanthophylls of leaves was adsorbed with the green chlorophyll when petroleum ether was used as the solvent. If a little alcohol was added to the petroleum ether, the xanthophyll was adsorbed below the chlorophyll. This phenomenon has been confirmed (Strain 10) and has been made the basis of a method for the separation of chlorophylls and xanthophylls (Seybold and Egle) (See page 124). The same effect has been observed when water is added to the solvent or to the adsorbent (Mackinney). In the case of ferric and zinc ions adsorbed upon columns of 8-hydroxyquinoline from aqueous solution the order is zinc (upper band) and ferric (lower band). Upon adsorption from acetic acid solution, the order is reversed (Erlenmeyer and Dahn). With different adsorbents, Cassidy has found that lauric acid may be adsorbed either above or below stearic acid (page 86).

Nearly every application of the adsorption method had as an immediate objective the preparation of pure compounds for use in other investigations. As a result, a great deal of fragmentary information concerning the use and applicability of adsorption methods soon accumulated. From this mass of information often pertaining to the preparation of uncommon and labile materials, empirical rules that enable experienced workers to obtain spectacular results have been established. Inexperienced workers on the other hand have not always been able to take the time necessary to learn by costly trial and error, and they have had to utilize other less efficient preparative methods.

Rules concerning the applications of the chromatographic adsorp-

tion method are valid only when unusually circumscribed conditions are maintained. Too much attention can not be paid to the detailed procedures that have been developed as the result of innumerable trials and that are discussed in Chapters III, IV and V. Many of these facts pertaining to the Tswett adsorption method have been reviewed in books and journals (Winterstein 1; Zechmeister and Cholnoky; Cassidy; Sørensen 3; Schwab; Meyer; Willstaedt 1; Stix; Valentin; Lederer 1, 12; Koschara 6; Armstrong; Celsi; Coffari; Cook; Dam; Hesse; Brockmann). References to the literature and specific examples are given in chapters VII, VIII and IX.

In the same year that Tswett first reported the use of adsorption columns, Goppelsroeder described a somewhat similar analytical method known as capillary analysis. According to this method, one end of strips of adsorptive paper are placed in solutions of the pigments or other materials to be resolved. As the liquid is drawn into the filter paper by capillary forces, the substances in solution gradually separate from one another forming a series of bands analogous to those observed in the Tswett columns. The rate of flow of the solvent into the paper strips can be increased by hanging them over the edge of the vessel containing the solution of the mixture. To some extent the bands may be separated from one another by placing the paper strips in a portion of the fresh solvent after some of the solution has been adsorbed.

In principle the capillary analysis procedure is similar to the columnar adsorption technique. However the development of the chromatogram is rather difficult and the preparation of materials in quantity is virtually impossible. As a consequence, capillary analysis has not found extensive use. A recent modification of the Goppelsroeder technique in which the paper alone or paper dusted with adsorptive powders is placed between glass plates (Brown) is subject to the same limitations as the original method. Various applications of the capillary analysis methods have been ably reviewed by Rheinboldt.

II. APPLICATIONS OF CHROMATOGRAPHIC ADSORPTION METHODS

Tswett's adsorption method is in effect a tool that finds application in all branches of science concerned with chemical compounds and their reactions. Its application to new problems necessitates knowledge of the ends attainable through its use in so far as these have been established or predicted. Thus far, a thorough theoretical treatment of chromatographic methods has not been presented so that it is difficult to consider the subject from this aspect. See page 30. Because the methods are in effect practical ones and because the major interest attaches to their application, chromatographic adsorption is treated here from the analyst's or technician's point of view.

The principal objectives attainable through use of the method are:

1. Resolution of mixtures into their constituents.
2. Determination of the homogeneity of chemical substances.
3. Comparison of substances suspected of being identical.
4. Purification of substances.
5. Concentration of materials from dilute solutions.
6. Recognition and control of technical products.
7. Quantitative separation of one or more constituents from complex mixtures.
8. Determination of molecular structure.
9. Combination with electrophoretic separations.
10. Regeneration of substances from complex addition compounds.

1. Resolution of Mixtures Into Their Constituents

By far the most important application of adsorption columns is the separation of mixtures into their several components. To this end, the procedure is similar to that employed by Tswett. A solution of the mixture is passed through the column until a considerable quantity of the dissolved substances is adsorbed near the top of the adsorbent. The column containing the adsorbed compounds is then washed with fresh portions of the pure solvent in order to complete the development of the chromatogram.

Satisfactory separation of the adsorbed compounds depends upon

a number of different conditions such as the concentration of the materials in solution before adsorption, the nature of the adsorbent and solvent, and the size of the adsorption column. The effect of these and of still other conditions are discussed in detail on pages 29 to 54.

After the chromatogram has been developed, each band of resolved material is usually removed separately from the glass tube, although several other procedures may be resorted to. See page 45. Substances adhering to the adsorbent are eluted with ethanol or similar polar solvents, and the adsorbent is removed from the solution by filtration or centrifugation. The resolved compounds in these solutions are isolated, identified or estimated by the accepted methods of analysis. Substances having physiological properties may be tested directly on plants or animals.

In this application of the adsorption method, preparation of sufficient quantities of materials for subsequent physical, chemical or biological investigations is usually the primary objective. Studies of this kind usually require a considerable supply of the pure compounds so that one might expect to find large columns in use. In practice a number of difficulties which do not appear insurmountable and which are discussed in detail on page 32 have prevented adoption of such apparatus.

The adsorption method lends itself readily to the preparation of extremely small quantities of chemical compounds. It involves smaller losses than the conventional fractional distillation and crystallization methods. Because the supply of raw materials for many investigations is often limited, this economy of the adsorption method is a distinct advantage.

Much smaller quantities of chemical substances can be resolved with diminutive or micro columns than can be isolated by crystallization or distillation (page 43). The minute quantities of resolved products may nevertheless be identified by special reactions, by spectrometry or spectrophotometry, or by comparison with authentic preparations of the same materials through readsorption on fresh columns as described on page 12.

Complex mixtures, such as those encountered in the extracts of plant and animal products, are often very difficult to separate into all their constituents with adsorption columns. One constituent may influence the adsorption of others to such an extent that a pre-

liminary, partial resolution of the mixture by crystallization, distribution between immiscible solvents, saponification or distillation is necessary. If this is impracticable, repeated adsorption of the mixture upon fresh columns must be resorted to. A typical illustration of the purification of a biological product through successive adsorption upon fresh columns is found in the preparation of vitamin K described on page 107.

When mixtures are purified or partially resolved before adsorption, care must be taken to prevent loss of some of the constituents in solutions, such as the mother liquors, that are to be discarded. Failure to observe this precaution has led to erroneous conclusions concerning the composition of several natural mixtures, for example that of the leaf xanthophylls (See page 143).

2. Determination of the Homogeneity of Chemical Substances

When examined with adsorption columns, many materials presumed to be homogeneous or chemically pure are often found to be mixtures of two or more compounds. This is indicated by the formation of two or more bands on the column. It results from the highly selective action of the adsorbent which makes possible the separation of contaminants not detectable by other methods.

In the absence of more selective, preparatory methods, chromatographic homogeneity is synonymous with chemical purity. It should be kept in mind, however, that each substance must be adsorbed under conditions favorable for the separation of its contaminants. This can be established only by the use of various adsorbents with different solvents.

So effective has this application of the columnar adsorption method become that its advocates have made it the basis of a new definition of chemical purity. Substances are said to be chromatographically homogeneous if they can not be resolved further with adsorption columns.

Some substances although pure are altered by reactive or by basic or acidic adsorbents and may give rise to two or more bands. An example is the decomposition of 2,4-dinitrophenylhydrazones on magnesia (Strain 4). Usually considerable experience with adsorption methods is necessary before one can rely upon them as a final criterion of purity. As a supplement to the more common analytical procedures, they are indispensable.

3. Comparison of Substances Suspected of Being Identical

The identity or nonidentity of two preparations may be established in the following way. Solutions of the materials are prepared and a portion of each is adsorbed on separate columns in order to make certain that the preparations are homogeneous (formation of one band). Portions of the two solutions are mixed, and this mixture is passed through a fresh column of the adsorbent. Formation of a single band indicates that the two preparations are identical. Formation of two bands demonstrates that the two preparations are different.

This application of the chromatographic adsorption method is analogous to the identification of substances through comparison of their melting points with those of their mixtures. In Germany, writers have called the comparison of two substances by adsorption of a mixture of them a *Misch-chromatogramm*, literally a mixed chromatogram. The method is also referred to as the *three column* or *three tube* test (Lederer; Scheer).

4. Purification of Substances

Often it is necessary to isolate only one or two substances from complex mixtures. For such preparations the use of adsorption columns offers many advantages. The mixtures may be adsorbed in the usual way and only the desired constituent is removed from the adsorption column. More commonly, adsorbents and solvents are so chosen that the constituents to be isolated are more firmly or less firmly adsorbed than the unwanted constituents of the mixtures. Under the former conditions the most strongly adsorbed constituent is held by the adsorbent while the contaminants are washed through the column. The adsorbed material is subsequently recovered by elution from the adsorbent. When the desired material is the least firmly adsorbed constituent of the mixture, it passes through the column while the contaminants remain bound to the adsorbent. In this purification procedure, adsorbents that react with the contaminants are occasionally used.

Although not known as chromatographic adsorption, removal of contaminants from technical products has long been effected by this procedure. Examples are the decolorization of lubrication oils by filtration through adsorptive earths, decolorization of sucrose solutions by filtration through towers of charcoal, removal of water vapor

from air with activated alumina, and preparation of potable water by adsorption of undesirable constituents on permutit (see chapter IX).

5. Concentration of Materials from Dilute Solutions

If a very dilute solution is passed through a column of adsorbent having a very great affinity for the solute, the latter will be concentrated in a band that moves slowly through the tube. The adsorbent acts like a filter capable of removing solute from the solution that passes through it. When the band is removed from the column and the adsorbed material is eluted, its solution is many times more concentrated than that from which it was adsorbed.

Through this application of the adsorption method, the solute in many liters of solution can be concentrated exceptionally rapidly. The method is especially useful in biological investigations because it may be used to displace or to supplement concentration by distillation, a process that is often troublesome. Interesting examples are the preparation of the pigments of urine, reported by Koschara (page 110) and the concentration of alkaloids described by Fink (page 101).

6. Recognition and Control of Technical Products

Chromatographic adsorption can be used to determine the source of some raw materials and the presence of certain adulterants in substances of technical importance. For example, adulteration of fats with red palm oil increases the proportion of α-carotene. This is readily detectable by adsorption of the saponified fat upon columns of magnesia from solutions in petroleum ether. Determination of the pigments contained in butterfat through the application of chromatographic methods enables one to draw conclusions concerning the rations of the cows from which the butter was obtained. In these applications of the adsorption method the procedures employed are analogous to those described in the preceding sections of this chapter. An example of the detection of artificial coloring matter in wine is described on page 161.

7. Quantitative Estimation of One or More Constituents of Complex Mixtures

Under favorable conditions one may resolve mixtures upon adsorption columns and estimate the quantities of the several constitu-

ents from the width and the intensity of the bands. More precise values for the quantities of material present may be obtained by elution of the resolved substances followed by determination of the quantity recovered in the elutriate by standard analytical methods. As a precaution, one should determine the proportion of the adsorbed materials that are eluted under the conditions employed. Occasionally losses of adsorbed compounds occur as the result of oxidation and through reaction with the adsorbent. As a consequence, better results are usually obtained when the adsorption procedure is so modified that the substance to be determined passes through the column without being adsorbed but with simultaneous separation from strongly adsorbed substances that interfere with its estimation. The material that passes through the column need not be separated from all the constituents of the original mixture.

Numerous examples of this application of Tswett's adsorption method are found in the literature pertaining to the leaf pigments. The carotene, least adsorbed of these colored materials, is separated from the green chlorophylls and yellow xanthophylls by passage of an extract of dried and powdered leaves through a column of calcium carbonate, (Tswett) soda ash (Kernohan) or magnesia (Strain 10). The carotene itself is estimated colorimetrically in the percolate. Besides the carotene, this percolate contains many substances that are colorless and therefore do not affect the colorimetric estimation (See page 132).

8. Determination of Molecular Structure

A vast amount of experience that can not be repeated here has brought to light a relation between the adsorbability of organic compounds and the architecture of their molecules. This permits deduction of the arrangement of the atoms in organic molecules from the positions of the adsorbed compounds relative to similar substances of known structure. In this respect, chromatographic adsorption is a valuable supplement to the tedious analytical methods of the organic chemist. Some of these relations between adsorbability and molecular structure are summarized and illustrated in the tables appended to this chapter.

The adsorbability of organic compounds is influenced primarily by the nature and the number of the polar groups in their molecules. This is illustrated by the following series in which the most strongly

adsorbed compounds are listed at the top, the least adsorbed compounds at the bottom.

Acids and bases
Hydroxyl, amino and thio compounds
Aldehydes, ketones and esters
Halogen compounds
Unsaturated hydrocarbons
Saturated hydrocarbons

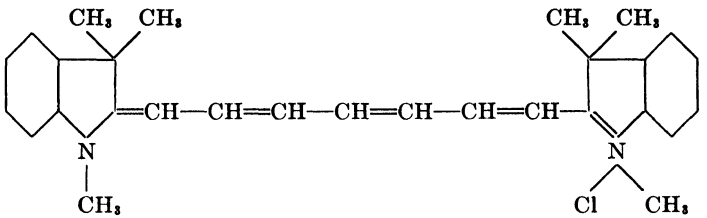
In practice, it has not been possible to separate all types of organic compounds into these several structural categories by the use of chromatographic adsorption methods. This is due in part to variations in adsorbability with the size of the molecule, large molecules usually being more strongly adsorbed than small molecules of the same class. It is complicated further by variations in adsorbability with stereoisomeric change, and with changes of the polarity of the substituent groups depending upon their positions in the molecule. The more complex the skeleton of the molecule and the greater the number of the polar substituent groups, the greater is the difficulty in deducing the structure from the position of the compound on the adsorption column. Among organic compounds of a given class, however, precise correlations may be made between the chemical structure and the adsorbability of the molecules.

In homologous series of organic compounds, the adsorbability increases with an increase in the number of double bonds as shown by tables 1, 2 and 3. Among compounds of the same type, adsorbability also increases with an increase in unsaturation as shown by tables 4 and 5. Among isomeric compounds such as the polycyclic hydrocarbons shown in tables 6 and 7, there is no simple relation between number of double bonds and adsorbability. The shape and the polarity of the molecule, as indicated by color, appear to exert greater influences than the number of double bonds alone.

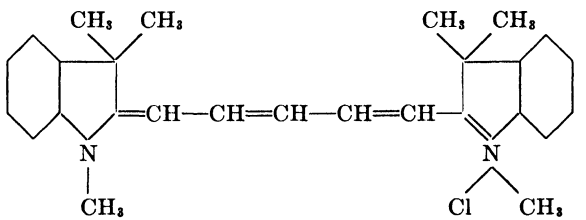
There is a very close relationship between the color, the adsorbability, and the number of conjugated double bonds of the diphenylpolyenes shown in table 8. A similar relation between the color, adsorbability and number of double bonds is also found among the isomeric carotenes illustrated in tables 9 and 10. In all these compounds, color or light absorption is intensified and shifted toward the red region of the spectrum by an increase in the number of double bonds. The effect is enhanced if the double bonds occur alternately

TABLE 1

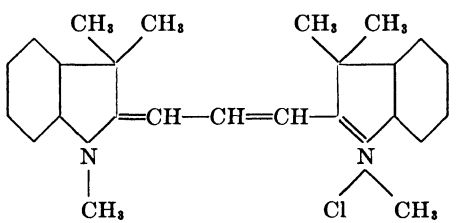
Adsorption series of some indolenin dyes (Ruggli and Jensen)



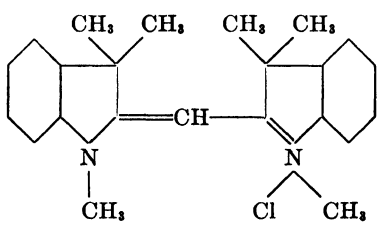
Indolenin-blue
(uppermost band)



Indolenin-violet



Indolenin-red



Indolenin-yellow
(lowest band)

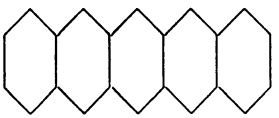
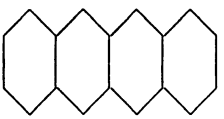
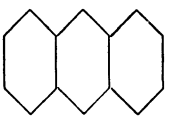
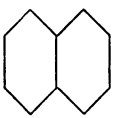
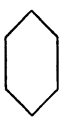
with single bonds. Dependence of adsorbability and color on the number of double bonds is especially pronounced here because of the absence of other polar groups.

When polar groups such as hydroxyls are added to the carotene molecules thus forming xanthophylls as illustrated in table 11, the

TABLE 2

Adsorption series of some linear polycyclic compounds

(Assembled from Winterstein and co-workers)

	2,3,6,7-Dibenzanthracene (uppermost band)
	Naphthacene
	Anthracene
	Naphthalene
	Benzene (lowest band)

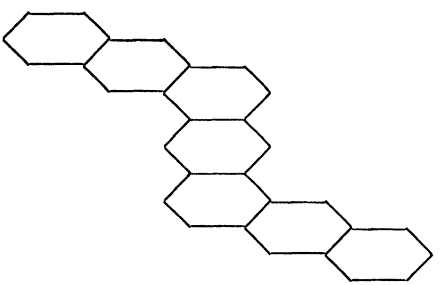
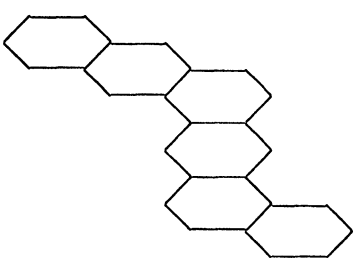
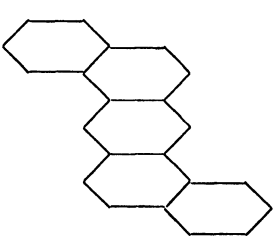
adsorbability of the compounds is increased a great deal, but the color, which depends primarily upon the double bonds, is unchanged. With isomeric dihydroxy polyenes such as lutein and zeaxanthin, the relative position of the two pigments on the adsorption column is still determined by the secondary effect of the double bonds. De-

pendence of the adsorbability of the xanthophylls upon the number of hydroxyl groups in the molecule is illustrated further by table 12.

The adsorbability of some stereoisomeric compounds such as position isomers and *cis* and *trans* isomers is related to their spacial con-

TABLE 3

Adsorption series of some benzanthracenes

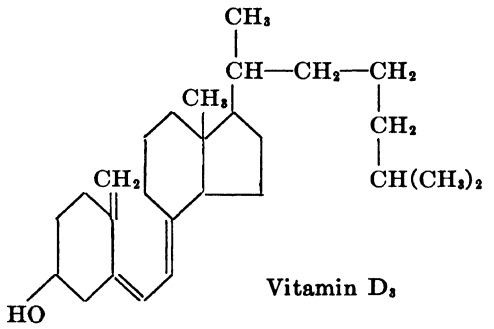
	1,2:2′,3′-5,6:2″,3″-Dinaphthanthracene (uppermost band)
	1,2-Benz-5,6-(2′,3′-naphth)-anthracene
	1,2,5,6-Dibenzanthracene (lowest band)

figuration (table 13). With compounds of both types, the isomer with the greatest dipole is usually most strongly adsorbed (Arnold).

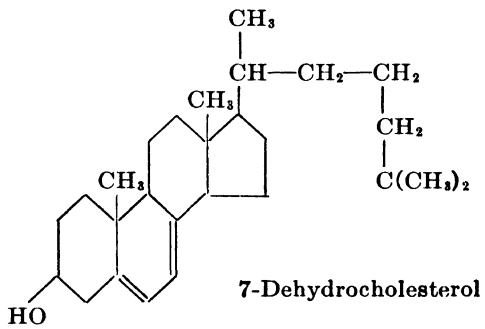
Optically isomeric or enantiomorphous compounds are separable upon columns composed of optically active adsorbents such as lactose (Henderson and Rule) (See page 92), but rules pertaining to the

TABLE 4

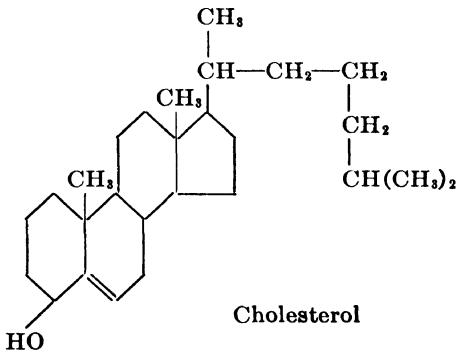
Adsorption series of some sterols. (*See page 97*)



Vitamin D_3



7-Dehydrocholesterol



Cholesterol

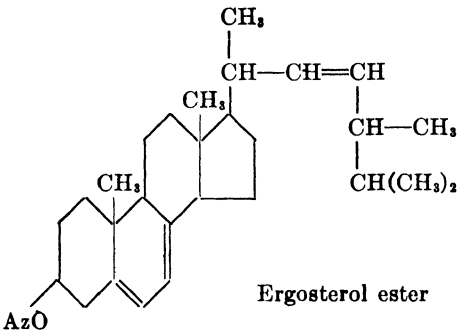
ability of these isomers in relation to their structure have not en formulated. It is interesting to note that a difference be- the adsorbability of optically isomeric dyes on wool was re-

TABLE 5

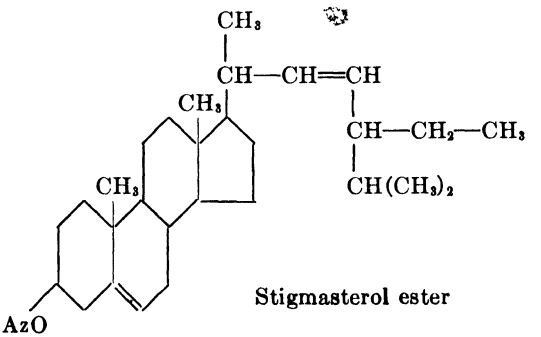
Adsorption series of sterol esters of azobenzene p-carboxylic acid

(*Az* = $C_6H_5N{=}NC_6H_4C(O)$ —)

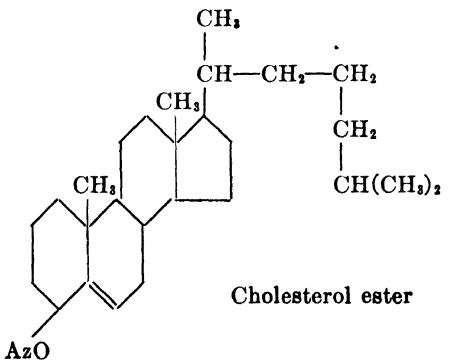
From Ladenburg, Fernholz and Wallis. (See page 98)



Ergosterol ester



Stigmasterol ester

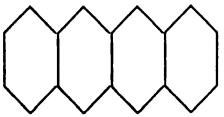
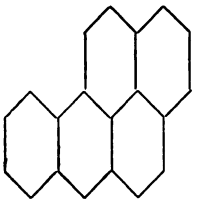
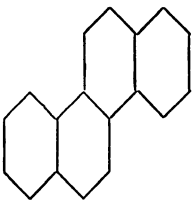
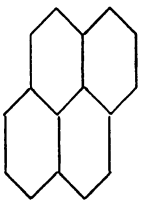
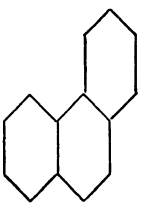


Cholesterol ester

TABLE 6

Adsorption series of some polycyclic compounds

(Assembled from Winterstein and co-workers)

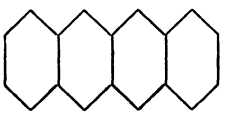
	Naphthacene (uppermost band)
	1,2-Benzpyrene
	Chrysene
	Pyrene
	Phenanthrene (lowest band)

ported a number of years ago (Porter et al.), although this observation was never used in the field of chromatographic investigations.

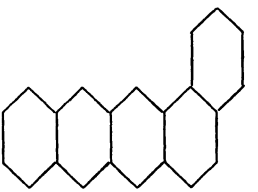
Changes in structure caused by chemical reaction may also be de-

TABLE 7

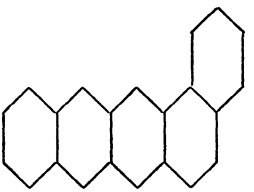
Relationship between adsorbability and color of certain pairs of polycyclic compounds (Winterstein and co-workers)



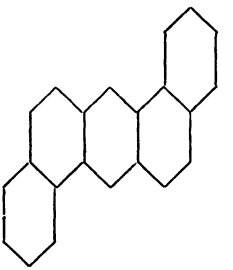
Naphthacene
(orange-red, upper band)



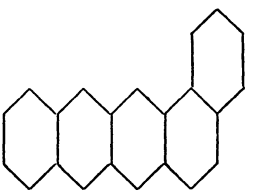
1,2,6,7-Dibenzanthracene
(orange-yellow, lower band)



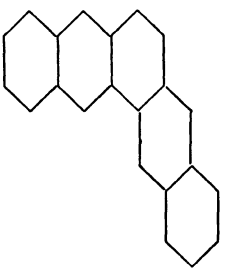
1,2,6,7-Dibenzanthracene
(orange-yellow, upper band)



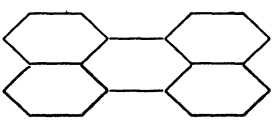
1,2,5,6-Dibenzanthracene
(colorless, lower band)



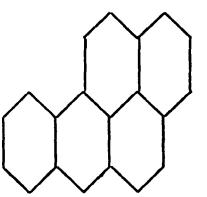
1,2,6,7-Dibenzanthracene
(orange-yellow, upper band)



1,2-(2′,3′-naphth)-anthracene
(yellow, lower band)



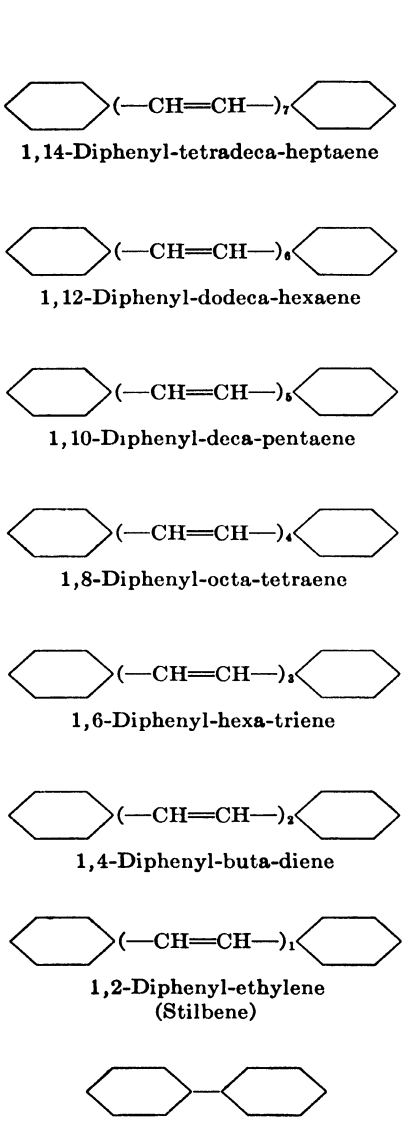
Perylene
(orange-yellow, upper band)



1,2-Benzpyrene
(yellow, lower band)

TABLE 8

Relationship between adsorbability, color and number of ethylene groups in the synthetic diphenylpolyenes. (Assembled from Kuhn and Winterstein 5; Winterstein and Schön 2; and Hausser. The three uppermost polyenes have not yet been tested in the adsorption column). Curves represent molar absorption coefficients; solvent, benzene; wave length in mμ.



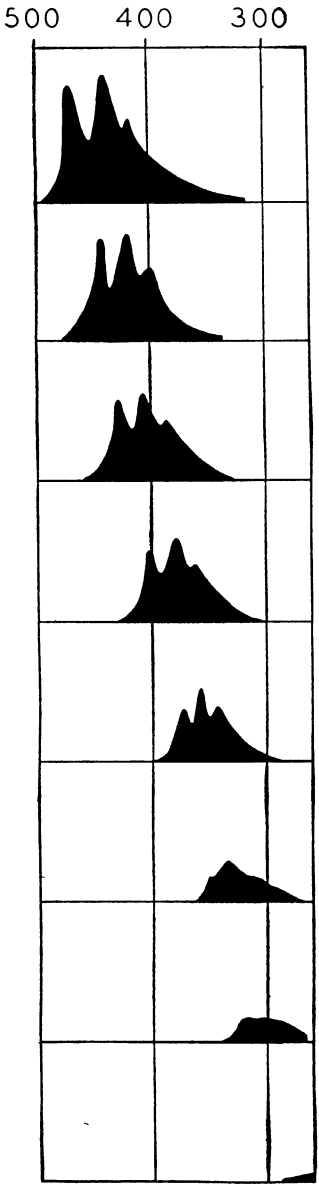
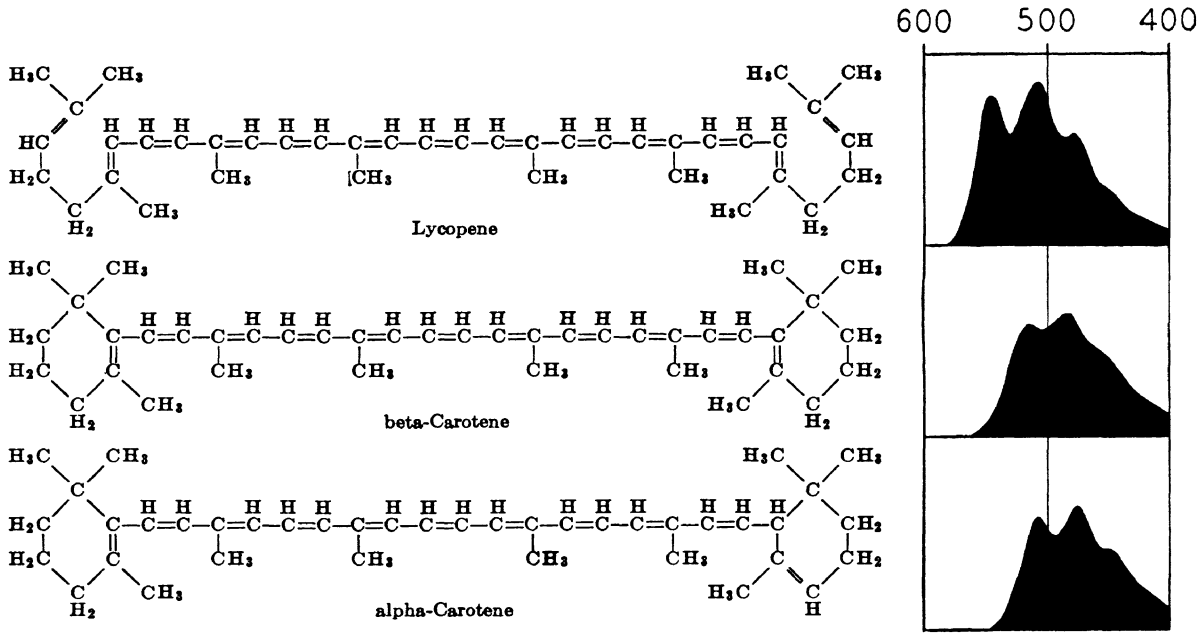


TABLE 9

Relationship between adsorbability, color and number and arrangement of double bonds of isomeric carotenes. *(See page 15).* *Curves represent molar absorption coefficients; solvent, carbon disulfide; wave length in mμ.*



tected by use of chromatographic methods. For example if polar groups are removed by a reaction, the resultant compound will not be so strongly adsorbed as the original one. Polar groups may be neutralized by reaction such as the formation of an ester from an alcohol and a fatty acid. In this case, the ester is not so strongly adsorbed as either the acid or the alcohol.

Conclusions regarding the chemical structure of organic compounds from their positions upon adsorption columns may be reached by the following and analogous procedures. If the compound is adsorbed below unsaturated hydrocarbons, it is probably a more saturated compound of this class. It is not likely to contain strongly polar groups like hydroxyl, amino or carboxyl. Materials adsorbed far above unsaturated hydrocarbons usually contain strongly polar groups.

TABLE 10

Adsorption series of the common carotenes. (*See page 128*)

Lycopene	$C_{40}H_{56}$	(13 double bonds, 11 conjugated)
gamma-Carotene	$C_{40}H_{56}$	(12 double bonds, 11 conjugated)
delta-Carotene	$C_{40}H_{56}$	(12 double bonds, 10 conjugated)
Flavoxanthin-like carotene (structure unknown)		
beta-Carotene	$C_{40}H_{56}$	(11 double bonds, 11 conjugated)
alpha-Carotene	$C_{40}H_{56}$	(11 double bonds, 10 conjugated)

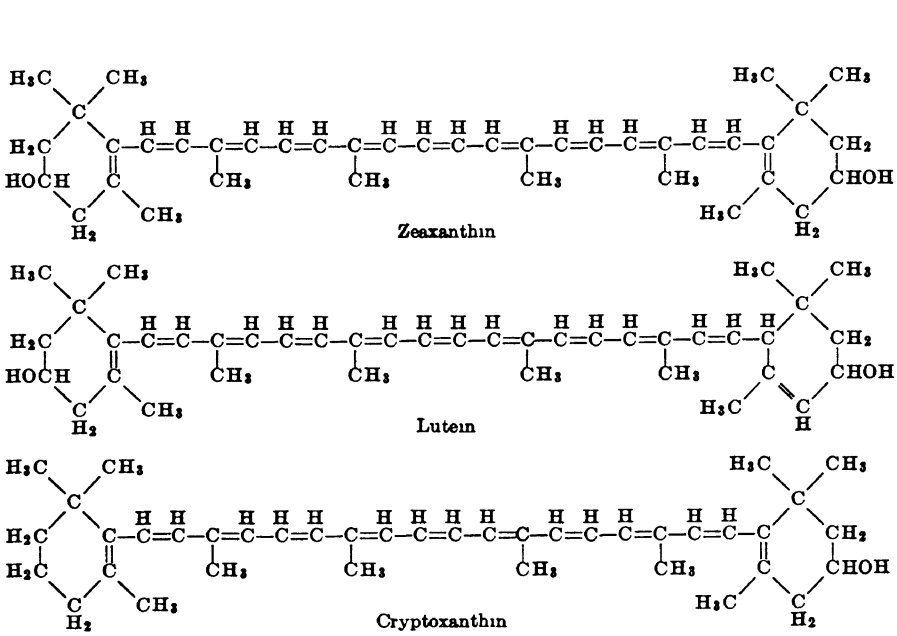
Changes in the adsorbability of organic molecules caused by chemical reaction also give clues to the structure of the molecules. It after treatment with alkalies, a substance is adsorbed at the same relative position on the column (formation of one band upon adsorption of the untreated and treated materials) one may conclude that the compound was not altered and therefore it is not an ester. If the compound is no longer adsorbed in the same position, it was altered. When treatment of a substance with the reagent that is specific for a given group does not alter the relative position of the substance upon the adsorption column, the group must not occur in the molecule. If the relative position on the column is altered, the specific group probably occurs in the molecule (Strain 6).

9. Regeneration of Substances From Their Complex Addition Compounds

Chromatographic adsorption has found some use in the separation of certain hydrocarbons and sterols from their addition compounds

TABLE 11

Relationship between absorbability, color, double bonds and hydroxyl groups of xanthophylls. Curves represent molar absorption coefficients; solvent, carbon disulfide; wave length in mμ.



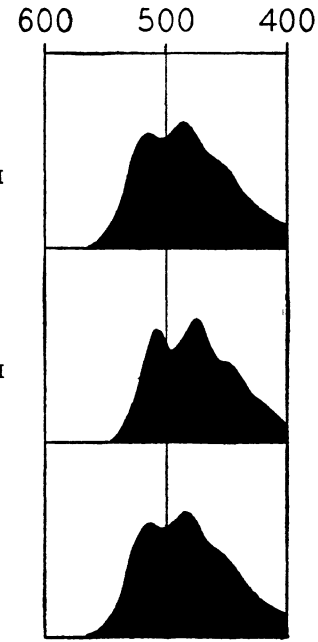


TABLE 12

Adsorption series of the common xanthophylls

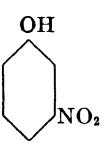
(Zechmeister and Cholnoky 10)

Fucoxanthin	$C_{40}H_{56}O_6$	
Capsorubin	$C_{40}H_{60}O_4$	(dihydroxy, diketo)
Capsanthin	$C_{40}H_{56}O_3$	(dihydroxy, monoketo)
Violaxanthin	$C_{40}H_{56}O_4$	(tri- or tetrahydroxy)
Taraxanthin	$C_{40}H_{56}O_4$	(tri- or tetrahydroxy)
Antheraxanthin	$C_{40}H_{56}O_3$	(trihydroxy)
Petaloxanthin	$C_{40}H_{56}O_3$	(trihydroxy)
Flavoxanthin	$C_{40}H_{56}O_3$	(trihydroxy)
Lycophyll	$C_{40}H_{56}O_2$	(dihydroxy)
Zeaxanthin	$C_{40}H_{56}O_2$	(dihydroxy)
Lutein	$C_{40}H_{56}O_2$	(dihydroxy)
Lycoxanthin	$C_{40}H_{56}O$	(monohydroxy)
Cryptoxanthin and Rubixanthin	$C_{40}H_{56}O$	(monohydroxy)
Rhodoxanthin	$C_{40}H_{50}O_2$	(diketo)

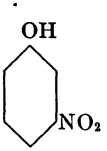
TABLE 13

Adsorbability of various pairs of isomeric compounds

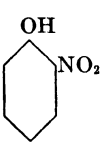
p-Nitrophenol (upper band)



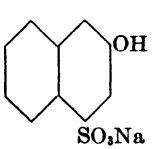
m-Nitrophenol (lower band) (see page 93)



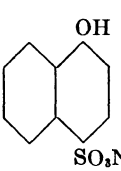
m-Nitrophenol (upper band)



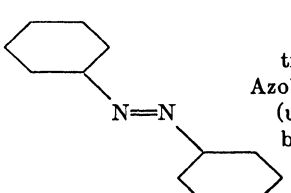
o-Nitrophenol (lower band)



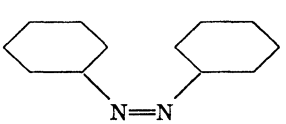
2-Naphthol-4-sulfonic acid sodium salt (upper band)



1-Naphthol-4-sulfonic acid sodium salt (lower band) (see page 151)



trans-Azobenzene (upper band)



cis-Azobenzene (lower band) (see page 93)

with trinitrobenzene or picric acid. These nitro compounds are held very strongly by the adsorbent, and the liberated material passes through the column and is recovered from the percolate. A typical example is described on page 153.

10. Combination of Chromatographic Adsorption and Electrophoretic Methods

Application of electromotive force to opposite ends of a Tswett column upon which charged or ionized substances have been adsorbed causes the adsorbed materials to migrate over and along the surface of the adsorbent. Under these conditions, the adsorbed compounds migrate at different rates. They gradually separate from one another forming a series of bands or a chromatogram although no liquid is permitted to flow through the column.

In principle, this technique combines many of the advantages of the electrophoretic or cataphoretic methods, in which charged particles migrate through a liquid under the influence of applied electromotive force, with those of the chromatographic method. It also eliminates some of the disadvantages of the electrophoretic method, particularly the disturbances of the boundaries of migrating substances through convection currents. The apparatus required for the separation of adsorbed compounds by this application of electromotive force is illustrated in figure 32, page 40. Typical experiments illustrating the separation of mixtures of ionized dyes on adsorption columns by the use of electromotive force are described on page 151.

III. APPARATUS AND PROCEDURE

1. General Considerations

Although the columnar adsorption procedure appears to be simple and straight forward, its efficiency and its applicability are influenced by many interrelated factors. Some of these must be given a great deal of attention; others affect the results in minor ways. Slight changes in the style of the apparatus or minor variations of the conditions affect the formation of chromatograms in several ways. On this account, discussion of these conditions or their effects under separate headings leads to undue repetition. An attempt has therefore been made to outline the method supplying such additional information as may assist the apprentice. Details of interest to those concerned with special fields of investigation are provided in the experimental section and through the index and bibliography.

Successful preparation of chemical compounds by chromatographic adsorption depends primarily upon the selection of adsorbents, and upon the choice of solvents for the adsorption, for development of the chromatogram, and for elution of the resolved compounds. The adsorbent should combine with sufficient quantities of the mixture to yield definite and detectable bands in the column. This adsorbed material should be bound firmly enough so that it can be washed with fresh solvent before it is carried into the percolate. It must not be bound or anchored too strongly because then it can not be washed along in the column and no resolution of the constituents will be obtained. Once the compounds have been separated upon the column, it is necessary to ascertain that they can be eluted with polar solvents. An empirical procedure for the selection of adsorbents, solvents and eluants is described in section 5 of chapter IV.

Other conditions that affect the Tswett adsorption procedure are the size and the porosity of the particles of the adsorbent. They determine the method employed to pack the columns. All these conditions influence the rate and evenness of percolation of the solvent through the column. This in turn affects the definition or uniformity of the bands. The quantity and concentration of the solution added

to the column before development of the chromatogram is commenced must be adjusted in relation to the size and shape of the column and also in relation to the activity of the adsorbent itself. Reactions between the adsorbent and the adsorbed compounds sometimes lead to spurious results. In the case of certain labile or oxidizable compounds, exposure of the adsorbed materials to air should be avoided. Impurities in the solvents and in the mixtures to be resolved occasionally reduce the activity of the adsorbent, often to the point where it is entirely inactive. All these complex conditions are discussed at greater length in this and the following chapters.

The formation of a chromatogram is a dynamic process. It depends upon the continuous and simultaneous adsorption, desorption or elution and readsorption of the substances being resolved upon the column. Differences between the rates of desorption of materials on the column as well as differences between the rates and the degree of their adsorption play an important role in the separation of bands from one another. These conditions also play a part in the formation of well-defined bands. Incomplete or slow desorption usually leads to the formation of bands whose upper boundaries are diffuse and whose width tends to increase rather rapidly. Slow desorption may depend upon the adsorptive properties and upon the porosity of the particles of the adsorbent. Molecules that have penetrated large porous particles diffuse out so slowly that they are not carried along with the bulk of the material as it moves through the column. At present it is not known whether or not the solute attains equilibrium with the adsorbent as it passes over the relatively large particles.

Concepts regarding the details of the adsorption process itself are not very clear (Bradley). Even in static systems where the adsorbed compounds come to equilibrium with the adsorbent, several types of adsorptive forces come into play (Brunauer, et al.). In adsorption columns, several kinds of reactions may play a role. Adsorption of hydrocarbons on unreactive solids appears to involve only surface forces. Resolution of ions by adsorption on columns of 8-hydroxyquinoline involves reversible, chemical reactions. Separation of isotopes on columns of permutit depends upon an exchange reaction (cf. page 82). Adsorption of certain dyes produces color changes indicative of the formation of complexes (Weitz and Schmidt).

An interesting attempt to interpret the formation of chromato-

grams in terms of the adsorption isotherm has been presented by Wilson. This adsorption isotherm, used to express the relation between adsorbent and adsorbate at equilibrium, has been determined for many combinations of solids and gases and solids and solutes (McBain; Freundlich). Wilson has assumed that equilibrium is attained as an adsorbate passes through the adsorption column, that the volume of the interstices between the particles of adsorbent are negligible and that the effects of diffusion can be neglected. On these assumptions he has derived a differential equation that relates the rate of migration of a band of a single adsorbed material to the volume of solvent that is passed through the column. He has also derived an equation to express the conditions that prevail at the boundaries of the band. When these equations are applied to the interpretation of the formation of several bands on the column, considerable difficulty is encountered because the adsorption isotherm does not take into account the effect of one adsorbate upon the adsorbability of others at varying concentrations of each one.

Wilson's theory accounts qualitatively for the separations effected in chromatographic analysis, for the uniformity of the color of the bands, and for the sharpness of the bands. The theory predicts that small quantities of each substance passing through the column will remain on the adsorbent in the upper portions of the tube. It also requires the substance with the lowest adsorbability to form the lowest band. Comparison of the adsorption isotherms of lauric and stearic acids with their separability on columns (Cassidy) has confirmed this last requirement for several adsorbents, but, for one adsorbent, the fatty acid with the greater adsorbability formed the lower band (page 86).

The effect of one adsorbed compound upon another has not yet been elucidated (Jones, Hudson and Jones). With mixtures of some substances such as those of isomeric α- and β-carotene, the more strongly adsorbed constitutent does not displace the less adsorbed one from the adsorbent (page 128). The formation of two bands in the column under these conditions appears to be due entirely to other factors. On the other hand, substances such as alcohols and hydrocarbons that exhibit great differences between their adsorbabilities will not be adsorbed together on the column because the strongly adsorbed constituents elute the weakly adsorbed ones. This latter effect becomes less and less evident as the constituents of the mixture approach each other in chemical and physical prop-

erties. With other substances such as tartaric acid and Bordeaux red, the adsorbability of the dye is increased by the presence of the acid (page 162). The effect appears to be analogous to that obtained with mordants in the dyeing of fabrics.

The effect of one adsorbed compound upon another is sometimes made use of to facilitate the separation of closely related compounds. For example, two substances that are adsorbed close together on the adsorption columns occasionally separate to greater distances in the presence of a third compound that is held between them. Separation of inorganic ions on columns of alumina has been accelerated in this way (page 80). Similar affects have been observed when extracts of plant and animal materials are adsorbed. Numerous examples may be found among the experiments relating to preparation of the carotenoid pigments (page 128).

2. Size and Shape of Adsorption Columns

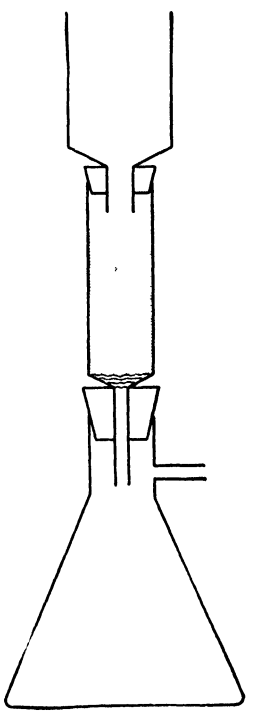


Fig 3. Large apparatus employed by Tswett.

The simplest and most widely used apparatus for preparation of chemical substances by chromatographic adsorption is illustrated by figure 1. It consists of a suction flask and a long cylindrical glass tube constricted at one end. On one or two occasions, conical glass tubes have been employed (Palmer and Eckles; Fischer and Hofmann).

The glass tubes or columns may be constructed of soft glass, pyrex or quartz. Columns lined with cellophane and columns of celluloid sheets rolled into tubes have also been used successfully. All these materials are transparent which facilitates observation of the bands formed in the column. Tubes of quartz, because of their high transmission of ultraviolet light, are advantageous for chromatographic adsorption of fluorescent materials. Square columns with quartz windows have also been used. (Karrer and Schopp). In industrial operations columns of opaque materials such as steel and porcelain have found extensive use.

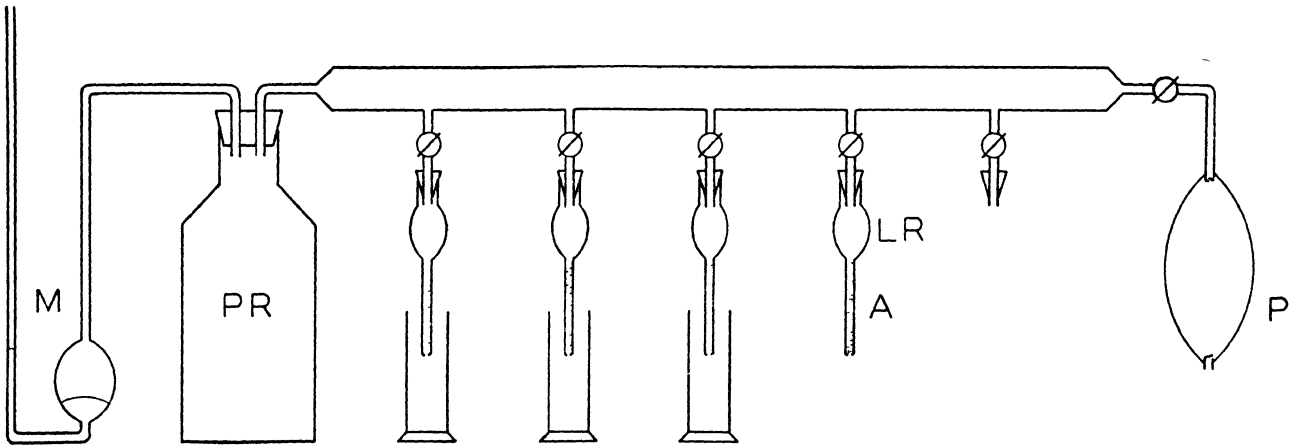


Fig 4 Adsorption apparatus modified after Tswett P, rubber aspirator bulb, A, adsorbent in tube 2–3 by 30–40 mm, LR, liquid reservoir, PR, pressure reservoir, M, mercury manometer.

The size of an adsorption column is determined by the quantity and the adsorbability of the materials to be resolved. It is dependent upon the solvents used for the adsorption and for the development of the chromatogram, and it is subject to considerable variation depending upon the skill and experience of the operator. In general, it can be determined only by trial and error.

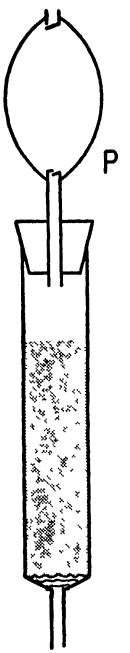


Fig 5 Apparatus for filtration with pressure P, rubber aspirator bulb

From a theoretical point of view, a given quantity of mixture adsorbed on a given quantity of adsorbent would be separated into bands in less time if the adsorbent were used in a short, wide column rather than in a long, narrow one. In wide columns, the same quantity of mixture would form much narrower bands than it would in the narrow columns, and the percolation rate would be much greater. However, for a given amount of percolate collected, the wider the column the narrower and closer together would be the bands, the greater the difficulty in seeing them and in separating them from one another, and the greater the chance of obtaining uneven filtration and poorly defined bands that could not be removed separately from the column. When some of the materials to be isolated occur in very small quantities relative to the major constituents of the mixture, use of wide columns should be avoided because the narrow bands of the minor constituents may not be visible. In practice a compromise is made with the result that the diameter of the columns is usually one-tenth to one-fourth of their length, but these proportions are by no means fixed. With improvements in the properties of adsorbents and with development of new methods for packing the columns to insure even percolation of the solutions through the adsorbent, it is probable that relatively much wider columns will come into use. For the separation of isotopes, very long and narrow columns have been used (page 82). For most laboratory work, columns varying in diameter from 0.5 to 10 cm. are usually employed.

Various types and arrangements of the apparatus have been de-

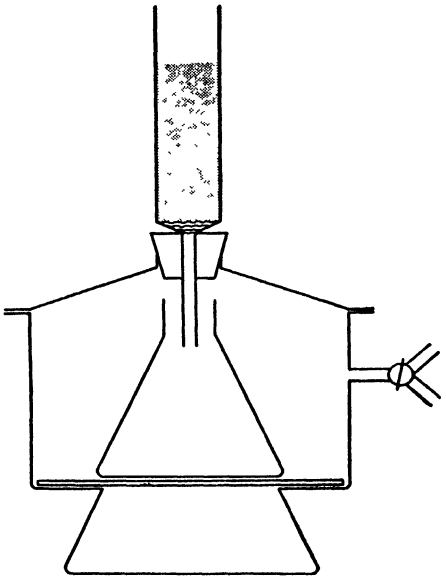


FIG. 6

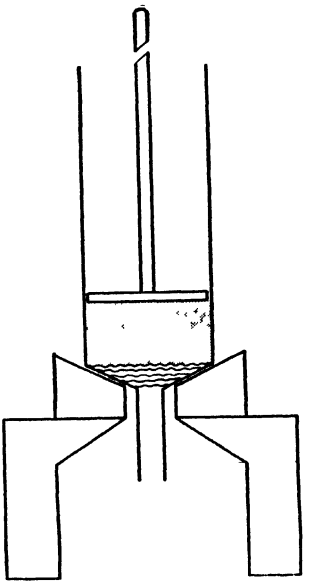


FIG. 8

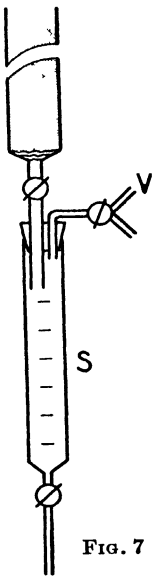


FIG. 7

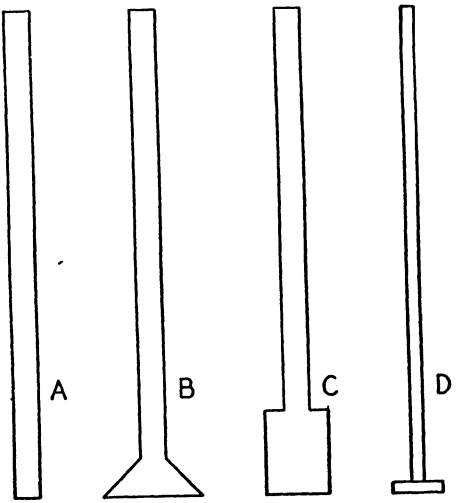


FIG. 9

Fig. 6. Arrangement for collection of several fractions of percolate (Trappe).

Fig. 7. Apparatus of Valentin and Franck. V, vacuum; S, separatory funnel.

Fig. 8. Cork ring support for use while packing adsorption columns.

Fig. 9. Plungers for use in packing adsorption columns. A, wood, plastic, glass or metal; B and C, wood; D, metal.

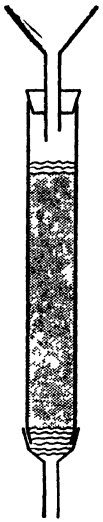


FIG. 10

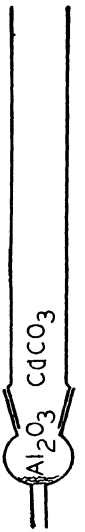


FIG. 11

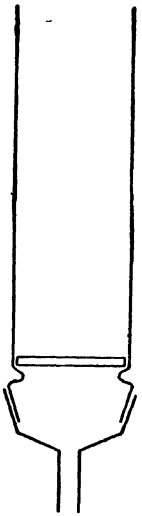


FIG. 12

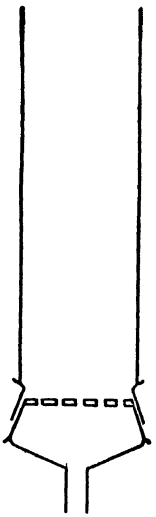


FIG. 13

FIG. 14

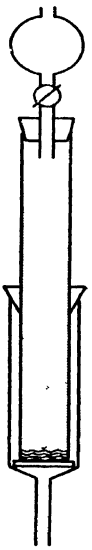


FIG. 15

FIG. 16

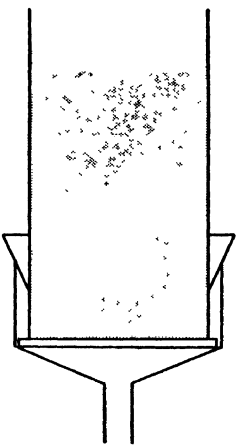


FIG. 17

Fig. 10 Apparatus after Mohler and Hammerle.
Fig. 11. Apparatus after Kuhn and Brockmann (3).
Fig 12. Apparatus of Zechmeister and Cholnoky.
Fig. 13. Apparatus of Zechmeister and Cholnoky.
Fig. 14. Apparatus after Dhéré and Vegezzi.
Fig. 15. Apparatus of Winterstein and Stein.
Fig. 16. Apparatus of Hesse.
Fig. 17. Apparatus for analysis of leaf pigments (Spohn).

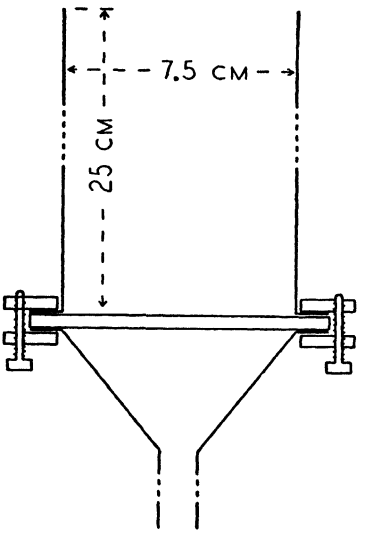


FIG. 18

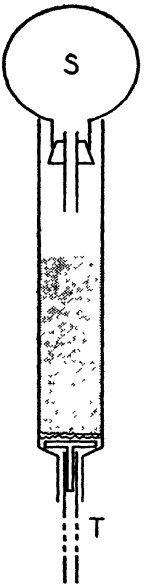


FIG. 20

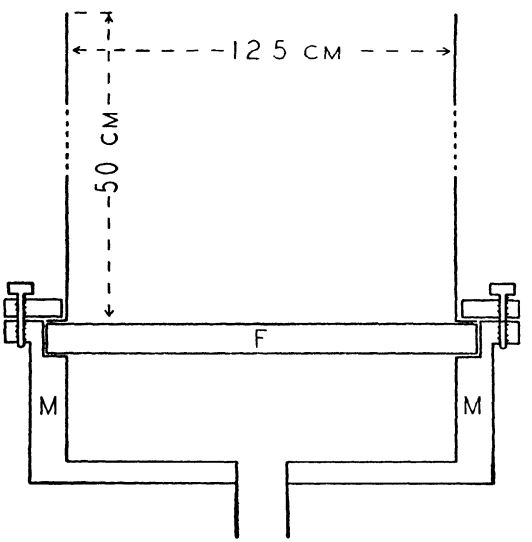


FIG. 19

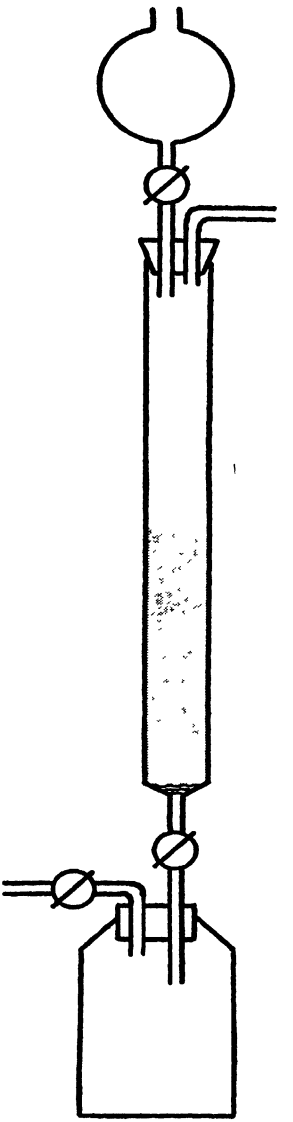


FIG. 21

Fig. 18. Medium-sized adsorption apparatus after Winterstein and Schon.

Fig. 19. Large adsorption apparatus for use with about 5 kg. of adsorbent. (After Winterstein and Schon). F, filter plate; M, metal support.

Fig. 20. Arrangement for addition of solvent and for removal of adsorbent. S, solvent; T, metal tube. (Modified from percolation apparatus of Békésy.)

Fig. 21. Apparatus for adsorption in the absence of air (Holmes, Cassidy, Manly and Hartzler).

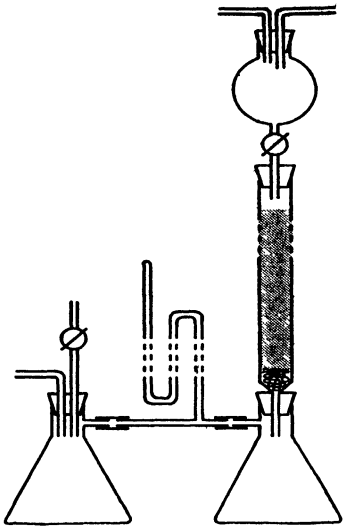


FIG. 22

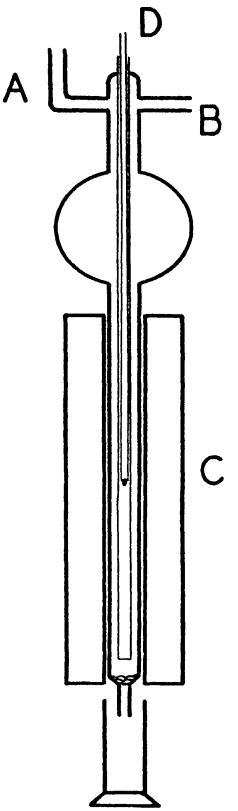


FIG. 23

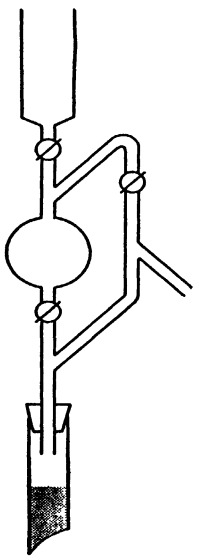


FIG. 24

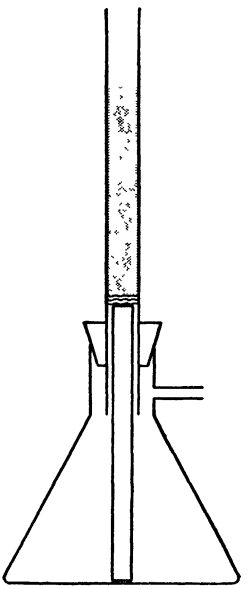


FIG. 25

Fig. 22. Apparatus for adsorption in the absence of air (Heilbron, Heslop, Morton, Webster, Rea and Drummond).

Fig. 23. Electrically heated column. A, addition of solution; B, nitrogen stream; C, electric jacket; D, leads to thermal junction (Ralston, Harwood and Pool).

Fig. 24. Apparatus for addition of liquid and application of pressure (Sannié).

Fig. 25. Micro adsorption column of Willstaedt and With.

signed for special purposes. Some of these are for use with suction; others are operated by pressure; some are employed with inert gases and a few are for use in ultraviolet light and at elevated temperatures.

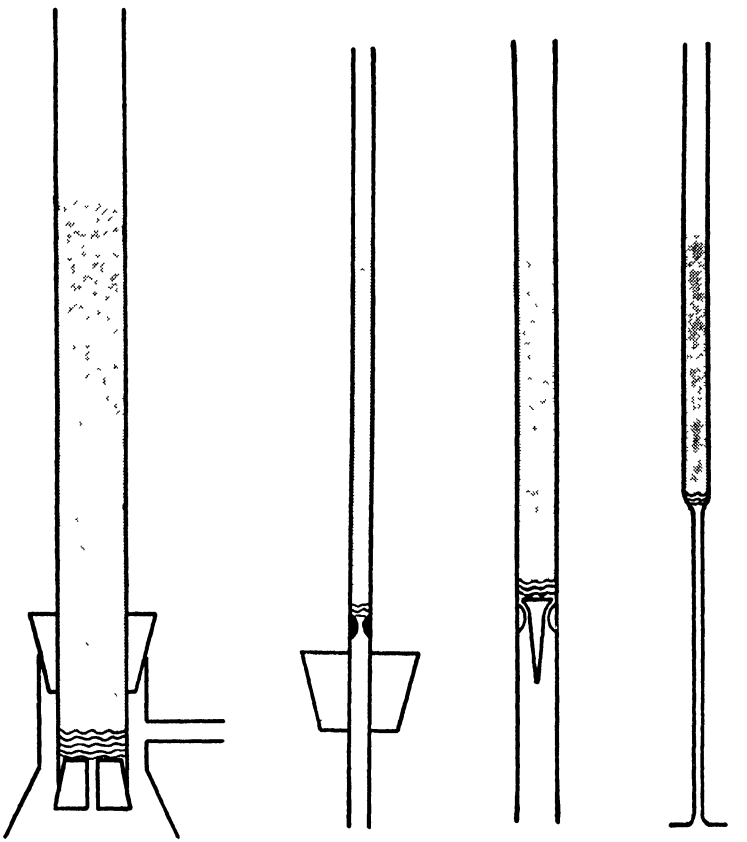


FIG. 26 FIG. 27 FIG 28 FIG. 29

Fig 26. Micro column (quartz) (Grassmann)
Fig 27 Micro column (Schopf and Becker)
Fig 28. Micro column (Hesse)
Fig. 29 Micro column (Becker and Schopf)

In addition to the apparatus shown in figure 1, a similar type was developed by Tswett for preparation of material on a larger scale, figure 3. Another apparatus for use with pressure was also described by Tswett, figure 4. With this apparatus several adsorption columns could be operated at once. Moreover the adsorption columns were

designed with a reservoir near the top so that a great deal of liquid could be drawn through the adsorbent before it was necessary to disconnect the tube for the addition of more solution or solvent.

FIG. 30

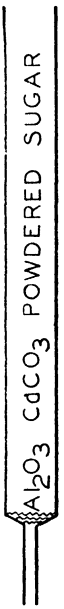


FIG. 31

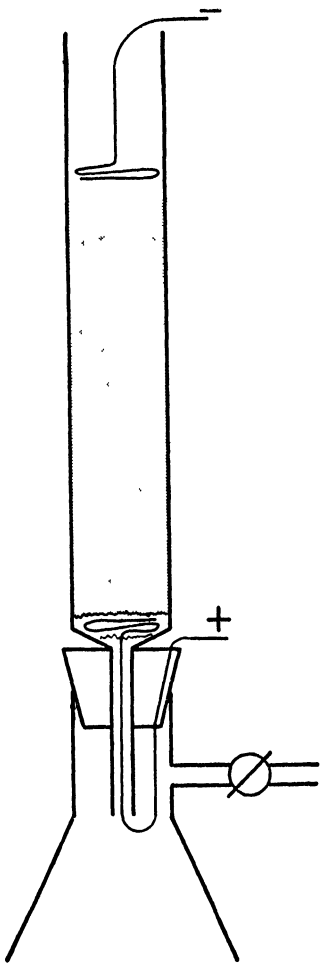


FIG. 32

Fig. 30 Capillary adsorption column (Strain).

Fig 31. Series of adsorbents for separation of leaf pigments. Sugar for chlorophyll a and b, $CaCO_3$ for xanthophyll, Al_2O_3 for carotene (Winterstein and Stein 1).

Fig 32 Arrangement of electrodes for separation of compounds by combination of electrophoresis and adsorption.

Modifications of this apparatus, figure 5, are useful for demonstration purposes especially when suction is not available.

Most of the columnar adsorption apparatus used at various times is represented by figures 6 to 32. It is believed that these illustrations are self explanatory. The apparatus represented by Figures 14 to 17 and 25 to 26 are recommended for use with quartz because the difficult operation of constricting the cylindrical tube is thus avoided. Figure 32 illustrates the apparatus employed for separation of mixtures by combination of electrophoretic and chromatographic methods.

3. Preparation of Adsorption Columns

Before the cleaned and dried adsorption columns can be filled, a support for the adsorbent must be placed in the constricted portion of the tubes. A wad of cotton pressed firmly into place is the usual support. However, if the cotton adsorbs the material to be resolved or if the cotton is attacked by the solutes in the percolate one may use glass wool or fritted glass, or perforated porcelain or metal discs. In order to insure even distribution of suction at the base of the column, this being essential for uniform percolation of the solution and for formation of definite bands, the support should extend above the constricted portion of the glass tube.

Adsorption tubes may be filled in several ways Under favorable conditions each method provides columns that exhibit uniform percolation and that yield well-defined bands of the resolved materials. As has been pointed out already, the choice of the method used to pack the adsorbent into the tubes depends to some extent upon the dimensions and properties of the particles. With granular adsorbents such as permutit having particles that fail to pass a 250 mesh sieve, one may pour the adsorbent into the tube with continuous tapping of the sides. Very large tubes may be filled by pouring the adsorbent through a funnel so that a pyramid is formed in the center of the column. After the requisite quantity of adsorbent has been added, the pyramid is leveled by jarring the tube.

Moderately fine powders such as precipitated chalk and powdered sugar are added to the tube in small successive portions, and each of these is tamped firmly into place. For this purpose one may use a rod of wood (dowel), metal or Bakelite (figure 9). Suction may be applied to the base of the column while the adsorbent is being tamped into place.

Very finely divided adsorbents form columns that exhibit extremely low filtration rates. In order to increase the percolation rate of columns of these materials, the powders are usually mixed with larger particles of the same adsorbent or with fibrous, inert fillers. A specially prepared very finely divided magnesium oxide, that has found extensive use for preparation of many natural products is often mixed with heat-treated siliceous earth (Strain 2, 10). Because of its great resilience, this mixture is usually pressed (not tamped) slowly but firmly into the tube in small layers (about 1 to 3 cm. deep). Most even packing of this adsorbent is obtained with a metal rod firmly affixed to a metal disc but slightly smaller than the tube itself. If the movement of the packing plunger is too rapid, a partial separation of the adsorbent mixture occurs at the upper surface with the result that lines or narrow zones appear at the boundaries between each batch of material packed into the tube. Peterson has recommended that these packing lines, once formed, be removed by loosening the topmost few millimeters of adsorbent before pressing the next portion into place.

The packing of these resilient adsorbents into columns 6 to 8 cm. in diameter may require about 150 pounds pressure on the plunger. During the pressing of the adsorbent, the column is supported in a cork ring attached around a hole in a heavy wooden box (figure 8). Adsorbents packed into columns in this way form a porous adherent mass that will not shrink from the tube after having been wet and sucked dry and that will not fall from the column if the latter is inverted and jarred. It is not necessary to wet these columns with solvent before addition of the mixture to be resolved.

Some adsorbents, notably sucrose, are often filled into the columns in the form of a slurry. The mixture of adsorbent and solvent is poured rapidly into the tube while gentle suction is applied at the base. Here care must be exercised to see that the adsorbent is never sucked dry else it will shrink from the tube and the column will be rendered useless.

Disturbance of the top layers of the adsorbent in the column by the addition of solvent or solution may be eliminated by placing a wad of cotton, a perforated disc or sheet of filter paper on top of the packed adsorbent. This practice is by no means universal. It has the disadvantage that pockets of the solution may form under the protective material and this leads to the formation of uneven bands of the adsorbed substances.

4. Concentration of Solutions To Be Adsorbed

For the resolution of mixtures with adsorption columns it is desirable to begin with relatively concentrated solutions. This has the advantage that much solute becomes adsorbed in a shallow band in the upper portions of the column, the remainder of the column being available for the development of the chromatogram. Under these conditions most substances form narrower, more easily discernible bands than those obtained if the same quantity of mixture is adsorbed from dilute solutions. This effect is most pronounced with the weakly adsorbed components of mixtures.

The quantity of mixture one should add to the column before the development of the chromatogram is begun varies greatly with different substances. Usually one wishes to resolve as much material as possible with the result that too much solution is added to the column. By the time the solute in the upper portions of the adsorbent has been washed down the column and adsorbed, no space remains for the development of the chromatogram with fresh solvent. *In preliminary experiments with unknown mixtures it is better to adsorb too little than too much of the mixture.* When columns of dry adsorbent are used, the amount of adsorption may be estimated by observing the quantity of clear liquid that collects below the adsorbed materials. If very little or no clear liquid appears at the base of the band of adsorbed material, a minimum quantity of solution should be added to the column. If considerable clear liquid appears more of the solution may be added.

In most adsorption experiments, the solution is passed through the column until the band of adsorbed material occupies from one-tenth to one-fourth of the adsorbent. The column is then ready for development of the chromatogram as described in section 6. The quantity of material adsorbed ranges from a few tenths of a milligram (with columns 0.5 to 1.0 cm. in diameter) to a few tenths of a gram (with columns 4 to 10 cm. in diameter).

5. Regulation of the Percolation Rate

Addition of solvents or solutions to columns of dry adsorbents often results in a loosening of the top portions of the adsorbent unless suction is maintained at the base of the column. With columns of coarse adsorbents the pressure may be reduced only 5 to 20 cm. of mercury. With columns of finely powdered materials, the pressure

may be reduced to 30 to 50 cm. After the addition of the solution, and during the development of the chromatogram, much suction may be used provided, of course, that it does not cause evaporation of the solvent in the column.

Because of mechanical difficulties, application of increased pressure to the top of the column has not often been resorted to (figures 4, 23, and 24) (Potts and Koch). With columns of finely divided adsorbents, this use of pressure may yet find important application.

6. Development of the Chromatogram

As soon as the solution of materials to be resolved has been drawn into the adsorbent, the column is washed with fresh portions of the same solvent as that from which the compounds were adsorbed. At first this solvent is added in small portions in order to wash residual solution into the column. Later, larger quantities of the solvent may be added to the column. This procedure improves the sharpness or definition of the upper boundary of the bands. During the addition of solution and of solvent, air should not be drawn into the upper portions of the adsorbent.

Passage of fresh portions of solvent through the column usually causes the adsorbed materials to move along at different rates and thus completes the development of the chromatogram. Occasionally however, the adsorbed compounds move through the column at such a slow rate that it becomes impracticable to develop the chromatogram with the solvent used for the adsorption. In such cases, it is customary to accelerate the development of the chromatogram through use of solvents from which the adsorbed compounds are not so strongly bound to the adsorbent. For example, substances adsorbed from petroleum ether may be washed subsequently with mixtures of this solvent and benzene or carbon tetrachloride. Increases in the concentration of the benzene or carbon tetrachloride accelerate the rate at which the adsorbed compounds are washed through the column.

Experience has shown that the adsorbability of most substances varies with the polarity of the solvent. It is possible therefore to select a series of solvents for the development of the chromatogram each of which causes the adsorbed compounds to move faster through the column than the preceding one. Several such series of solvents are described in chapter V.

When the development of the chromatogram is to be accelerated

by use of a more polar solvent, it is important to choose a liquid that is only slightly more polar than the solvent and that does not cause the adsorbed compounds to move too rapidly. It is better to use successively increased quantities of the liquid that decreases the adsorption rather than to use too much of it at the beginning or to use a solvent that virtually elutes the adsorbed compounds. By the use of gradually increasing quantities of the more polar solvent, the development of the chromatogram can be controlled. If under these conditions development of the chromatogram proceeds too rapidly, it can be decelerated by decreasing the proportion of the more polar solvent; but if too much of a strongly polar liquid is added, the adsorbed compounds migrate through the column before they can be reached again with the less polar liquid and before they have separated into distinct bands.

When a column with a partially developed chromatogram is to be washed with a more polar solvent or solvent mixture, care should be taken that weakly adsorbed substances have already been washed well away from the bands of strongly adsorbed materials. If this precaution is not taken, the bands of strongly adsorbed compounds may be carried into the bands of the weakly adsorbed ones before the latter can be carried along by the more polar solvent. Such effects are often observed with extracts of plant and animal products They are most troublesome when a relatively small section of column remains below the weakly adsorbed bands.

Inasmuch as many compounds may be identified on the basis of their colors and positions on the columns, it is customary to record the order, the color and the width of the bands This is often done by reproducing a column drawn to scale with the position and color of each band plainly labeled (page 144). A more convenient and economical method consists in reporting only the width and color of the band with the name or some characteristic property of the compound beginning at the top of the column (page 90).

7. Recovery of Resolved Compounds

Substances resolved upon adsorption columns may be isolated in several ways. By one procedure, the column is washed with fresh portions of the solvent until each compound is successively carried into the percolate. The percolates containing single compounds are collected separately and the dissolved materials are isolated by

evaporation of the solvent. In German, this technique is called a "flüssiges Chromatogramm", a flowing chromatogram.

More commonly the zones of adsorbent containing the resolved, adsorbed compounds are removed separately from the tube. This is accomplished in one of two ways. According to one method, each band of adsorbent is loosened with a long, straight spatula. It is then dumped from the tube or scraped out with a hoe-shaped spatula. (These spatulas may be hammered from silver or brass rod.) The other procedure consists of removal of all the adsorbent as a moist cylinder that is then cut into sections, each section containing a single adsorbed compound. In order to facilitate removal of the intact cylinder of adsorbent, most of the solvent is first removed with suction. This causes the adsorbent to shrink so that it can be shaken or pressed from the tube (figure 20).

After each zone of adsorbent has been removed separately from the tube, the adsorbent is agitated with a polar solvent that elutes the desired compound from the adsorbent. These solvents or eluants are described in chapter V. The solutions obtained by the action of the polar solvents are separated from the adsorbent by filtration or by centrifugation, and the dissolved substances are prepared by evaporation of the solvent or by other standard procedures.

With mixtures of compounds that exhibit great differences between their adsorbabilities, the resolved compounds may be isolated by a combination of methods. The weakly adsorbed constituents are washed through the column. The more strongly adsorbed ones are isolated from the respective portions of the adsorbent after these have been removed from the tube. When soluble adsorbents are used, these may be dissolved and the liberated compounds recovered from the resulting solution (Dam and co-workers) (page 108) (Duschinsky and Lederer) (page 112).

The yields of resolved compounds recovered from adsorption columns are subject to great variation. In the absence of reactions with the adsorbent and with efficient eluants as much as 98 per cent of the carotene adsorbed on columns of specially prepared magnesia has been recovered. With some other adsorbents such as the adsorptive clays, the yield may decrease to a few per cent. Because of this great variation in the recovery of adsorbed compounds, great care must be exercised in the use of chromatographic methods for quantitative estimations, and if possible tests for the per cent recovery of the adsorbed compounds should be performed.

IV. ADSORBENTS

1. General Considerations

No universal adsorbent has yet been found nor has a perfect adsorbent for any given purpose been developed. Ever since the first studies by Tswett, researchers have tested substance after substance with the hope of discovering more suitable fillers for the adsorption columns. Tswett (10) himself tested about one hundred materials, and subsequent workers have examined hundreds more. In view of the fact that the conditions determining the activation of these compounds were seldom understood or described, it is now impossible to evaluate the results. Experimenters have had to make the best of the materials at hand and to improve these by trial and error. As a result, some adsorbents, originally considered to be weak and nonselective, have, with improved methods of preparation, become most efficient fillers for Tswett columns. Others once regarded as the best have been superceded by the improved kinds. Because the adsorptive characteristics of each solid vary with the method of preparation and activation and since each preparation may be used with several solvents, the possibility for variation in procedure and in selection of adsorbents is enormous.

As a result of experience with many different solids, some of the properties desired in an absorbent for use in adsorption columns have been discovered. The ideal adsorbent should hold relatively large quantities of the materials to be resolved. These materials should be carried through the column with fresh portions of the solvent in order to permit development of the chromatogram. The adsorbed substances should be eluted completely from the adsorbent with polar solvents. The adsorbent must not decompose the adsorbed substances nor the solvents used for the adsorption, for the development of the chromatogram, or for the elution of the separated compounds. Under the usual conditions, it should not be soluble in any of these solvents. The adsorbent should be of particle size conducive to rapid and uniform percolation. The particles themselves should not be porous because solute that has penetrated the interstices is removed very slowly. Colorless or white adsorbents are preferable to colored

ones. Those that exhibit highly selective action for closely related chemical substances are most efficient.

Although many fibrous and granular substances both organic and inorganic are active adsorbents, only a few of these have been widely used. Colored, oxidizing, and strongly acidic or basic solids are unsuitable for separation of many compounds. The most satisfactory adsorbents are the alkaline earth oxides, hydroxides and their salts, such as carbonates and sulfates. Talc, silica, soda ash and various silicates have been found useful for special purposes. For the separation of labile organic compounds, carbohydrates have found extensive use. Of these starch, inulin, sucrose and lactose have been used most. Advances have also been made in the utilization of organic adsorbents for the resolution of inorganic compounds. An indication of the various adsorbents used for special purposes is given in table 14. Arranged in series according to their adsorptive capacities, the commonly used adsorbents are found to be a strange medley of chemical compounds (table 15). Because of variations in the activity of the solids with different methods of preparation and activation, the order of the compounds is not an absolute one.

2. Conditions That Influence Adsorptive Properties

Extensive experience with numerous adsorbents has brought out many important facts concerning their adsorptive properties. The adsorptive capacity of any given substance depends upon the method of preparation, upon the treatment or activation, upon the size of particles, upon the solvents used for the adsorption, upon the presence of impurities and probably upon many as yet unknown factors including exchange reactions (Kolthoff and coworkers).

Dependence of the adsorptive properties upon the method of preparation of the adsorbent has too often been overlooked. Many widely used adsorbents as alumina, lime and magnesia may vary several thousand fold in their adsorptive capacity depending primarily upon their method of preparation or activation. Magnesia, for example, has been prepared in such form that it will decompose nearly all the carotene absorbed upon it; yet when prepared under other conditions it will cause no decomposition of adsorbed carotene; prepared under still other conditions it is not adsorptive (Strain 2). Activated alumina may be further activated by treatment with a solution of lime water (Franck; Ruggli and Jensen). Irradiation of cadmium sulfide decreased its adsorption capacity for phenolphthalein (Hed-

vall and Cohn). No relationship between the chemical structure and the adsorption capacity of organic molecules has been discovered. It is noteworthy, however, that the presence of many polar groups

TABLE 14

Various substances and the adsorbents and solvents used for their preparation and purification by chromatographic methods

SUBSTANCE	ADSORBENT	SOLVENT
Inorganic ions	alumina	water
	8-hydroxyquinoline	water
Enzymes	bauxite	water
Sugars	bauxite	water
	bone ash	water
Flavins	Frankonit	water
Anthocyanins	alumina	water
Alkaloids	alumina	benzene or water
Amino acids	titania	water
Chlorophylls	saccharides	petroleum ether and benzene
	magnesium citrate	ether and petroleum ether
Chlorophyll derivatives	talc or saccharides	benzene, ether or alcohols
Nitro compounds	talc	benzene
Oranic acids	talc	benzene
Phenols	lime	alcohols
	alumina	benzene
Ethers, ketones and esters	alumina	petroleum ether and benzene
Xanthophylls	alumina	benzene
	magnesia	1,2-dichloroethane
	alkaline earth carbonates	petroleum ether or benzene or carbon disulfide
Aliphatic alcohols and sterols	alumina	petroleum ether and benzene
Unsaturated hydrocarbons	lime, alumina or magnesia	petroleum ether, carbon tetrachloride or benzene
Saturated hydrocarbons	alumina or magnesia	petroleum ether

in the adsorbent, as in sugars and urea, does not prevent the adsorption of substances like chlorophyll normally eluted by polar solvents.

On account of the great variation in the adsorptive properties of solids prepared in the laboratory, most workers have preferred

to use adsorbents prepared commercially. Some of these have been developed especially for use in adsorption columns and are remarkably uniform. Further research will undoubtedly lead to the preparation of more active and selective adsorption agents.

Some organic materials such as sucrose must be ground before use in adsorption columns. Many of these take up water with a simultaneous decrease in adsorption capacity. Restoration of activity or reactivation is effected by dehydration with heat or vacuum.

TABLE 15

Adsorbents arranged in approximate order of their adsorption capacities

(Least active adsorbents are first in the series)

Sucrose, starch
Inulin
Magnesium citrate
Talc
Sodium carbonate
Potassium carbonate
Calcium carbonate
Calcium phosphate
Magnesium carbonate
Magnesia (Merck)
Lime (freshly and partially slaked)
Activated silic acid
Activated magnesium silicates
Activated alumina, charcoal and magnesia (Micron Brand)
Fuller's earths

The adsorption capacity of all solids depends upon the solvents used for the adsorption. Adsorption is greatest from saturated hydrocarbons, less from unsaturated and cyclic hydrocarbons, chlorinated hydrocarbons, ketones, and esters, still less from alcohols, nitrogeneous bases, and least from aqueous solutions particularly from acidic and basic solutions. This is discussed at greater length on page 53 and in chapter V. Substances that are weakly adsorbed from nonpolar solvents are eluted completely with strongly polar solvents. For this reason it is important that solvents and solutions be pure and dry before passage through the columns.

Some commercial, activated adsorbents bind adsorbed compounds

so firmly that a chromatogram can not be developed. Others decompose many organic compounds. These undesirable effects may be ameliorated by partial deactivation of the adsorbent with water or alcohol. Maximum inactivation of the adsorbent is obtained by treatment with water followed by dehydration in air at room temperature. Dehydration at higher temperatures increases the adsorptive capacity often to that of the original preparation. Exposure of the technical adsorbents to cool, moist air also decreases their adsorptive capacity. Similar results are obtained when the adsorbents are inactivated with alcohol, but the initial inactivation is usually not as great as that obtained by the use of water. Examples of the preparation of alumina of various adsorptive activity are described on page 55.

Of the many activated adsorbents that have been used alumina and magnesia are most easily inactivated and reactivated. Some adsorbents such as charcoal and the fuller's earths are only slightly inactivated with polar solvents.

The adsorptive properties of most substances are augmented with a decrease in particle size; that is, with an increase in surface. It does not follow, however, that every finely divided substance is an active adsorbent. Although the surface area and the adsorptive capacity per unit weight of adsorbent may be increased enormously by pulverization, the character or quality of the adsorption is not altered a great deal thereby.

Particle size of the adsorbents has a tremendous influence on the rate and evenness of percolation of the solutions through the columns. The larger the particles the greater is the rate of filtration. The smaller the particles the more uniform is the flow of liquid through the column. It is possible that very finely divided adsorbents could be sintered or cemented together in loose aggregates retaining the desirable characteristics of the fine powders and at the same time forming columns with high rates of percolation.

According to Zechmeister and Cholnoky (10), the particles of most adsorbents vary in size between one and ten microns. Our experience indicates that preparations of some adsorbents such as alumina standardized according to Brockmann (Merck) have particles about ten times this size; namely 200 mesh or about one hundred microns (page 55). For most laboratory purposes, adsorbents with larger particles are not desirable.

3. Recovery of Adsorbents

After many uses of Tswett adsorption columns, recovery of the adsorbent is not economical. On the other hand, large preparative undertakings may involve such quantities of costly adsorptive materials that their recovery is imperative. Most of the alkaline earth oxides, hydroxides and their salts can be freed of the last portions of adsorbed materials by prolonged extraction with alcohol. After drying in air, the inactive solid may be revivified with heat. Many of the organic adsorbents may be recovered by recrystallization.

4. Mixtures of Adsorbents

In order to preserve the advantages inherent in the use of finely divided adsorbents and to increase the rate of percolation it has been found advantageous to mix finely powdered adsorbents with a filter aid before packing the column. One may use large and small particles of the same adsorbent or one may select a filter aid that exhibits weak adsorptive properties. A specially prepared, heat-treated siliceous earth (Hyflo Super Cel, F.A. 501) sold by Johns-Manville is an excellent, weakly adsorptive filter aid. Adsorbent and filter aid are mixed together in a tightly closed can or ballmill and this mixture is packed into columns with pressure as described already on page 42. Columns formed from mixtures exhibit remarkably uniform surfaces and the boundaries of the bands formed on them are sharp and even. The adsorption capacity of the column varies with the proportions of inactive diluent added to the adsorbent.

Columns composed of layers of different adsorbents have found occasional use. The most active adsorbents are packed in the bottom of the column, the least active near the top (figures 11 and 31). Because it is impossible to foretell at what position substances will be completely separated and because substances already separated upon one layer may form a single band when washed onto a more active region, columns of different adsorbents have not found extensive use in research. They may be used to advantage for demonstration purposes.

Instead of utilizing a column composed of layers of different adsorbents, one may pass the mixtures to be resolved through several columns, each successive column containing a more active adsorbent. In this way, one can be certain that all the constituents have been

adsorbed. In order to keep the volumes of solution as small as possible and thus to insure the formation of relatively narrow bands, the percolate from each column is collected in small portions and these are passed through the succeeding columns. The percolates may also be concentrated before passage through subsequent columns.

5. Selection of Adsorbents

In spite of the mass of information that has been collected regarding the adsorptive properties of various solids, the final selection of an adsorbent for the separation of a mixture of unknown compounds must be made by empirical methods. The theory of chromatography based upon knowledge of the adsorption isotherm and discussed on page 31, has not made possible the prediction of whether or not a given adsorbent may be used for the chromatographic separation of a given set of solutes. However, knowing the relative activities of various adsorbents (table 15), their effect on adsorbed compounds (table 14 and pages 54 to 63), the effect of solvents on this activity (chapter V), and the relation between molecular structure and adsorbability, one may select likely adsorbents for the separation of mixtures of given types of compounds.

Various solids may be tested quickly as adsorbents in the following way. Small tubes, 0.5 by 10 cm., are partially filled with the adsorbents. The materials to be adsorbed are then dissolved in a nonpolar solvent such as petroleum ether and portions of the solution are passed through the columns. If the percolation rate is too low it may be necessary to mix the adsorbent with a filter aid. Solids upon which no adsorption occurs are useless. Those upon which the solute has become adsorbed may be of use providing the adsorbed material can be washed along in the column. To accomplish this it may be necessary to use several solvents in succession, each fresh solvent used being slightly more polar than the preceding one. Such a series of solvents is petroleum ether, carbon tetrachloride or carbon disulfide, benzene, 1,2-dichloroethane, alcohols and organic acids. (See chapter V, table 16). Finally it must be ascertained that the material carried through the column is unchanged. Materials remaining on the column must be elutable with polar solvents such as alcohol, water, or solutions of organic acids, pyridine or ammonia in water or organic solvents.

6. Properties of Various Adsorbents

Alumina. Partially hydrated alumina is one of the best and most widely used adsorbents. Like other adsorptive substances, it exhibits great variations in properties depending upon its method of preparation.

Fibrous alumina may be prepared in several ways. According to Wislicenus, aluminum groats about 0.5 cm. in diameter (200 g.) were treated with 200 ml. of 10 per cent sodium hydroxide solution until rapid evolution of hydrogen ensued. The alkali was removed rapidly with water and the process was repeated. To the aluminum cleaned in this way, there was added about 40 ml. of a cold, saturated solution of mercuric chloride. This was mixed thoroughly with the aluminum and removed with water, the last portions of the water being drained off quickly. A mixture of 2–2.5 g. of nitrobenzene, 18–20 g. of ether, 20 g. of 90 per cent alcohol and 10–12 g. of water was added to the aluminum, and the mixture was permitted to stand several hours. Alumina that was formed was separated by suspension in alcohol and collected on a filter. It was activated by calcination in a muffle at red heat and screened to the desired size.

For preparation of fibrous alumina, Renz cleaned the aluminum with alkali, amalgamated it with mercuric acetate solution, drained off this solution and then sprinkled water onto the aluminum as it became hot from the heat of reaction. In this way a dry, fluffy gray powder was obtained. A similar product was obtained by hanging a freshly cleaned and amalgamated aluminum bar in air. As soon as feathery masses of the hydroxide fell from the bar and were collected on a sheet of paper, the aluminum bar was recleaned in alkali, reamalgamated and again hung in air. The alumina was activated by heat. This alumina is finely divided and forms columns having very slow filtration rates. Occasionally, it is used in admixture with the granular forms of alumina thus providing columns that appear homogeneous and that filter more rapidly than those composed of fibrous alumina alone. Preparation of fibrous alumina is an exceptionally tedious process and the commercial preparations are far too expensive for most chromatographic investigations.

Granular activated alumina is prepared commercially and finds extensive use in research and technology. In Germany the firm of Merck sells an excellent preparation labeled "standardisiert nach Brockmann." This preparation exhibits unusually uniform adsorp-

tive properties. About one half of the material which is of nearly equal particle size passes through a 200 mesh sieve. It forms uniform columns through which the solvent percolates evenly and rapidly. It has been used more extensively than any other adsorbent, and it is applicable to the resolution of a great variety of substances. This wide applicability depends in part upon the slight solubility of the preparation in various solvents and upon its slightly alkaline reaction (Cahn and Phipers). It is occasionally necessary to neutralize this alkali with acid (Schwab and co-workers) (page 78). Alumina standardized according to Brockmann is expensive in Germany and is difficult to obtain elsewhere.

For preparation of alumina of various adsorptive capacities, Brockmann and Schodder activated the technical alumina of E. Merck in different ways. Aluminum oxide I was prepared by calcination of small portions of the technical product. Aluminum oxide II was obtained by heating the technical preparation in a layer 3 cm. deep in a large pot over a burner for 4-6 hours. The heated material was cooled in air for about 30 minutes. Aluminum oxides III, IV and V were prepared from aluminum oxide II by exposure to moist air (in a cellar) until the desired activity was obtained.

In order to measure the adsorptive activity of these five activated aluminas, Brockmann and Schodder observed the separation of various dyes on columns 1.5 by 10 cm. For each test, two dyes (about 20 mg. each) were dissolved in 10 ml. of the purest benzene and diluted with 50 ml. of petroleum ether. Ten ml. of this solution were passed through the columns, followed by 20 ml. of solvent (benzene + petroleum ether, 1:4).

Aluminum oxide I was of the proper activity when *p*-methoxy-azobenzene (upper) and azobenzene were separated before the latter had passed through the column. With these two dyes and aluminum oxide II, the azobenzene was washed completely through the column; sudan yellow (upper) and *p*-methoxy-azobenzene were separated on the column. Aluminum oxide III had reached the correct activity when sudan red (upper) and sudan yellow were separated in the upper regions of the column. With these two dyes and aluminum oxide IV, most if not all the sudan yellow was carried into the percolate. With this alumina, amino-azobenzene formed a distinct band above and adjoining sudan red. Aluminum oxide V was of suitable activity when *p*-hydroxy-azobenzene (upper) and *p*-amino-azobenzene were separable on the columns.

When alumina is treated with acid, some of the ions are adsorbed. These adsorbed ions may be exchanged subsequently with other solutes that are passed through columns of the treated alumina. Kuhn and Wieland have observed that a technical preparation of alumina washed with hydrochloric acid adsorbs panthothenic acid with a simultaneous liberation of chloride ion. The same alumina washed with sulfuric acid instead of hydrochloric acid did not adsorb pantothenic acid and there was no liberation of sulfate ions.

In the United States granular activated alumina is prepared on a large scale for technical operations. According to information published by Aluminum Ore Company (East St. Louis, Illinois) and its parent company, Aluminum Company of America, a special activated alumina is obtained from aluminum trihydrate by a patented process (U. S. Patents 1,868,869 and 2,015,593). This product is essentially aluminum oxide in porous, amorphous form, with minor amounts of hydrated alumina and very small amounts of soda, oxides of iron, silicon and titanium. Substantially all the soda is combined with alumina as an insoluble compound. Analytical results published by Aluminum Ore Company for its Grade A product are:

Alumina (Al_2O_3)		92 00%
Loss on ignition (H_2O)		7 00
Soda (Na_2O)	less than	1 00
Silica (SiO_2)	less than	0 10
Ferric oxide (Fe_2O_3)	less than	0 10
Titania (TiO_2)	less than	0 10

This activated alumina is available in granules ranging from 1.5 inches to those that pass a 200 mesh sieve. The larger particles find use in technical processes such as the decolorization of oil and the dehydration of air and other gases (Chapter IX). The regular Activated Alumina, Grade A, when crushed to pass 100 or 200 mesh does not form a free filtering bed when used in Tswett columns. However, the Aluminum Ore Company is now supplying a minus 80 mesh Activated Alumina, special for chromatographic analyses, which contains particles of uniform size and which ranges between 80 and 300 mesh. This grade is now being employed extensively. With respect to its adsorptive properties, this alumina is similar to that standardized according to Brockmann. Its adsorption capacity for many substances compares with that of the best grades of charcoal. It is neutral and nearly insoluble in water. It is deactivated by water and alcohols which remove most adsorbed compounds. In-

activated and used samples may be partially reactivated by drying in air at room temperature or completely reactivated by drying within the temperature range of 177 to 316°.

For some time Baker Chemical Company, Phillipsburg, New Jersey, has sold an activated alumina, known as "Hydralo," that is manufactured for them by the Aluminum Ore Company. When this alumina is ground and screened to the proper size, it is also an effective adsorbent for use in Tswett columns.

Those who desire to prepare activated alumina will derive much valuable information from the papers of Holmes and his co-workers. There are described various methods for the preparation and activation of alumina as well as procedures for the removal of free alkalies.

For some purposes, as for example the resolution of anthocyanins and algal pigments, alumina of reduced activity is required. These preparations may be made by activation of alumina at low temperatures or activated alumina may be partially deactivated with water or alcohol (Heilbron and Phipers). For separation of certain dyes, alumina treated with lime water has been used (Ruggli and Jensen) (page 149).

A native hydrated alumina, known as "bauxite," has been used for preparation of enzymes (page 115). Bauxite activated by heat has been found useful for purification of sugar solutions (page 159).

Magnesia. As first reported by Tswett and later by Euler and Gard, the usual preparations of magnesium oxide exhibit weak adsorptive properties. Subsequently it was discovered that magnesia prepared by dehydration of the hydroxide is a very active adsorbent. Experiments carried out by the California Chemical Company (now Westvaco Chlorine Products Company) Newark, California, where magnesium hydroxide is prepared from salt bitterns, showed that the adsorptive capacity of magnesia for carotenes varies with the temperature at which the hydroxide is dehydrated. Some preparations caused marked decomposition of the pigments others exhibited about the same adsorptive capacity but did not destroy the adsorbed carotene. By comparison of samples prepared in different ways, a preparation was selected that exhibited great adsorption capacity yet caused minimum decomposition of the carotenes (Strain 2). This magnesia is now sold under the trade name of Micron Brand Magnesium Oxide, No. 2641.

This specially prepared magnesia which is comparatively cheap has been used for the resolution of a variety of compounds (hydro-

carbons, alcohols, ketones and ethers). It has been found especially efficient for the resolution of mixtures of xanthophylls (Strain 10) making possible the preparation of pigments from mixtures not resolvable with other adsorbents. It is strongly alkaline and can not be used for the resolution of nitro compounds, organic acids and other substances that are easily altered by alkalies. In this respect, it is not nearly so widely applicable as an adsorbent as is activated alumina.

Micron Brand magnesium oxide is finely pulverized and for use in adsorption columns it must be mixed with Hyflo Super Cel, a special heat-treated siliceous earth. It is difficult to use columns of this magnesia with water as a solvent. The magnesia also decomposes carbon disulfide and it is discolored by commercial gasolines. Like alumina, magnesia is deactivated by alcohols and water and is reactivated by dehydration.

Comparative experiments on the elution of adsorbed carotenes from columns of powdered magnesia mixed with siliceous earth and from columns of granular alumina have shown that for a given volume of eluant, the carotenes are more completely removed from the magnesia. Slower diffusion of the pigments from the interstices of the larger alumina granules appears to be the cause of this.

Reagent magnesium oxide (Merck and Company) is a highly selective adsorbent. Its adsorptive capacity lies between that of the activated magnesia and that of the alkaline earth carbonates. Limitations to its use because of its alkaline reaction are the same as those for activated magnesia. Magnesium oxide prepared by combustion of the metal in air is a very weak adsorbent.

Lime. Both quick lime and slaked lime are active and selective adsorbents (Karrer and Walker). Their adsorptive capacity lies between that of activated alumina or magnesia and that of the alkaline earth carbonates. The activity of most technical preparations of these compounds is subject to enormous variation due possibly to the presence of impurities and to the method of preparation.

Freshly slaked lime usually exhibits more uniform adsorptive properties than old preparations. It is prepared from quick lime by the addition of water in small portions until the mass falls apart. This product is passed through a sieve to yield particles of the desired size (Zechmeister and Cholnoky 10).

Limitations to the use of lime as an adsorbent are similar to those

for magnesia. It can not be used readily with water, and it is strongly alkaline. In view of its cheapness, however, lime may often be used in preference to other more active and efficient adsorbents.

Titania. Adsorptive titania, used for separation of amino acids (Johnson), was prepared in the following way. Titanium tetrachloride (1 lb.) was diluted with six volumes of water and the resulting solution was filtered. The filtrate, kept in agitation with a mechanical stirrer, was treated with a solution of potassium carbonate (300 g. per l.) which was added from a dropping funnel until the titania set to a vibrant gel. This gel was immediately stirred into a large volume of water and washed repeatedly by decantation until the supernatant fluid no longer cleared. The precipitate was dried on a water bath and then in an oven at 110° for 1 hour. The friable solid that resulted was ground and sieved.

Calcium Carbonate. Tswett often used calcium carbonate as a mild adsorbent for leaf pigments. Like other adsorbents, this carbonate exhibits great variations in its adsorptive properties. The presence of very small quantities of moisture greatly diminishes the activity of all preparations. In order to obtain even moderately active preparations, the powdered material should be dried at about 150° immediately before use.

Barium Carbonate. Most preparations of barium carbonate are better adsorbents than calcium carbonate. Many of these are very finely divided and form columns with much lower percolation rates than columns of calcium carbonate. The adsorptive activity of barium carbonate is also increased by dehydration at elevated temperatures.

Magnesium Carbonate. Both the normal and basic carbonates of magnesium are active and selective adsorbents. They are slightly more active than barium carbonate and are usually somewhat more selective. Most preparations are so finely divided that they must be mixed with a filtration aid like Hyflo Super Cel before they can be used in columns.

Calcium Sulfate. Hydrated calcium sulfate is a weak, neutral adsorbent. It has been recommended for the adsorption of anthocyanins from aqueous solution and for the adsorption of vitamin K_1 from petroleum ether solutions (Karrer and Weber).

Soda Ash. Anhydrous sodium carbonate has been used as an adsorbent for the removal of chlorophylls and xanthophylls from

solutions of carotene in the extracts of leaf material (Kernohan). It does not exhibit selective adsorption properties for closely related chemical compounds.

Magnesium Trisilicate. A highly adsorptive, finely powdered magnesium trisilicate is prepared by the Philadelphia Quartz Company at Berkeley, California. With many solvents such as carbon tetrachloride and benzene, this adsorbent forms translucent, viscous gels that are nearly impenetrable to solvent on the columns. When mixed with four parts of Hyflo Super Cel, magnesium trisilicate can be packed into columns that filter rapidly and upon which free halogens may be separated from one another (page 77).

Although magnesium trisilicate is neutral in reaction when suspended in water, it decomposes adsorbed carotenoids as do the fuller's earths. Carotenes are strongly adsorbed from petroleum ether solutions and are gradually converted into gray products, the β-isomer being decomposed more rapidly than the α-isomer. There are some indications that the magnesium silicates may be used as adsorbents for acidic substances (page 87).

Magnesium Sesquisilicate. Preparations of magnesium sesquisilicate have also been distributed by the Philadelphia Quartz Company. The adsorptive properties of this compound are similar to those of the trisilicate. Both compounds behave like talc with respect to their effect on adsorbed organic compounds although their adsorption capacity is much greater.

Talc. Talcum powder is an excellent adsorbent for organic acids and other weakly acidic compounds such as the phenols and polynitro derivatives that are too strongly adsorbed upon magnesia and alumina. Although its adsorptive capacity is small as compared to the activated adsorbents, talcum exhibits a marked selective action and it is relatively inert. Most preparations of talcum are so finely powdered that it is advantageous to mix them with a filter aid before packing the adsorption column. Columns of talc are efficient for the resolution of the colored hydrazones formed by treating carbonyl compounds with 2,4-dinitrophenylhydrazine.

Permutit. Zeolites developed originally for the removal of hardness from water are finding extensive use in Tswett columns. In the removal of alkaline earths from water, the adsorption is accompanied by a replacement or exchange reaction. In the resolution of organic materials, the adsorption appears to be purely a surface reaction.

Two preparations, Permutit and Decalso are sold by the Permutit Company, New York City.

Adsorptive Clays. A number of earths, some of which are prepared by mechanical treatment others of which are purified by chemical treatment, are available for use in adsorption columns. Among the best known of these products are

Frankonit KL	activated with hydrochloric acid (Pfirschinger Mineralwerke, Kitzingen-am-Main, Germany)
Floridin XXF	finely powdered
Floridin XF	larger granules than Floridin XXF

All these products are active adsorbents and retain much of their activity even in the presence of water. For this reason they are suitable for the resolution of many water-soluble compounds. (Koschara; Schopf and Becker).

The adsorptive clays exhibit two undesirable characteristics. They are sufficiently acid to destroy many adsorbed substances and they absorb nearly all substances in such a way that subsequent elution is difficult and incomplete. Advantage may be taken of the second undesirable property in the preparation or purification of materials from which it is desired to remove a strongly adsorbed contaminant as for example in the decolorization of oils.

Fuller's Earth. Many natural products used as Fuller's earth are active adsorbents. The best known of these are "Lloyd's reagent" and various similar products known as "Bentonite" These slightly acid adsorbents find extensive use in technological operations for the removal of colored contaminants from many different products.

Charcoal. The adsorptive properties of few substances are better known than are those of the activated charcoals (McBain). Nevertheless, charcoal is not readily adaptable to use in adsorption columns. Many organic compounds are oxidized when in contact with activated carbon. Most substances adsorbed upon charcoal are more difficult to elute than when adsorbed upon activated alumina or magnesia. Under the most favorable conditions, elution is usually incomplete. Moreover substances adsorbed upon columns of charcoal are difficult or impossible to see. By collecting the percolate from columns of charcoal in successive portions it is possible to resolve many mixtures into their constituents, as for example, the separation of α- and β-carotene (Strain 2). The better known commercial preparations

of charcoal are sold under the trade names of Norit, Norit A (iron free), Nuchar, Carboraffin and blood charcoal. In water, some of these preparations are strongly acid, others are alkaline.

Sucrose. Among organic compounds, cane sugar has found extensive use as an adsorbent for chlorophylls and similar compounds. Commercial preparations of powdered sugar exhibit great variations in their adsorptive properties as do many samples that are ground in the laboratory. The causes of these variations are not known. Powdered sugar is hygroscopic and its adsorption capacity is reduced upon the adsorption of water. For this reason sugar should be powdered in closed vessels and thoroughly dried before use if a product of maximum adsorptive capacity is to be obtained. Sugar that has been used for adsorption may be recovered by recrystallization from water.

Starch. Technical preparations of starch have been used for the resolution of many substances that are also resolvable on sucrose. The adsorptive capacity of starch also varies, but none of the conditions that contribute to this variability have been determined.

Inulin. Among the many substances tested by Tswett as adsorbents for leaf pigments was inulin. Interest in this material has again been stimulated as the result of its use for the resolution of the chlorophylls (Mackinney) and in consequence of improvements in the methods for its preparation and recovery after use (Spoehr). The inulin was extracted from dahlia tubers with hot water. This extract was filtered and frozen. After several days the mass was thawed, and the precipitated inulin was collected by filtration. It was dried, ground thoroughly and stored in vacuum. The activity of the product was decreased by the presence of small quantities of water. Inulin that has been used in adsorption columns was recovered by dissolution in water followed by freezing the solutions as just described. Columns of inulin are more efficient for the resolution of the chlorophylls than are columns of sucrose or starch. The inulin itself appears to be without action upon the adsorbed pigments.

Magnesium Citrate. Metallic salts of organic acids represent a new type of adsorbent that promises to find wide application in adsorption columns. Through the use of magnesium citrate Mackinney has shown that chlorophylls may be readily separated from one another in the presence of considerable quantities of impurities. The activity of the magnesium citrate is altered by the same conditions that affect the activity of other adsorbents.

8-Hydroxyquinoline. Utilization of 8-hydroxyquinoline as an adsorbent for the resolution of mixtures of inorganic ions from aqueous solution portends new developments in the field of adsorbents (Erlenmeyer and Dahn). Here one is apparently dealing with a reversible chemical reaction on the surface of the adsorbent (page 00). Before use in columns, most preparations of 8-hydroxyquinoline must be powdered.

V. SOLVENTS AND ELUANTS

1. Selection of Solvents and Eluants

Choice of a solvent is determined to a large extent by the solubility of the materials to be adsorbed and by the activity and solubility of the adsorbent. Selection of solvent and adsorbent and of eluant and adsorbent are interdependent as has been pointed out on page 50.

There is no sharp demarcation between solvents and eluants. Liquids that serve for the adsorption of some compounds may be used for the elution of other substances with lower adsorbabilities. In contrast to the effect on solvents, contamination of eluants with polar substances does not affect adversely the properties of these liquids.

Eluants, as a rule, are chosen with the object of obtaining rapid and complete liberation of adsorbed compounds. It is desirable that adsorbed compounds be quite soluble in the eluant else they may crystallize after liberation from the surface of the adsorbent and before they are washed through the column. For example, if carotenes adsorbed upon magnesia are eluted with methanol, the eluted pigments crystallize in the adsorbent and are not removed upon prolonged washing of the column. If they are eluted with petroleum ether containing a little alcohol, or with benzene, crystallization does not occur and the eluted pigments are rapidly washed from the column.

Most liquids more polar than the adsorbed substances will displace them from the adsorbent. Chlorinated compounds will usually liberate hydrocarbons, and alcohols will liberate many other organic substances. Acids and bases will remove all but the most strongly adsorbed compounds. If the eluant is sufficiently polar, small quantities of it added to the solvent will suffice to remove all the adsorbed substances. The presence of about 1 per cent methanol in petroleum ether liberates carotenes adsorbed on alumina or magnesia. Similar effects have been obtained by the addition of water to ketones and to chlorinated compounds. The eluant should not react with the adsorbent or with the adsorbate.

Eluants that have found extensive use are alcohols, ethers, chlorinated compounds and benzene. Mixtures of alcohols with a variety of organic solvents have also been used. Some of the most efficient eluants are mixtures of alcohols and organic acids in pyridine and mixtures of all these in water.

Liquids used for adsorption and elution may be arranged in a series in respect to their action on adsorbed compounds. This has been done in table 16, the solvents from which adsorption is strongest being tabulated first, those from which adsorption is weakest being

TABLE 16

Liquids arranged in approximate order of their effect on adsorption

(Adsorption is greatest from those materials listed first in the series)

Petroleum ether, b.p. 30–50°
" " " 50–70°
" " " 70–100°
Carbon tetrachloride
Cyclohexane
Carbon disulfide
Ether (anhydrous, alcohol-free)
Acetone (anhydrous, alcohol-free)
Benzene
Toluene
Esters of organic acids
1,2-Dichloroethane, chloroform, dichloromethane
Alcohols
Water (variations with changes in pH and salt concentration)
Pyridine
Organic acids
Mixtures of acids or bases with water, alcohol or pyridine

recorded last. The former are most likely to be used as solvents, the latter as eluants, depending, of course, upon the activity of the adsorbent and upon the nature of the adsorbed compounds. The order of the liquids in this series is also subject to slight variation depending upon the adsorbents and adsorbed compounds.

This arrangement of liquids has recently been called an *elutropic* series of solvents (Trappe). Knowledge of the order of various solvents in this series is of great aid in the selection of liquids for separation of adsorbed compounds by the flowing chromatogram method (page 46). In order to obtain gradual changes in the effect of solvents on adsorbed compounds, mixtures of two nearby solvents

in the series may be used as already described in chapter III, section 6 page 44.

Nearly all commercial solvents should be purified before use with adsorption columns. Usually the liquids are dried, separated from the dehydrating agent, and distilled in an all glass apparatus. This avoids contamination with rubber and other substances, traces of which might contaminate the adsorption columns. Traces of acids that occur in some solvents may be removed with lime or with activated adsorbents. Solvents that form peroxides upon standing in contact with air should be redistilled immediately before use. Occasionally solvents like benzene, ether and petroleum ether are contaminated with alcohols. These are removed by extraction of the solvent with distilled water before dehydration and distillation.

Once solvents have been purified, care must be taken to prevent their contamination with substances that are difficult or impossible to remove. Extracts of plant and animal materials often contain volatile compounds that influence the adsorption of many mixtures. When working with such materials it is desirable to keep two lots of each solvent, one for the extraction of the natural product, the other for exclusive use with adsorption columns.

2. Properties of Various Solvents and Eluants

Petroleum Ether. Light petroleum, ligroin, or benzine is one of the best, cheapest and most accessible of the organic solvents used with adsorption columns. By distillation it is separable into fractions boiling from 30° to 100° and needs no further purification. From petroleum ether, all substances are strongly adsorbed, maximum adsorption being obtained from the fractions of lowest boiling point. As solvent for the adsorption of weakly adsorbed compounds there is no substitute for low-boiling petroleum ether. Its great stability renders it usable with all adsorbents and it is easily recoverable. The inflammability and the high vapor pressure of petroleum ether render the use of this solvent in crowded laboratories extremely dangerous.

Products similar to petroleum ether such as pentane (b.p. 36°) and *n*-heptane (b.p. 98.4°) (Westvaco Chlorine Products Company, Newark, California) have also been used as solvents. Commercial gasolines, due to their great boiling point range and to the presence of reactive substances that discolor magnesia, are unsuitable for use in adsorption columns.

Carbon Tetrachloride (b.p. 76°). With respect to its effect upon adsorbed substances, carbon tetrachloride resembles the higher boiling fractions of petroleum ether. Carbon tetrachloride is the only noninflammable organic solvent that has been used with adsorption columns. This solvent is readily purified by washing with water followed by dehydration with lime. In the future carbon tetrachloride will undoubtedly find more extensive use than it has thus far.

Carbon Disulfide (b.p. 46°). Although used extensively as a solvent for the resolution of xanthophylls by adsorption on columns of alkaline carbonates, carbon disulfide exhibits numerous disadvantageous properties. Technical preparations contain impurities that are difficult to remove. The compound itself is poisonous, relatively unstable and highly inflammable. It is decomposed by activated magnesia with the formation of dark colored products. Xanthophylls adsorbed from it on Tswett columns do not separate into bands as readily as they do when adsorbed from other solvents. (Strain 10).

Benzene (b.p. 80°). Benzene or benzole, the hydrocarbon distilled from coal, is frequently used as solvent for the resolution of organic compounds on Tswett columns. It can be purified by dehydration with lime or activated magnesia followed by distillation. Substances dissolved in benzene are not so strongly adsorbed as when dissolved in petroleum ether or carbon tetrachloride.

For the resolution of many substances such as the highly unsaturated carotenes and the monohydroxy xanthophylls that are too strongly adsorbed from petroleum ether and too weakly adsorbed from benzene, mixtures of benzene and petroleum ether may be used to advantage. By gradually varying the proportion of the benzene in the solvent used for the development of the chromatogram, the rate at which the bands move through the column may be regulated.

1,2-Dichloroethane (b.p. 84°). Of the solvents recently introduced for use in adsorption columns, *sym*-dichloroethane, also known as ethylene dichloride and glycol dichloride, exhibits many desirable properties. This compound is manufactured on a large scale (E. I. duPont de Nemours and Company) and different preparations are remarkably uniform. Dichloroethane is relatively cheap; it is readily recoverable; and it keeps well. It may be purified by washing with water, by dehydration with calcium chloride and lime and finally by distillation after removal of the dehydration agent.

of charcoal are sold under the trade names of Norit, Norit A (iron free), Nuchar, Carboraffin and blood charcoal. In water, some of these preparations are strongly acid, others are alkaline.

Sucrose. Among organic compounds, cane sugar has found extensive use as an adsorbent for chlorophylls and similar compounds. Commercial preparations of powdered sugar exhibit great variations in their adsorptive properties as do many samples that are ground in the laboratory. The causes of these variations are not known. Powdered sugar is hygroscopic and its adsorption capacity is reduced upon the adsorption of water. For this reason sugar should be powdered in closed vessels and thoroughly dried before use if a product of maximum adsorptive capacity is to be obtained. Sugar that has been used for adsorption may be recovered by recrystallization from water.

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VI. LOCATION OF COLORLESS ADSORBED SUBSTANCES

Resolution of colorless compounds upon adsorption columns necessitates the use of special methods to locate each band of adsorbed substance. A number of such methods have been developed, and in a relatively short time they have been applied to investigations of a variety of colorless compounds. The procedure employed depends upon the properties of the compounds themselves.

It is significant that resolution of colorless materials upon adsorption columns has led to a complex terminology. Tswett regarded the pattern of colorless compounds upon a column as a *colorless chromatogram.* Koschara (4) has suggested that *analysis by adsorption* be substituted for *chromatographic analysis* because color is not an essential feature of the chromatogram. This suggestion has not been adopted and it seems probable that *chromatogram* shall continue to apply to the bands of colorless as well as of colored substances.

The following procedures are the principal ones used to locate colorless substances upon adsorption columns:

1. Empirical sectioning of the column.
2. Collection of successive portions of the percolate (flowing chromatogram).
3. Colored indicators.
4. Observation in ultraviolet light (ultra-chromatogram).
5. Formation of colored derivatives before adsorption.
6. Formation of colored compounds after adsorption.
7. Use of adsorbents that form colored products.
8. Untried methods.

1. Empirical Sectioning of the Column

Columns containing colorless substances that have been adsorbed and washed in the usual way are divided into sections from which the adsorbed materials are eluted and determined by appropriate physical, chemical or biological methods. Having established the relation between position on the column and the quantity of material adsorbed, one can then repeat the separations quite as precisely as if the adsorbed substances were colored.

2. Collection of Successive Portions of the Percolate (Flowing Chromatogram)

For the separation of colorless materials that are not too strongly adsorbed, the solution is passed through an adsorption column and the percolate is collected in successive portions. These portions are then subjected to analysis. If none of the materials appear in the percolate the column may be washed with solvents from which the compounds are less strongly adsorbed and successive portions of the percolate are again collected and analysed (Trappe). Instead of adsorbing the mixture upon one column and washing this with a series of solvents, the solution of the mixture may be passed successively through a number of columns each one of which contains a slightly more active adsorbent than that contained in the preceding column. Portions of the percolate from each column are analysed before the solution is passed through succeeding columns.

3. Colored Indicators

If the adsorbability of a colorless substance is determined with respect to a colored substance, then adsorption of the colored compound with the colorless one will serve to determine the position of the latter either above or below the former. The closer together two such substances are adsorbed, the more precisely one can locate the position of the colorless compound.

Selection of a suitable indicator of this type often involves a great deal of preliminary, empirical experimentation. Nevertheless, the use of this indicator method may be necessary for the isolation of many rare, naturally occurring substances such as vitamin D_3 (Brockmann 3) (page 105). In many instances, natural mixtures contain pigments that serve as indicators for the location of their colorless constituents. A striking example is the occurrence of a pigment in toad poison that is adsorbed with the toxic principle bufotalin (Wieland, Hesse and Hüttel).

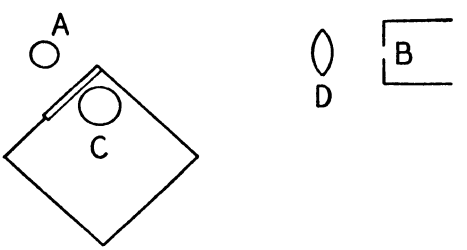
4. Observation in Ultraviolet Light (Ultra-Chromatogram)

Colorless compounds that fluoresce in light of short wave lengths can often be located upon adsorption columns by examination of these with ultraviolet light in a dark room (Karrer and Schopp, Winterstein and Schön). In order to obtain high transmission of the radiant energy, columns of quartz or of pyrex are usually employed. The

bent containing the resolved materials may also be pressed the tube and examined directly in ultraviolet light.

ıny adsorbents such as magnesia and Hyflo Super Cel are ıscent and this fluorescence must not be confused with that due e presence of adsorbed compounds. A hand spectroscope is l for the differentiation between the light emitted by many ounds and that emitted by the adsorbent. Larger spectro- s may also be used as indicated by the arrangement shown in ı 33.

e fluorescence of adsorbed materials depends upon their con- ıation on the adsorbent and upon the presence of other sub- es. According to Winterstein, Schön and Vetter, the fluores- of anthracene is completely quenched by 1/30,000 per cent of ːhacene. In aqueous solutions, the color and the intensity of



33. Arrangement for determination of the fluorescence spectrum of a atogram A, adsorption tube, B, spectroscope, C, source of ultraviolet D, quartz lens (Almasy)

uorescent light of some compounds varies with the hydrogen ion ntration. Uropterin from urine fluoresces red in strong mineral , not at all in normal mineral acid, slightly yellow in acetic acid, w-green at pH 4, sky blue at pH 7 to 11, and green in soda ıon.

a source of ultraviolet light one may use a quartz, mercury vapor with a Corning filter that absorbs the visible light. A cheaper, gh somewhat less intense source of ultraviolet light consists of a *flood* light bulb encased in a large metal can provided with a ing glass window that transmits only the light of short wave hs.

5. Formation of Colored Products Before Adsorption

colorless substances are converted into colored derivatives these ıcts may then be adsorbed upon Tswett columns in the usual

way. Colorless carbonyl compounds may be combined with 2,4-dinitrophenylhydrazine to yield yellow derivatives that are readily adsorbable on columns of talc from benzene or 1,2-dichloroethane solutions (Strain 4) (page 87). Amines may be combined with picric acid, and phenols may be converted into colored compounds with ferric chloride before adsorption (Zechmeister and Cholnoky 10). Azobenzene-*p*-carboxylic acid chloride has been used to form colored derivatives with hydroxy compounds such as sterols (Ladenburg, Fernholz and Wallis) (page 98) and sugars (Reich) (page 89). Dinitrobenzoic acid has been used to form adsorbable colored derivatives of sterols (Brockmann). According to private reports from Dr. O. H. Emerson at the University of California, 3,5-dinitro-4-dimethylamino-benzoic acid may also be used for formation of colored derivatives of hydroxy compounds. This use of colored acids to form colored derivates can undoubtedly be applied to a variety of other

Fig. 34. Location of colorless adsorbed compounds by brushing on a reagent that forms a colored product (Zechmeister, Cholnoky and Ujhelyi).

organic compounds such as amines, amino acids, thiols and their analogues.

6. Formation of Colored Products After Adsorption

Colorless substances adsorbed upon a column may be rendered visible by treatment of the column with a reagent that converts the compounds into colored products. The adsorbent containing the materials to be located is pressed from the tube. A solution of the reagent is then applied in a narrow stripe along the long axis of the moist cylinder of adsorbent as shown in figure 34. As soon as the position of the adsorbed materials has been determined, the column is cut into sections, the stripe containing the reagent is removed with a knife or spatula and the adsorbate is recovered in the usual way by elution with a polar solvent (Zechmeister, Cholnoky and Ujhelyi).

This method has the disadvantage that it is impossible to follow the development of the chromatogram. In order to determine the movement of the compounds in the column it is necessary to adsorb

portions of the mixture on a number of columns and to examine these after they have been washed with solvent for increasing periods. The main portion of the solution is then adsorbed under the conditions found to give a satisfactory resolution of the compounds in the preliminary tests.

A great variety of reagents may be used to locate the colorless compounds of the column. Vitamin A and similar polyenes may be detected with antimony trichloride dissolved in chloroform. Reducing substances may often be located with iodine or potassium permanganate. Carbonyl compounds that color fuchsin sulfurous acid are readily detectable with this reagent. Many inorganic ions are rendered visible on the columns by use of hydrogen sulfide.

In many instances, the concentration of the adsorbed compounds is very low. As a consequence the color formed by the reagent acting upon the colorless material is frequently so pale as to be undetectable. In this event, one must start with more concentrated solutions or else use reagents that yield more highly colored products.

Instead of applying the reagent to the cylinder of adsorbent, a solution of the reagent may be passed through the column. For example, ions of the metallic elements that have been resolved by adsorption can be rendered visible by washing the column with a solution of hydrogen sulfide. Under these conditions, the colorless chromatogram is rendered visible just as the latent image on an exposed film is developed with reducing agents. This colorization of the chromatogram by reagents has also been called "development of the chromatogram" (Schwab and Jockers), even though the same term had already been used in reference to the separation of the bands of adsorbed compounds by washing the column with additional solvent. For want of better terms, this ambiguity is likely to persist a long time.

Another procedure for separation of the bands of colorless chromatograms consists in elution of the adsorbed compounds from small portions of the adsorbent. The elutriate is tested for the presence of the colorless compounds. After these have been located on the column, they are eluted from the appropriate section of the adsorbent (page 113).

7. Use of Adsorbents That Yield Colored Products

Metallic ions, many of which react with 8-hydroxyquinoline to form colored, slightly dissociated products, may be separated from one another when adsorbed upon columns of this quinoline derivate

(Erlenmeyer and Dahn) (page 77). Should other adsorbents be found upon which organic compounds are adsorbed with the production of colored bands, this application of the method will undoubtedly find wide application.

8. Untried Methods

It is highly probable that a number of new methods will become available for the location of colorless compounds upon adsorption columns. For example, radioactive elements may be combined with the colorless materials before the latter are adsorbed, the radioactivity providing the spoor by which the position of the compound on the column is determined. Fluorescent compounds may also be combined with the materials to be adsorbed, the fluorescence serving as the means for the location of the resolved compounds. Certain adsorbents become translucent when wet with organic solvents so that it may become possible to determine the position of the bands of adsorbed compounds from changes in the light transmission at the several bands or zones (Trappe). Tiselius has recently utilized the Toepler-Schlieren optical method for analysis of the percolates.

VII. CHROMATOGRAPHY OF INORGANIC COMPOUNDS

1. Elements

Bromine and iodine are readily separable by adsorption upon columns of magnesium trisilicate or sesquisilicate. According to this procedure which has not been described elsewhere, a column (2 by 10 cm.) is prepared from a mixture of the magnesium silicate (1 part) and Hyflo Super Cel (4 parts). The halogens dissolved in carbon tetrachloride at a concentration of 2 to 4 mg. per ml. are passed through the column until a 2 to 3 cm. band is formed. The column is then washed with fresh carbon tetrachloride which soon causes the separation of three distinct bands. The uppermost is purple-brown; the middle one is violet and contains the iodine; the lowest is reddish-brown and contains the bromine.

In order to isolate the resolved pigments, the bands are removed separately from the column with a spatula, and each compound is eluted with 1,2-dichloroethane. This elution is not complete, due presumably to reaction of the halogens with the adsorbent. The longer the halogens have remained in contact with the adsorbent the lower is the quantity recovered by elution.

2. Ions of Metals

Adsorption on 8-Hydroxyquinoline. Erlenmeyer and Dahn have shown that a number of cations dissolved in water are readily bound upon columns of 8-hydroxyquinoline or of this reagent and non-adsorptive siliceous earth. Because many of the ions form colored bands on the column, the separated compounds are easily located even when present in minute quantities. Adsorption of neutral solutions containing two, three or four compounds, has established that the following ions form bands in the order and of the color given here beginning at the top of the column:

VO_3'	grey-black
WO_4''	yellow
$Cu^{\cdot\cdot}$	green

$Bi^{\cdots}$	yellow
$Ni^{\cdot\cdot}$	green
$Co^{\cdot\cdot}$	reddish
Zn	yellow
$Fe^{\cdots}$	black
$UO_2^{\cdot\cdot}$	red-orange

If the adsorption is made from aqueous acetic acid solution positions of iron and zinc on the column are reversed. Develop of the chromatogram with aqueous acetic acid facilitates the re tion of nickel and cobalt. The method is fairly sensitive, 2 μ Fe being detectable. It was suggested that this procedure r be adapted to the semiquantitative analysis of alloys.

Adsorption on Alumina. Numerous procedures have beer veloped for the resolution of various mixtures of inorganic subst (Lange and Nadel; Schwab and co-workers). Of the many ad ents tested, alumina (Brockmann) gave the most consistent reliable results. This alumina was made into a slurry with w heated to 70 to 80° in order to remove air, and filled into the r tubes described by Hesse (page 36). Often it was necessary to the column with acids thereby neutralizing the alkaline condi prevailing upon the surface of the adsorbent. Percolation wa duced by gravity only. When materials were adsorbed from r solutions, the band formed on the adsorbent was adjusted to a one-seventh of the length of the column before development o chromatogram was commenced.

Completion of the separation of the bands on the columns usually accomplished with water or diluted acids. These called the *wash liquids.* They did not always effect a com separation of the bands of adsorbed materials from one anc The separated bands were further separated from one anothe rendered visible by washing the column with reagents or devel such as ammonia saturated with hydrogen sulfide, potassium cyanide, or alkalies. This development of the chromatogram m illustrated by the following example. A solution of ferric, c and cobalt nitrates adsorbed on a column of alumina and w with water formed this chromatogram,

Ferric	brown
Cupric	blue
Cobalt	rose

After the column was developed with potassium ferricyanide, the chromatogram was

Ferric	blue
Cupric	brown
Cobalt	greenish

The order of the common cations on columns of alumina is given below. This order may be varied a bit by adsorption of ions from solutions in which complexes are formed. Substances that were not readily separable are tabulated horizontally.

$As^{\cdots}$
$Sb^{\cdot\cdot}$
$Bi^{\cdots}$
$Cr^{\cdot\cdot}$, $Fe^{\cdots}$, $Hg^{\cdot\cdot}$
$UO_2^{\cdot\cdot}$
$Pb^{\cdot\cdot}$
$Cu^{\cdot\cdot}$
$Ag^{\cdot}$
$Zn^{\cdot\cdot}$
$Co^{\cdot\cdot}$, Ni , $Cd^{\cdot\cdot}$, $Fe^{\cdot\cdot}$
$Tl^{\cdot}$
$Mn^{\cdot\cdot}$

Separation of the cations of a great many pairs of compounds is described by Schwab and Jockers (2). All were adsorbed on columns of alumina. The results are summarized in table 17. In the first two mixtures, the ions were adsorbed from solutions containing tartaric acid; the next three solutions contained dilute nitric acid; whereas the remaining solutions contained no acid.

Alumina alters some adsorbed ions. Mercurous compounds are changed into metallic mercury and mercuric ions. This adsorbent also causes oxidation reduction reactions between certain ions such as cupric and ferrous and mercuric and palladous. Ions of a few elements tend to precipitate on alumina as the hydroxides unless acid is present.

Ions of metals in the form of special complexes are separable upon columns of alumina. Here the order of the metals on the column is quite different from that observed with the normal ions. Com-

plexes of the cations with ammonia were adsorbed in the following order. The change in the relative position of silver ion is especially noteworthy.

Co··
Zn··
Cd··; Cu··
Ni··
Ag·

Complexes of the cations with tartrates in alkaline solution are reported to be useful for the difficult separation of ferric and chromic ions. Metals in these complexes are adsorbed in the following order:

Mn··
Cd··
Zn ·, Pb··, Cu··, Bi , Fe···, Cr···
Co··
Ni··

A more complete resolution of two compounds of similar adsorbabilities is occasionally effected by the presence of a third compound that is adsorbed between them. Cobalt and nickel nitrates dissolved in ammonium hydroxide and adsorbed on a column of alumina are washed with a solution of zinc acetate dissolved in an excess of ammonia. When the column is subsequently washed with colorless ammonium sulfide, an upper black band of cobalt sulfide is found to be separated from a lower black band of nickel sulfide by a white band of zinc sulfide. In the absence of the zinc ions, the separation of the cobalt and nickel is incomplete.

Detection of Cations. Adsorption of solutions of salts on columns of alumina is sufficiently sensitive for detection of traces of many elements in the presence of others. As little as 1 μg. of ferric iron in 0.2 ml. of 0.000,1 molal solution can be observed on a column after development with ferrocyanide solution. This observation is possible even if the solution is molal with respect to copper or cobalt. Likewise, 1 μg. of copper can be detected in the presence of cobalt or cadmium. Experiments on the detection of these small quantities of ions were performed by diluting the solutions of the salt to be tested for with molal solutions of the salts that were present in excess.

TABLE 17

Pairs of cations separated by adsorption on alumina with solutions utilized for development of the chromatograms and with the color of the completed chromatograms (Schwab and Jockers 2)

COMPOUNDS ADSORBED		WASH LIQUID	DEVELOPER	COLORS OF CHROMATOGRAM	
Upper	Lower			Upper	Lower
$AsCl_3$	$SbCl_3$	dil. HCl	$H_2S—H_2O$	yellow	orange-red
$SbCl_3$	$Bi(NO_3)_2$	dil. HCl	$H_2S—H_2O$	orange-red	dark brown
$Bi(NO_3)_2$	$Cr(NO_3)_3$	dil. HCl	NH_4OH	dark brown	grey-green
			$NH_4OH—H_2S$		
$Bi(NO_3)_2$	$Fe(NO_3)_3$	dil HNO_3	NaOH	dark brown	green-black
			$(NH_4)_2S$		
$Bi(NO_3)_2$	$Hg(NO_3)_2$	dil. HNO_3	NaOH	dark brown	deep black
			$(NH_4)_2S$		
$Cr(NO_3)_3$	$UO_2(NO_3)_2$	H_2O	$(NH_4)_2S$	grey-green	deep black
$Fe(NO_3)_3$	$UO_2(NO_3)_2$	H_2O	$K_4Fe(CN)$	blue	brown
$Hg(NO_3)_2$	$UO_2(NO_3)_2$	H_2O	$(NH_4)_2S$	black	brown
$UO_2(NO_3)_2$	$Pb(NO_3)_2$	H_2O	$(NH_4)_2S$	brownish	black (indef.)
$Pb(NO_3)_2$	$Cu(NO_3)_2$	H_2O	$(NH_4)_2S$	black	greenish-black
$Cu(NO_3)_2$	$AgNO_3$	H_2O	NaOH	green-black	brown
$AgNO_3$	$Zn(NO_3)_2$	H_2O	$NaOH—H_2S$	grey	———
$Zn(NO_3)_2$	$Cd(NO_3)_2$	H_2O	$(NH_4)_2S$	white	yellow

Only 0.2 ml. of the diluted solutions were adsorbed on columns 3 to 4 mm. in diameter.

3. Anions

According to Schwab and Dattler, many inorganic anions may be resolved upon adsorption columns, but this method is unsuitable for systematic analysis because it is impossible to convert a sufficient number of the anions into colored products. For most of the adsorptions reported by Schwab and Dattler, columns of alumina that had been washed with molal nitric acid and water were used. Various metallic ions were employed to color the bands of the resolved, adsorbed anions. As an example, solutions of silver salts were

utilized for the detection of adsorbed phosphate, chromate, dichromate and ferricyanide ions. Some of the anions investigated by Schwab and Dattler were adsorbed in the following order:

OH'
PO_4''
F'
$Fe(CN)_6'''$, CrO_4''
SO_4''
$Fe(CN)_6''$, Cr_2O_7''
Cl'
NO_3'
MnO_4'
ClO_4'
S''

4. Purification of Inorganic Compounds

Inorganic compounds have been purified by passage of a solution of the material through a column that removes the impurities. In this way, Schwab and Jockers (2) removed traces of iron, lead, copper and alumina from their reagents. Traces of acid were also removed by this technique.

Practical applications of this adsorption procedure have long been practiced. An example is the removal of alkaline earths from water with zeolites. A more recent application is the use of bone ash for purification of the fluorine containing waters of the Southwestern United States. The adsorption and elution procedure utilized for purification of organic compounds has not been widely applicable to inorganic materials because the latter are not readily eluted once they have been adsorbed.

5. Isotopes

Partial separation of the isotopes of lithium, potassium and nitrogen by passage of solutions of lithium, potassium and ammonium chlorides through columns of zeolites was obtained by Taylor and Urey. For this separation, columns of three sizes were employed. One was 0.75 inch by 30 feet and contained 2 kg. of sodium zeolite; the second was 1.25 inches by 30 feet and contained 4 kg. of sodium zeolite; the third column was 1.25 inches by 100 feet and contained 13 kg. of the sodium zeolite. These columns were constructed in 10 foot sections. They were filled with water, and a slurry of the zeolite was then added

In a series of experiments in which only lithium chloride solutions (3 per cent and 1 per cent) were passed through the smallest adsorption column at a rate of 2 to 3 ml. per minute, the leading portion of the lithium chloride solution was shown by spectrographic analysis to contain Li^7 and Li^6 varying between the rations of 12.3 to 1 and 13.3 to 1. The normal ratio of Li^7 to Li^6 is about 11.7 to 1.

One experiment in which lithium chloride (15 g. in 10 per cent solution) was passed into the column and washed through with 0.5 per cent sodium chloride solution resulted in a change in the isotopic proportions of both the leading and the trailing lithium solution. The ratio of Li^7 to Li^6 in the leading fraction was 12.6; that in the trailing fraction was 8.9.

Passage of 15 g. of lithium chloride (in 10 per cent solution) through the 100 foot column using 3 per cent sodium chloride to develop the chromatogram resulted in Li^7 to Li^6 ratio of 14.1 in the leading fraction and of 8.8 in the trailing fraction. In this experiment, less than 2 per cent of the lithium chloride solution passing through the column showed any considerable change in isotope composition. The large middle portion was unresolved.

In similar experiments with potassium chloride, less satisfactory results were obtained. However, after washing the column with sodium chloride solution, there was a slight increase in K^{41} in the trailing fraction and an increase of K^{39} in the leading fraction. With ammonium chloride, there was preferential adsorption of the heavier isotope.

Taylor and Urey also reported that in a closed system, 10 to 20 minutes is required for dissolved salts to come to equilibrium with the sodium zeolite. Because the order of light and heavy isotopes on the column is different for lithium and potassium, they conclude that the chromatographic separations do not depend upon differences between the rate of diffusion of the isotopes. Some of the newer prospects in the separation of isotopes have been discussed by H. S. Taylor.

VIII. CHROMATOGRAPHY OF ORGANIC COMPOUNDS

1. Aliphatic Compounds

Hydrocarbons. Hentriacontane, which occurs in plant material, is often contaminated with dipalmityl ketone. This mixture of hydrocarbon and ketone was separated on columns of alumina (Winterstein and Stein 1). Partially purified hentriacontane (300 mg.) was dissolved in petroleum ether (100 ml.) and the resultant solution was passed through an adsorption column (1 by 6 cm.) composed of activated alumina and fibrous alumina (5 to 1). The column was then washed with 200 ml. of petroleum ether. From the filtrate, hentriacontane of the correct melting point was obtained. Extraction of the column with alcohol followed by evaporation of the solution provided 2 mg. of dipalmityl ketone.

By a similar procedure in which a mixture of chloroform, petroleum ether and benzene was used as solvent, a plant paraffin of the formula $C_{23}H_{48}$ was obtained from roots of *Sarsaparilla* (Simpson and Williams). Hydrocarbons contaminated with aliphatic nitriles have been purified by adsorption of the latter on heated columns of silica gel (Ralston, Harwood and Pool) (See figure 23, page 38), and preliminary experiments on the chromatography of rubber solutions have been reported (Levi and Cajelli; Cajelli). Some of the hydrocarbons and higher alcohols contained in the unsaponifiable matter of liver oil have also been separated by chromatographic adsorption (Nakamiya).

Acids. Partial resolution of mixtures of oleic and stearic or oleic and palmitic acids was obtained by Kondo. About 0.5 g. of acid was dissolved in benzene plus petroleum ether (1:1, 40 ml.) and passed through a column of alumina 1.2 by 20 cm. After development of the chromatogram with 500 ml. of benzene, the oleic acid was found in the upper 6 cm. of the column, stearic or palmitic acid in the lower 8 cm. and the unresolved mixture in the central portion. Analogous results were reported by Manunta for adsorption of these same acid mixtures on columns of magnesium sulfate or Frankonit and by Kaufmann for adsorption of the acids on alumina or silica gel.

Cassidy has made a careful comparison of the relation between the adsorption isotherms for various fatty acids and the resolvability of these acids upon columns of various adsorbents. On the basis of measurements of the adsorption isotherms, adsorbents were divided into three groups: a. solids that adsorbed acids of high molecular weight more strongly than those of lower molecular weight (decolorizing, activated carbon, Merck); b. solids that adsorbed acids of low molecular weight better than those of higher molecular weight (Silica Gel I, Howe and French, Inc., and charcoal, Cliffchar R-Fine, Cliffs Dow Chemical Company); c. solids that did not differentiate appreciably between acids of different molecular weights (activated carbon Grade 5P, Carbide and Carbon Chemicals Corporation). Mixtures of lauric and stearic acid were adsorbed upon columns of these adsorbents from solution in petroleum ether (100 ml.), and the columns were then washed with petroleum ether. The percolate was collected in portions of 100 ml. from which the acids were recovered by evaporation of the solvent.

One g. of carbon Merck was packed into a column 1.3 by 3 cm. Lauric acid (0.151 g.) and stearic acid (0.156 g.) in solution in petroleum ether were passed through this column using air pressure of about 20 cm. of mercury to facilitate filtration. The first 300 ml. of percolate contained 0.137 g. of pure lauric acid. The next 400 ml. of percolate contained 0.026 g. of a mixture of lauric and stearic acids. When the column was washed with 200 ml. of petroleum ether containing 2 per cent methanol, 0.120 g. of pure stearic acid was removed. This experiment showed that the adsorption of the acids on the column was consistent with that predicted from the adsorption isotherms. It also demonstrated that resolution of the two acids was nearly complete on the short column employed.

A somewhat similar experiment with silica gel as adsorbent resulted in an incomplete separation of the two acids. Here stearic acid formed the lower band. With columns of Cliffchar R-Fine carbon, resolution of the acid mixture was also incomplete, but in this case lauric acid formed the lower band. This relation of lauric and stearic acid in the column is the opposite of that predicted from a comparison of the adsorption isotherms of the pure acids.

A partial separation of lauric and stearic acids was obtained on a column of activated carbon Grade 5P in spite of the fact that there was no appreciable difference between the adsorption isotherm of this

acid and that of stearic acid. Here lauric acid formed the lower band.

Phthioic acid in the form of its tribromanilide has been freed of impurities by passing it, in solution in benzene, through a column of activated alumina (Wagner-Jauregg).

The aliphatic keto acids levulinic acid, $CH_3C(O)CH_2CH_2COOH$, and geronic acid, $CH_3C(O)CH_2CH_2CH_2C(CH_3)_2COOH$, were separated by adsorption of their 2,4-dinitrophenylhydrazones upon a column of talc. Benzene was used as solvent for the adsorption and for development of the chromatogram. Alcohol was used for elution of the resolved compounds. Under these conditions, the geronic acid derivative formed the lower band on the column (Strain 4).

Nearly everyone who has tested various adsorbents for the separation of organic acids has found that the alkaline solids like magnesia and lime are unsuitable for this purpose. Except for its weak adsorptive capacity, talc exhibits many desirable properties. In some respects it resembles the more active, synthetic magnesium silicates described on page 60. Further investigation of this latter group of compounds may lead to the development of better adsorbents for the resolution of acids and of compounds that are decomposed or too strongly adsorbed by alkaline solids.

Fats. Trappe has observed that mixtures of some fatty compounds may be separated on adsorption columns through use of the flowing chromatogram method provided the adsorbed materials are washed with a series of solvents of increasing polarity. Alumina (Brockmann) and silicic acid (Schering) were used as adsorbents, in spite of the fact that the former tended to hydrolyze esters and accelerated oxidation of unsaturated fats and the latter caused isomerization of unsaturated fats. The following example is typical of the results obtained. Cetene (40.7 mg.), cholesterol stearate (71.2 mg.), triolein (48.1 mg.), cholesterol (72.2 mg.) and oleic acid (32.1 mg.) were dissolved in petroleum ether (10 ml.) and the solution was passed through a column of silicic acid (4.0 g.) 2 cm. by 2.1 cm. The column was then washed with 100 ml. portions of petroleum ether, trichloroethylene and ether. The percolate was collected in portions of 25 ml. in the apparatus shown in figure 6 on page 35. Evaporation and analysis of these fractions revealed that 98.5 per cent of the cetene was carried through with the first 25 ml. of petroleum ether, 99.3 per cent of the cholesterol stearate was removed in the first 50 ml. of

trichloroethane and 100.3 per cent of the triolein, cholesterol and oleic acid was removed with the first 50 ml. of ether. Under other conditions separation of the triolein from the oleic acid and cholesterol was also accomplished, and the method was extended to separation of the fatty compounds of serum.

Amino Acids. Adsorbability of the amino acids on most adsorbents varies with the pH of the solution. Of a number of adsorbents tested, titania exhibited the greatest selectivity. At pH 3.2 glutamic acid was strongly adsorbed on titania while histidine was weakly adsorbed. At pH 10, histidine was also strongly adsorbed (Johnson).

For the separation of amino acids, a column of titania was prepared by packing about 4.5 g. of 40 mesh titania (see page 59) in a tube 8 mm. in diameter thus forming a layer 6 cm. deep. This was washed with water until the percolate was clear and dried in the oven at 110° for several hours. Through this column there was filtered 25 ml. of a solution containing 0.235 g. glutamic acid and 0.216 g. histidine monohydrochloride in 50 ml. of water adjusted to pH 3.2. The column was then washed with dilute hydrochloric acid pH 3.2 until the volume of the percolate was 25 ml. Analysis of this percolate by the Kjeldahl and Van Slyke methods revealed that all the glutamic acid and about 15 per cent of the histidine remained upon the adsorbent, the filtrate containing 85 per cent of the pure histidine.

Kuhn and Wieland have isolated pantothenic acid from fish liver by adsorption of the partially purified extracts upon columns of technical alumina that had been washed with hydrochloric acid. For this preparation, it was necessary to maintain the pH of the solution at about 8.5. The adsorbed pantothenic acid was eluted from the lower portion of the column with barium hydroxide solution.

A micromethod for the separation of glycocyamine (guanylglycine) from arginine in biological fluids depends upon the greater adsorbability of arginine on permutit (Dubnoff and Borsook). A column 0.5 by 10 cm. was packed with 0.9 g. of permutit forming a layer 0.85 cm. deep. This column was attached to a larger tube that served as a reservoir for the solutions. Five ml. of the serum or urine to be analyzed was passed through the column by gravity and the adsorbent was washed with 5 ml. portions of 0.3 per cent sodium chloride. When the second 5 ml. portion had passed through the column, the filtrate was made up to a definite volume and the glycocyamine was determined by means of the Sakaguchi reaction. Glycocyamine added to blood or urine was recovered quantitatively.

It was essential to determine by preliminary experiments that glycocyamine was not retained by the adsorbent and that arginine was not carried through the column.

Carbohydrates. Little progress has been made in the chromatography of the carbohydrates themselves (Hayashi). However, when the sugars are combined with acids in the form of esters, they are readily resolvable upon adsorption columns. An example is the separation of mixtures of the colored azobenzene-*p*-carboxylates of glucose and fructose (W. S. Reich).

Azobenzene-*p*-carboxyl chloride was prepared in quantitative yield from the free acid (50 g. in 150 ml. dry benzene) and $SOCl_2$ (40 g. in 100 ml. benzene). The dark red solution obtained by heating the mixture under reflux for 1 hour was concentrated in vacuum. Benzene was added and evaporated and this was repeated again. Finally the residue was dissolved in petroleum ether (b.p. 60–80°), and the resultant solution was filtered and cooled to 0°. Dark red crystals melting at 93° were obtained.

For esterification of glucose and fructose, 8.2 g. of the azoyl chloride were added to 50 ml. of dry pyridine and cooled to −20°. The mixture was shaken at −20° for 30 minutes and 1 g. of pure, pulverized and dried sugar was added. After storage for 4 days at −12° with occasional shaking, the mixture was then cooled to −20°, and 4 ml. of absolute methanol were added. After 3 hours at −20°, the mass was stored at 0° for an additional 12 hours. At this stage, the solid ester of glucose had separated. It was collected, dissolved in chloroform, precipitated with absolute ethanol, again collected, washed with ethanol, and recrystallized from dioxane. The yield was about 80 per cent of the theoretical; m.p. 234–236°.

The fructose ester, prepared under the conditions just described, did not crystallize from pyridine; hence, this solvent was removed in vacuum at room temperature. The residual ester was dissolved in a minimum quantity of benzene and precipitated with ethanol. Recrystallized from carbon tetrachloride it formed red crystals that melted at 135–136°.

A mixture of the esters of glucose and fructose (0.200 g. of each compound) was dissolved in 40 ml. of chloroform, 80 ml. of benzene and 80 ml. of petroleum ether. This solution was passed through a column formed by adding a slurry of silica (70 g. pure precipitated, British Drug Houses) and 300 ml. of benzene (25 per cent) in light petroleum into a tube 3 by 30 cm. When all the solution of the

azoyl esters had been added to the column, the following chromatogram was observed:

3.3 cm.	red
3.3 cm.	orange
2.2 cm.	red

The chromatogram was developed with 800–900 ml. of the 25 per cent benzene in petroleum ether. Each band was finally removed separately with a spatula and adsorbed compounds were eluted with a mixture of methanol and chloroform (1:4) during a period of 12 hours. Solutions obtained by filtration of these mixtures were evaporated in vacuum, the resulting residue was dissolved in carbon tetrachloride and precipitated with petroleum ether (b.p. 40-60°). The appearance of the final chromatogram and the weights of the recovered pigments were as follows:

40 cm.	dark orange	172 mg.	fructose ester
65 "	nearly colorless	17 "	
2 "	orange	16 "	
6 "	nearly colorless		
30 "	dark orange	166 "	glucose ester
105 "	nearly colorless	9 "	
2 "	orange	1 "	
		381 mg	of 400 mg. adsorbed.

Polysaccharides like α- and β-amylose have been found to be separable by adsorption on cotton. The β-amylose was more firmly bound than the α-amylose (Pacsu and Mullen).

Acetyl cellulose (1.5 g.) was resolved into fractions of different viscosity when dissolved in acetone (300 ml.) and passed through a column of blood charcoal (3 by 42 cm.). The adsorbed esters in three sections of the column were eluted by long contact with dioxane. Solutions obtained in this way were filtered from the adsorbent through a layer of starch on asbestos. Analysis of the eluted cellulose acetate revealed no change in acetyl content but a considerable increase in the viscosity of the fractions that formed the lower bands on the column (Mark and Saito). Similar separations were obtained by adsorption of cellulose diacetate but not by adsorption of cellulose triacetate (Levi and Giera).

2. Terpenes

Aliphatic Hydrocarbons and Alcohols. Geraniol and limonene (4 g.) dissolved in petroleum ether (700 ml. b.p. 70°) were separated

by adsorption on a column of alumina (5.5 by 12 cm.). For development of the chromatogram, 300 ml. of petroleum ether were used. Limonene was recovered by fractional distillation of the percolate. Geraniol was eluted from the column with petroleum ether containing a little methanol and isolated by fractional distillation of the eluate.

Cineol and diptentene were separated by a similar procedure (Winterstein and Stein 1).

Purification of the chloride and bromide of citonellol by removal of impurities on a column of alumina was reported by Wagner-Jauregg and Arnold. Geraniol bromide was decomposed by the adsorbent.

Cyclic Compounds. Camphor and β-ionone, in the form of their 2,4-dinitrophenylhydrazones, are readily separable on columns of talc when petroleum ether is used as solvent and developing agent. Alcohol in petroleum ether elutes the adsorbed compounds, which were isolated by crystallization after concentration of the solvent. The camphor derivative formed the lowest band on the column (Strain 4).

According to Ruzicka and Schellenberg, β-amyrin, $C_{30}H_{50}O$, is not so strongly adsorbed as erythro-diol, $C_{30}H_{50}O_2$. A mixture of the two compounds was dissolved in petroleum ether and benzene (1:1) and adsorbed on column of alumina (Brockmann). Passage of benzene through the column carried the β-amyrin into the percolate. When the column was subsequently washed with ether, the erythro-diol was likewise carried through the tube.

Limetin, found in Bergamott oil, has been observed as a blue fluorescent band, when the fraction of oil boiling at 110–140° was adsorbed on alumina from a mixture of benzene and petroleum ether. The limetin was eluted with ether (Späth and Kainrath).

The terpenoids leucotylin and zeorin, found in the nonsaponifiable residue of extracts of the Japanese lichen *Parmelia leucotylia*, have been separated by adsorption from benzene on alumina. Under the conditions employed, two successive adsorptions were required to complete the separations. Leucotylin was adsorbed in the uppermost portions of the column (Asahina and Akagi).

Filtration through columns of alumina have been used to remove impurities from solution of the following terpenes:

2-Hydroxy-10-methyl-$\Delta^{1:9}$-octalin benzoate and dinitrobenzoate (Huber),

9-Hydroxy-13-methyl-$\Delta^{10\ 11}$-dodecahydro-phenanthrene benzoate (Huber),

9-Keto-13-methyl-$\Delta^{10:11}$-dodecahydro-phenanthrene (Huber),

Tetrahydroxy-abietic acid methyl ester (Ruzicka and Sternbach).

Many ethereal oils like the limonenes are altered with simultaneous evolution of heat when adsorbed on columns of adsorptive Floridin XXF. The reaction is inhibited by strongly adsorbed compounds such as alcohols (Carlsohn and Müller 2).

Partial resolution of *d,l*-camphor was accomplished by adsorption of the Schiff's base formed from two molecules of camphor and one molecule of *p*-phenylene diamine. A dilute solution of this *p*-phenylene-bis-imino-*d,l*-camphor in petroleum ether plus benzene (8:1) was passed through a column of lactose and the chromatogram was developed with petroleum ether until the colored band occupied most of the tube. Separation of the column into four sections followed by elution of the pigment and examination of the elutriate in the polariscope revealed that the *d*-compound was more strongly adsorbed than the *l*-compound. Material from the uppermost section exhibited a specific rotation of $+90°$; that from the lowest region a rotation of $-50°$. The specific rotation of the antipodes was $+1500°$ and $-1500°$ (Henderson and Rule).

3. Benzene Derivatives

Phenols. Hydroxyl derivatives of benzene such as phenol, pyrocatechol, resorcinol and gallic acid yield homogeneous bands when adsorbed on columns of alumina or magnesia from solution in methanol. These bands exhibit blue to violet fluorescence in ultraviolet-light. Adsorbed phloroglucinol exhibits yellow fluorescence (Grassmann and Lang). It has also been reported that phenol, resorcinol, pyrocatechol, and phloroglucinol may be converted into colored compounds with ferric chloride, and that these products are then resolvable by chromatography (Zechmeister and Cholnoky 10).

A mixture of the mono- and dicetyl ethers of durohydroquinone, $(CH_3)_4C_6(OH)_2$, was separated by passage of the benzene solution through a column of alumina. The dicetyl ether passed into the percolate; the monocetyl ether remained on the column from which it was eluted with alcohol and ether (John, Dietzel and Günther).

Amines. The acetyl derivatives of ethoxyaniline (acetphenetidide) and of aniline are readily separable upon columns of alumina. A benzene solution (100 ml.) containing about 0.5 g. of each compound was passed through a column 1.8 by 4 cm., and the chromatogram was developed with 200 ml. of benzene. The column was

divided into five sections, each of which was eluted with a mixture of ether and methanol (7 to 3). Material eluted in this way was crystallized by the addition of water to the concentrated eluates. Pure acetphenetidide was obtained from the upper two-fifths of the column; acetanilide from the lowest fifth (Kondo).

Azo Compounds. Azobenzene and each of its derivatives occur in two interconvertible forms, the *cis* and *trans* isomers (Hartley). In the case of azobenzene, the two isomers were readily separable by adsorption on alumina from solution in petroleum ether and benzene. The *cis*-azobenzene formed the lower band (Zechmeister, Frehden, and Jörgensen; Cook).

Nitro Compounds. The three isomeric nitroanilines were separated from one another by adsorption of a petroleum ether solution of the mixture on a column of lime (Karrer and Nielsen). Complete resolution of the mixture necessitated a second adsorption of the compounds eluted from the first column. The nitroanilines formed the following chromatogram:

p-nitroaniline	bright yellow
m-nitroaniline	yellow
o-nitroaniline	dark yellow to brown

Isomeric nitrophenols adsorbed on columns of alumina or calcium carbonate formed bands in the same order as the corresponding nitroanilines; namely, para, meta and ortho (Kuhn and Strobele). The adsorbabilities of these isomers is proportional to their dipoles, the isomer with the greatest dipole forming the upper band (Arnold).

In the course of the synthesis of the flavines it became necessary to prepare as intermediates a number of the Schiff's bases of dimethylnitroanilines and sugars. Several of the free anilines as well as the bases themselves were purified by adsorption methods. The Schiff's bases, because of the many hydroxyl groups in the sugars, were much more strongly adsorbed than the amines from which they were prepared.

3,5-Dimethyl-6-nitroaniline in solution in petroleum ether was freed of impurities by adsorption on a column of lime (Karrer and Strong 2). It was also prepared by adsorption on a column of alumina, the subsequent elution being performed with methanol (Kuhn, Desnuelle and Weygand).

3,5-Dimethyl-6-nitroaniline base of *l*-arabinose was adsorbed on a column of alumina with absolute methanol as solvent and developer.

The adsorbed base was eluted with hot 80 per cent methanol (Karrer and Strong 2). A similar method was used for purification of the 4,5-dimethyl-2-nitroaniline base of *l*-arabinose (Kuhn, Reinemund, Weygand and Ströble).

The following compounds were purified by adsorption from alcohol on alumina with subsequent elution by a mixture of methanol, water and pyridine:

1,2-dimethyl-4-nitro-5-amino-benzene-*d*-glucoside (Kuhn and Dansi),

1,3-dimethyl-4-*d*-arabitylamino-5-nitrobenzene (Kuhn, Desnuelle and Weygand),

l-arabinose-2-nitro-3,5-dimethyl-anilide (Kuhn, Desnuelle and Weygand),

2-nitro-3-amino-5,6,7,8-tetrahydro-naphthalene-N-*l*-arabinoside (Kuhn, Vetter and Rzeppa),

d-arabinose-2-nitro-4,5-dimethyl-anilide,

d-ribose-2-nitro-4,5-dimethyl-anilide,

d-glucose-2-nitro-anilide,

trityl-*d*-mannose-2-nitro-4,5-dimethyl-anilide (Kuhn and Ströbele).

Adsorption of 2,4-dinitrophenylhydrazones has already been described on pages 87 and 91.

4. Aromatic-Aliphatic Compounds

Compounds in this class, like triphenyl-methane and triphenyl-carbinol, are separable upon columns of alumina. Here, as is usually the case, the hydrocarbon passes through the column faster than the alcohol (Wieland, Ploetz and Indest). Several other compounds in this group have been freed of impurities by passage of the solutions through columns of alumina. Compounds purified in this way are:

α-(7-methyl-naphthyl-1)-β-(5-methyl-naphthyl-1) ethane (Ruzicka and Hofmann),

α-(5-methyl-6-methoxy-naphthyl-1)-β-(7-methyl-naphthyl-1) ethane (Ruzicka and Hofmann),

bis-6-methoxy-3,4-dihydro-naphthyl-1, 1′-acetylene (Dane, Höss, Bindseil and Schmitt).

Some triaryl methyl halides and triphenyl-methane dyes form colored products when adsorbed on columns of silica. Because the colored products are similar to those formed from these organic compounds with acids, the phenomenon has been attributed to

olarization of the dye on the polar surface of the adsorbents. Elu-on of the adsorbed compounds with alcohol results in re-formation of ıe colorless or weakly colored, nonpolarized molecules (Weitz and hmidt).

Diphenyl polyenes, formulas and the adsorption order of which ere given in table 8 page 23, were adsorbed on alumina from pe-oleum ether. The adsorbed compounds were easily located on the olumn by examination in ultraviolet light. The two uppermost ınds exhibited different yellow fluorescence; the three lower bands hibited different blue fluoroescence (Winterstein and Schon 2).

Indolenin dyes, polyenes containing heterocyclic groups at the ıds of the aliphatic chain as shown in table 1 on page 16, were parated by adsorption on alumina from solution in water (Ruggli ıd Jensen 1).

5. Condensed Polycyclic Compounds

The relation between structure and the adsorbability of a number polycyclic compounds has already been indicated in tables 2,3,6 ıd 7 on pages 17 to 22. In nearly every instance the compounds ere adsorbed on alumina from solutions in petroleum ether or enzene. Positions of the bands of adsorbed compounds on the olumns were determined in ultraviolet light.

In the following experiment, selected from the work of Winterstein ıd his associates, is to be found a typical example of the separation polycyclic hydrocarbons. Naphthalene (150 mg) and anthracene 0 mg.) in solution in petroleum ether were adsorbed on a column ntaining 150 g. of activated alumina. The adsorbed materials ere washed with 500 ml. of petroleum ether. At this stage, the oper portion of the column contained the anthracene; the napthalene ıd already been carried into the percolate. After removal from the lsorbent by extraction with ether, the anthracene was recovered by aporation of the solvent. Naphthalene in the percolate was olated by evaporation of the petroleum ether.

Numerous experiments by Winterstein and his co-workers have vealed impurities such as carbazole, naphthacene and anthra-inone in commercial preparations of anthracene. 1,2-Benz-rbazole was found in a preparation of chrysene. 2,3,5, Dibenzo-cumarone was separated from technical pyrene.

Small quantities of impurities have been found to exert a great fluence upon the fluorescence of several polycyclic hydrocarbons.

As mentioned already, the intense blue fluorescence of purified anthracene was quenched by 1/30,000 per cent of naphthacene (Winterstein, Schön and Vetter). Acridine exhibited blue fluorescence before purification by adsorption on alumina and yellow fluorescence after purification (Wagner-Jauregg).

Because of the carcinogenic action of 1,2-benzpyrene (Cook, Hewett and Hieger) and some other polycyclic hydrocarbons, a great deal of attention has been given to the synthesis and purification of this group of complex organic substances. In many of the investigations, the materials were purified by adsorption of the contaminants upon columns of alumina. Some of these columns contained an upper layer of charcoal in order to remove strongly adsorbed constituents of the mixtures. Examples of these separations are to be found in the papers by Winterstein, by Windaus with Rennhak, by Fieser and his co-workers, by Newman, by Wieland and Probst and by Bachmann and Chemerda. Isomeric nitro and amino derivatives of some of the polycyclic compounds have been resolved by adsorption (Rossner; Kuhn, Vetter and Desnuelle).

Polycyclic hydrocarbons of value in the elucidation of molecular structure are produced when some sterols and other natural products are dehydrogenated. For purification of these hydrocarbons, adsorption columns of alumina have found extensive use. Among the compounds purified in this way are methyl-cyclopenteno-phenanthrene obtained by selenium dehydrogenation of cholesterol chloride (Diels and Rickert), a ketone and a quinone obtained by oxidation of the dehydrogenation products of ergosterol (Ruzicka and Goldberg), and hydrocarbons and a quinone obtained from quinovaic acid and from pyroquinovaic acid (Wieland, Hartmann and Dietrich).

Hydroxy-carboxy-anthraquinone pigments, boletol and isoboletol, found in the molds *Boletus satanas* and *B. badius* were separated from one another by adsorption on alumina from solutions in ethanol. Development of the chromatogram was accomplished with ethanol, benzene and xylene. For elution of the adsorbed pigments, 1 per cent potassium hydroxide was employed. Even after a second adsorption, a complete separation of the two red-brown bands was not achieved (Kogl and Deijs).

Alkannin, a substituted dihydroxy-naphthoquinone found in *Alkanna* roots has been purified by adsorption on siliceous earth from solution in benzene. It formed a red band located above the

bright red band of the associated alkannan. The latter, although representing only about 0.05 per cent of the mixture was readily separable on the column. Impurities contained in the tetrahydromethyl ether of alkannin were removed by passage of the solution in petroleum ether through a column of calcium carbonate (Brockmann 2).

Phthiocol, 2-hydroxy-3-methyl-1,4-naphthoquinone, occurs in the brown fats of the tubercle bacillus. It has been purified by adsorption on alumina from solution in benzene followed by elution with water, dilute alkalies or alcohol (Wagner-Jauregg).

Dichloro-quinones have been purified by Criegee. Several naphthol derivatives and dyes containing them have been separated by adsorption on alumina (page 149).

6. Sterols and Related Compounds

Sterols. (See also vitamin D, page 104, and formulas on pages 19 and 20). Most sterols, due to the influence of the hydroxyl group, are strongly adsorbed on alumina from solutions in petroleum ether or benzene. This property has enabled investigators to separate sterols from the hydrocarbons contained in the nonsaponifiable constituents of various natural products. The sterols have also been partially purified by precipitation as the digitonides before as well as after adsorption.

Cryptosterol was obtained by filtration of the nonsaponifiable, noncrystalline residue of yeast fat (500 g. in benzene) through a column of alumina (1.2 m. long containing 1.5 kg. alumina). The fraction of the percolate containing the cryptosterol was concentrated. Crystals that separated were redissolved in benzene, and this solution was passed through another column of alumina. Most of the cryptosterol remained on the alumina from which it was eluted with ether (Wieland, Pasedach and Ballauf). Zymosterol and ascosterol, prepared in small quantities from yeast, have been purified by adsorption (Wieland and Kanaoka).

Many of the natural fats contain colored or fluorescent compounds that are adsorbed with or near the sterols in the adsorption columns. This has facilitated the location of adsorbed sterols obtained from wheat germ oil and from rice germ oil. For example, a dark colored, methanol-insoluble oil from the nonsaponifiable residue of wheat germ oil was adsorbed on a column of alumina from petroleum ether

and washed with this solvent until the percolate became colorless. The following chromatogram then appeared on the column:

20–30 mm.	green-yellow
100 mm.	sandy
150–180 mm.	red-brown to yellow
350–400 mm.	sandy

The lowest sandy zone contained the bulk of the tritisterols. These were eluted with methanol and ether (4:1) and the crude sterols were purified further by readsorption and by formation of the digitonides. (Drummond, Singer and MacWalter).

Orysterols from rice germ oil were accompanied by strongly adsorbed fluorescent materials. Most of the sterols were adsorbed with a substance that exhibited greenish fluorescence on the alumina column (Karrer and Salomon 1; Todd, Bergel, Waldmann and Work).

Other sterols investigated by use of chromatographic adsorption on alumina are: γ-sitosterol from the poisonous secretion of the toad, *Bufo vulgaris* (Huttel and Behringer); lanosterol from wool fat (Dorée and Petrow); cholestadienol and 2,4-dibromcholestenone (Dane and Wang; Dane, Wang and Schulte), iso-ergosterone (Wetter and Dimroth).

In order to facilitate location of the adsorbed sterols on the adsorption columns, Ladenburg, Fernholz and Wallis have esterified the hydroxyl groups with the colored azobenzene-*p*-carboxyl chloride and have adsorbed the colored esters. The azoyl chloride was prepared from the acid and $SOCl_2$ in the presence of sodium carbonate. It was crystallized from petroleum ether. This chloride was permitted to react with the sterols in the presence of pyridine at 100° for 1 hour. Esters formed in the pyridine were precipitated by the addition of methanol, collected and recrystallized from benzene and alcohol.

Ladenburg, Fernholz and Wallis described the separation of cholesterol and ergosterol esters as follows: Cotton was placed in the lower end of a glass tube 1.4 by 70 cm. This was covered with a layer of sand about 3 cm. in height. Anhydrous aluminum oxide was then introduced in small portions. Each portion was packed firmly. The last 10 cm. of the tube was left empty. During the filling, suction was applied, and after each portion had been packed, the tube was tapped lightly to smooth out the surface. A mixture of benzene and petroleum ether (1:1) was poured into the tube and allowed to

run through the column until only a small layer of solvent remained over the aluminum oxide. A solution of 0.05 g. of the cholesterol ester and 0.05 g. of the ergosterol ester in 10 cc. of benzene was then added, and the suction was turned off. When only a small amount of solution remained on top of the column, a few cubic centimeters of pure benzene was added to wash down the mixture. A liter funnel was then stoppered tightly into the tube, and pure high-boiling petroleum ether was allowed to drop onto the column at the same rate as it was running through the adsorption tube (10–20 drops per minute). The petroleum ether was introduced only after all colored solution had disappeared from the top of the column. Care was taken not to allow the surface of the column ever to become dry once the experiment had been started.

Before the development of the chromatogram, the adsorbed ester mixture formed a red-brown zone at the top of the column (about 6 cm.). On washing with petroleum ether, this zone began to travel down, slowly lengthening and decreasing in color intensity. After 3 hours, it had reached a length of 15 cm , having travelled about halfway down the column. About that time a break in the middle became noticeable, and widened as the washing was continued. After 4 hours the development was stopped, and the glass tube was cut in the middle of the noncolored zone which had reached a length of about 7 cm. The upper and lower layers were eluted separately by shaking with a mixture of benzene, ether, and alcohol (5:5:1). The filtered solutions were evaporated, and the residues were dried.

Upper layer: 0.048 gram, melting at 199–201° (ergosteryl ester).

Lower layer: 0.047 gram, melting at 187–189° (cholesteryl ester).

By procedures similar to that just described, mixtures of the esters of the following sterols were separated on adsorption columns; cholesterol and stigmasterol; stigmasterol and ergosterol; cholesterol and ergosterol; cholesterol, stigmasterol and ergosterol. The ester of β-sitosterol could not be separated from that of cholesterol, and only incompletely from those of stigmasterol and ergosterol. It was concluded that separability of sterols on alumina columns depends primarily upon differences between the number of double bonds in the molecule and not upon the position of the double bonds or the shape of the side chains. It should also be pointed out that the relative positions on the column of the sterols themselves and of their esters was the same.

Bile Acids. Products derived from bile acids and related structur-

ally to the sterols have been purified by adsoprtion on alumina. In many instances, this purification involved filtration of solutions of the compounds through the columns, the strongly adsorbed contaminants being retained by the adsorbent. For example, 1,1-diphenyl-methyl-(3-acetoxy-etiocholyl)-ethylene was freed from contaminants by washing it through a column of alumina with pentane (Sawlewicz and Reichstein). This procedure has recently been applied by Reichstein and his co-workers to the purification of numerous products of this type descriptions of which are to be found in the *Helvetica Chimica Acta.* Isomeric cholenic acids have been partially resolved by chromatographic methods (Wieland, Kraus, Keller and Ottawa).

Sapogenins and Sapogenols. Columns of alumina have been widely used for purification of the sapogenins from various plant sources. For purification of sarsa-sapogenin, the benzene solution was passed through the column, the impurities remaining on the alumina (Askew, Farmer and Kon) (Fieser and Jacobsen). The soy bean sapogenol was separated into four components by a combination of crystallization and adsorption methods. Benzene was used as solvent. (Ochiai, Tsuda and Kitagawa 1,2; Tsuda and Kitagawa). Decarboxylation products of olcanolic acid, oleanol and oleanylene were resolved by adsorption on alumina from benzene solution, the hydrocarbons passing into the percolate, the alcohol remaining on the column. Ether and methanol were used for elution (Winterstein and Stein 1).

Toad Poisons. Bufotalin ($C_{26}H_{36}O_6$) the active principle in the poison of *Bufo vulgaris* was readily isolated by adsorption on alumina. The partially purified material from about 33,000 individuals was dissolved in 10 parts of acetone and adsorbed on a column 7 by 16 cm. This column was washed with chloroform until a yellow pigment adsorbed with the bufotalin was nearly all carried into the percolate. From the filtrate and from the lower yellow band which was 2 cm. deep, 9.2 g. of bufotalin was recovered (Wieland, Hesse and Hüttel).

Cinobufagin, ($C_{25}H_{32}O_6$) a constituent of the Chinese drug "Ch'an Su," is obtained from *Bufo gargarizans* and is readily purified by adsorption on alumina from benzene solution (Tschesche and Offe 1,2) or from chloroform solution (Kotake and Kuwada 1, 2).

Plant Poisons. Examination of the arrow poisons from the plants *Adenium somalense* and *Calotropis procera* has been facilitated by use of adsorption columns (Hartmann and Schlittler; Hesse and Reicheneder). Sarmentocymarin, a heart poison from *Strophanthus*

eds (Jacobs and Heidelberger) was purified by adsorption on umina from benzene and chloroform (1:1). The poison was grad-ılly washed through the column and was recovered from the per-ılate (Tschesche and Bohle).

Rotenone. Rotenone, contained in the crude resin extracted from ırris root, has been purified for further analysis by chromatographic lsorption (Meijer and Koolhaas). About 2.5 g. of the resin were ssolved in 25 ml. of benzene and this solution was passed through column of Frankonit KL (2 by 15 cm.). Most of the impurities mained on the adsorbent when the column was washed with benzene ıtil a yellow-green zone was carried into the percolate. Benzene as removed from the percolate by distillation and the rotenone in .e residue was determined gravimetrically after extraction with her and crystallization of the extract.

7. Heterocyclic Nitrogenous Bases

Simple Compounds. According to Kondo, a number of hetero-rclic bases are readily separable on columns of alumina. Pyridine ıd α-picoline were adsorbed from petroleum ether, the column was parated into 5 parts and the bases recovered by elution with ether ıd methanol (2:1). Pyridine formed the upper band, picoline the wer. With ether as solvent, quinoline was weakly adsorbed on umina, whereas hydroxyquinoline was strongly adsorbed. Anti-rrene, which was weakly adsorbed, was removed from strongly ɔund chloral when the column was washed with benzene. Pyrami-ne and veronal were separated by adsorption from benzene solution, rramidine passing through the column. Removal of carbazole ɔm polycyclic aromatic compounds with Tswett columns has al-ady been described.

Alkaloids. Thus far, three ends have been attained through use chromatographic methods for investigation of the alkaloids; ımely, concentration of these bases from dilute solution, separation synthetic mixtures of the bases and preparation of the bases from ıeir natural sources.

Through the use of asbestos and kaolin as adsorbent, Fink (2) ɔncentrated cinchonine and quinine from aqueous solutions contain-g 1 part of the alkaloid per million parts of water. These solutions ere adsorbed at pH 8–8.2. Hydrochloric acid was used for elution the adsorbed bases.

Synthetic mixtures of morphine (upper band) and thebain, of

narcotine (upper) and thebain, of codein (upper) and thebain, and of *d*-lupanin (upper) and spartein were separated by adsorption on columns of alumina. The solvent employed was either benzene or ether (Kondo). Quinine and cinchonine were separated by adsorption from benzene and chloroform on Floridin XXF (Karrer and Nielsen). Impurities contained in vomicidin were removed by percolation of the ether solution through columns of alumina (Wieland and Horner).

Alkaloids isolated in small quantity from natural sources by adsorption on alumina were: sanguinarin (Späth, Schlemmer, Schenck, and Gempp), methyl-isochondodendrin (Kondo, Tomita and Uyeo) the constituents of *Amanita* toxin (Renz; and Lynen and Wieland) and the ergot alkaloids, ergotamin, ergotaminin, ergotinin and ergotoxin. The separation of the latter compounds has been patented (Sandoz) (Stoll and Hofmann). In the preparation of all these compounds alumina was used as adsorbent and benzene, chloroform, ether or alcohols were used as solvents.

An interesting modification of the adsorption procedure is the isolation of toxiferin in the form of its Reinecke salt (Reinecke acid = $H(NH_3)_3Cr(SCN)_4$, (Wieland, Konz and Sonderhoff). This salt of the alkaloid was dissolved in acetone, and passed through a column of alumina, which removed the impurities. The hydrochloride of the base was recovered from the percolate after acidification with HCl and extraction of the Reinecke acid with ether.

A mixture of two alkaloids contained in the loco week *Astragalus Earlei* was separated by adsorption of the picrates. The extracted and partially purified mixture of α- and β-earleine was neutralized with picric acid in alcohol, diluted with benzene and passed into a column of alumina. β-Earleine picrate passed through the column and was isolated by concentration of the percolate. α-Earleine picrate remained on the upper two-thirds of the column from which it was extracted with hot alcohol (Pease and Elderfeld).

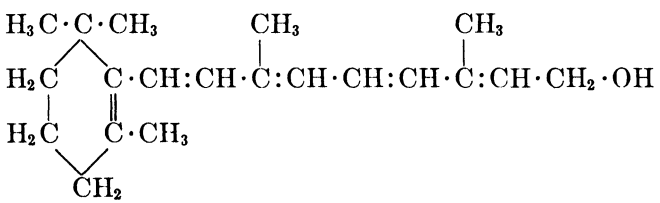
8. Fat-Soluble Vitamins

Vitamin A. Fat-soluble, colorless vitamin A is found widely distributed in fish, birds and mammals. It occurs in highest concentration in the liver. Often it is found in association with the yellow carotenoids some of which are convertible into the vitamin in the living organism. These carotenoids and the vitamin itself play a

role in the process of vision, increasing visual perception in faint light and thus relieving the condition known as "night blindness."

For a long time, criteria for the purity of vitamin A preparations were wanting. This led to much confusion concerning the chemical properties and the physiological activity of the vitamin. It now appears, as the result of extensive chemical and physiological investigations, that the vitamin can be prepared in the same degree of purity either by chromatographic adsorption or by distillation and crystallization (Karrer).

In the absence of carotenoids, vitamin A can be detected by its formation of a blue color with a chloroform solution of antimony trichloride (Carr-Price reagent). This reaction may be used to locate the vitamin on adsorption columns by painting a streak of the reagent along the adsorbent after the latter has been extruded from the tube (Zechmeister, Cholnoky and Ujhelyi; Willstaedt and With 1). The



Vitamin A

vitamin has also been identified by mixing it with a standard preparation followed by readsorption of the mixture (Kuhn and Morris).

Vitamin A is most easily prepared from fish-liver oils by adsorption (Karrer, Morf and Schopp). The nonsaponifiable fraction of the oil is dissolved in methanol and chilled in order to crystallize sterols and related compounds that affect the adsorption. After removal of these substances by filtration, the vitamin is transferred to petroleum ether; the methanol is removed with water; and the petroleum ether solution is passed through a column of alumina. Some impurities remain near the top of the column; the vitamin is adsorbed near the middle; and hydrocarbon contaminants are carried into the percolate. Methanol and petroleum ether are used for elution of the adsorbed vitamin.

If partially purified vitamin A in solution in petroleum ether is readsorbed on calcium hydroxide, it separates into two bands that can be differentiated by means of the Carr-Price reagent. The mate-

rial from the upper band, called β-vitamin A, forms a blue color with maximum absorption at 622 mμ; that from the lower band, called α-vitamin A, yields a blue color with maximum absorption at 580 mμ. (Karrer and Morf; Karrer, Walker, Schöpp and Morf; Castle, Gillam, Heilbron and Thompson).

Vitamin A has also been purified by adsorption on magnesia after preliminary adsorption on activated charcoal (Holmes, Cassidy, Manly and Hartzler; Bowden and Bastow), by adsorption on fuller's earth (van Eekelen, Emmerie, Julius and Wolff), and by adsorption on calcium carbonate (Wald and Zussman). Petroleum ether was usually used as solvent.

Separation of vitamin A from the carotenoids is of importance in biological investigations and may be accomplished by chromatographic adsorption. Carotenes dissolved in petroleum ether are less firmly bound by alumina than is the vitamin and are washed through the column leaving the colorless vitamin on the adsorbent (Karrer and Schöpp). For separation of the xanthophylls lutein and zeaxanthin from the vitamin, a petroleum ether or petroleum ether and benzene solution of the mixture is passed through a column of calcium carbonate. Under these conditions, the pigments remain on the column and the vitamin passes into the percolate (Gillam and Heilbron).

Because several of the 60 odd known carotenoids exhibit vitamin A activity, it is frequently necessary to estimate each of these compounds in plant and animal materials. This can be done only by the use of chromatographic adsorption methods as described in the section of this publication devoted to the carotenoids (page 128).

Adsorption of the substances associated with vitamin A in natural products has been employed for their purification (Pritchard, Wilkinson, Edisbury and Morton). Alteration products of vitamin A such as the condensation product with acetone have also been purified in this way (Batty, Buraway, Harper, Heilbron and Jones).

Vitamin D. The antirachitic vitamin, D_3, occurs in fish liver oil from which it has been isolated by adsorption on alumina. It is related to the sterols and has been synthesized by irradiation of 7-dehydro-cholesterol. The irradiation products were also purified by adsorption on alumina from solution in petroleum ether and benzene (Windaus, Schenck and Werder).

Both 7-dehydro-cholesterol and ergosterol may be regarded as provitamins D_3. These two compounds with 2 and 3 double bonds

respectively are more strongly adsorbed than cholesterol with only 1 double bond (See formulas on page 19 and adsorption of sterols on page 97).

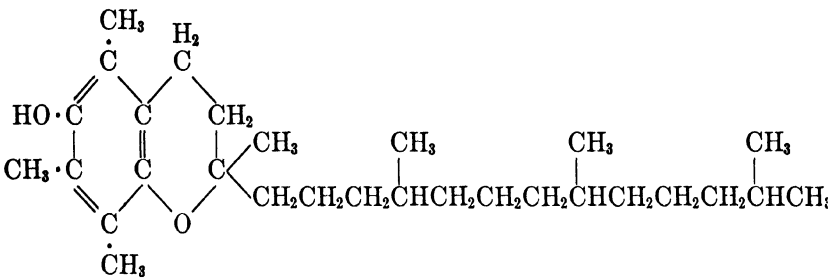
For isolation of vitamin D_3 from fish liver oils, the vitamin concentrates were dissolved in petroleum ether and the vitamin was extracted with 90 per cent methanol. It was again transferred to petroleum ether by addition of this solvent and water. Evaporation of the petroleum ether solution to dryness yielded a residue that was redissolved in petroleum ether and benzene and passed through a column of aluminum hydroxide. Most of the vitamin appeared in the percolate. The crude vitamin obtained by evaporation of the solvent was separated into 10 g. portions each of which was treated with 0.1 g. of indicator red 33 (sudan III) in 600 ml. of benzene and petroleum ether (1:4). This mixture was passed through a fresh column of alumina and washed with 3 l. of solvent. The following chromatogram was thus developed.

bright yellow
rose
red
brownish

From the red band, 2 9 g. of oil was obtained by elution followed by evaporation of the eluate. This oil was redissolved in 200 ml. of the solvent mixture used above and readsorbed on another column of alumina. Here a red band appeared between two yellow bands. The oil (0.9 g.) obtained by elution and concentration of the eluate of the red band was dissolved in petroleum ether and the indicator red was removed by extraction with 20 per cent potassium hydroxide in 80 per cent methanol. The solvent was again evaporated and the residue was dissolved in methanol. When this solution was cooled, most of the cholesterol that was present crystallized and was removed by filtration. In order to remove the remaining cholesterol, the solution was treated with digitonin and evaporated to dryness. The residue obtained in this way was extracted with petroleum ether, and the extract was also evaporated to dryness. The resulting residue was dissolved in pyridine and esterified with 3,5-dinitrobenzoyl chloride (2 days at 20°). The ester was then transferred to benzene and the solution was extracted with sodium bicarbonate solution, with dilute acetic acid and with water. After removal of the benzene by distillation, the residue was dissolved in 70 ml. of benzene and

petroleum ether (1:4); and the solution was passed through a column of alumina. The eluate from the lowest of four zones was evaporated to dryness and the residue was dissolved in acetone and diluted with methanol. It yielded yellow crystals. These were reconverted into viscous vitamin D_3 by saponification with 5 per cent potassium hydroxide in methanol in the absence of air (Brockmann 3,4; Brockmann and Busse).

Vitamin D_3, prepared from 7-dehydro-cholesterol by irradiation and partially purified by chemical methods, was adsorbed on alumina from solution in benzene and petroleum ether (1:4). The material eluted from the middle portion of the column with methanol and benzene was converted into the 3,5-dinitrobenzoate of the vitamin which was purified further by repeated recrystallization. (Windaus, Schenck and Werder).



Vitamin E (α-tocopherol)

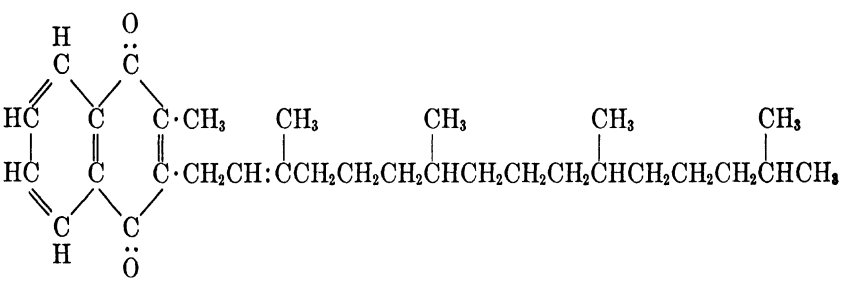
Adsorption on alumina has been used for preparation of ergosterol from commercial preparations of cholesterol (Winterstein and Stein 1); for the separation of artificial mixtures of ergosterol and cholesterol (Karrer and Nielsen); for the preparation of ergosterol from eggyolk (Windaus and Stange) and from other sources (Windaus and Bock). The acetate of ergosterol has also been purified by adsorption on alumina from solution in benzene and petroleum ether (1:1) (Philips Patentverwaltung, German Pat. 673,277). Esters of lumisterol$_3$ have been purified by filtration of their solutions through a column of alumina (Windaus, Deppe and Wunderlich).

Vitamin E. Several compounds that exhibit antisterility activity have been prepared from various plant sources (I. G. Farbenindustrie; John; Karrer, Salomon and Fritzsche; Drummond and Hoover; Emerson, Emerson, Mohammad and Evans). These compounds are now generally known as α-, β- and γ-tocopherols. They are conven-

iently prepared from the nonsaponifiable residues of the oils obtained from the germs of cereals. The purification may involve adsorption of the tocopherols themselves or of their allophanates (Evans, Emerson and Emerson).

The principal constituent of most vitamin E preparations is α-tocopherol which is readily separable from the β-tocopherol by crystallization (I. G. Farbenindustrie) or by direct adsorption (Karrer and Salomon 2). The β-tocopherol has been prepared by adsorption on alumina after preliminary resolution of the vitamins by partition between methanol and petroleum ether (Todd, Bergel and Work). John has purified this vitamin by adsorption of a mixture of the *p*-nitrobenzoates on alumina from benzene.

A synthetic tocopherol, believed to be *d*,*l*-α-tocopherol was prepared by condensation of trimethyl-hydroquinone with phytylbromide



Vitamin K_1

(Karrer, Fritzsche, Ringier and Salomon). This condensation product was purified by adsorption on alumina from light petroleum followed by elution with ether and methanol. It was observed subsequently that oxidation of the vitamin was accelerated by its adsorption. (Isler; Smith and Ungnade).

Vitamin K (Phylloquinone). Two forms of antihemoragic vitamin K have been found in natural products Both of these, vitamin K_1 or 2-methyl-3-phytyl-1,4-naphthoquinone and vitamin K_2 or 2,3-difarnesyl-1,4-naphthoquinone, have been prepared with the aid of adsorption methods.

Vitamin K_1 is most readily prepared from leaves (Dam and co-workers, Karrer, Geiger, Legler, Rüegger and Salomon; Binkley, MacCorquodale, Thayer and Doisy). Binkley and his co-workers extracted 1000 pounds of kiln dried alfalfa with about 500 gallons of petroleum ether for 12 hours. The resultant extract was concen-

trated (to about 120 gallons) and passed through a 5 foot column containing 500 to 750 pounds of Decalso. As soon as the solution had passed through it, the column was washed with petroleum ether and with this solvent containing 10 per cent benzene. In this way, there were obtained a series of percolates that exhibited greatly increased concentration of vitamin per unit weight of dissolved material. These fractions were readsorbed on a fresh column of Decalso and the portion of percolate exhibiting vitamin K activity was re-adsorbed on a column of Permutit. Fractions of the percolate were again collected and the one exhibiting the highest vitamin concentration was passed through a second column of Permutit. Final purification was accomplished by adsorption on Darco (charcoal) using as solvent, successively, ethanol, petroleum ether, ethanol and benzene (1:1), and benzene. Several fractions of the percolate yielded vitamin of the same activity per mg.

In their early experiments, Dam and his assistants devised a unique procedure for removal of adsorbed vitamin K_1 from the adsorbent. They adsorbed the vitamin on sugar, dissolved the sugar in water and extracted the vitamin from the aqueous solution with an immiscible organic solvent. In the course of their investigations they observed that vitamin K_1 was decomposed by magnesia and alumina.

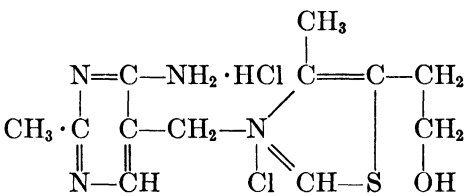
For the preparation of vitamin K_2 from putrefied fish meal, McKee with Binkley, MacCorquodale, Thayer and Doisy employed a procedure similar to that described for preparation of vitamin K_1. First, the petroleum ether extract of the putrified meal was passed through a column of Decalso, and the column was washed with benzene (20 per cent) in petroleum ether. The portion of the percolate containing most of the vitamin was concentrated; the residue was distilled at low pressure, dissolved in petroleum ether and passed through a fresh column of Permutit. After the vitamin had been carried through the column by washing with petroleum ether and benzene, the percolate was concentrated, and the vitamin was crystallized from the concentrate.

9. Water-Soluble Vitamins

Vitamin B_1. Cerecedo and his co-workers have isolated vitamin B_1 (aneurin) from various sources such as beer yeast and rice hulls by adsorption on the zeolite "Decalso." A metal column 28 by 150 cm. was filled to a depth of 46 cm. with the adsorbent that had been washed with dilute sulfuric acid at pH 4. The column was then

washed with warm water at 75°. An extract from 30 kg. of rice hulls was partially purified with barium hydroxide, brought to pH 4.5 and a temperature of 75°, and passed through the column at the rate of 1 l. per minute. The 300 l. of extract were followed by 152 l. of warm water. Vitamin B_1 that remained on the column was eluted with hot, molar ammonium nitrate solution (76 l.) and water (19 l.). After the eluate had been brought to pH 7.5 with ammonia, the vitamin was isolated as the silver salt. About 40 mg. of the vitamin were obtained.

Separation of vitamin B_1 from the pigments of urine has been achieved by adsorption on siliceous earth (Widenbauer, Huhn and Becker). A method for estimation of the vitamin by adsorption of the colored product formed with diazotized 2,4-dichloroaniline has been devised by Willstaedt (10) (Willstaedt and Bárány). Lime was used as adsorbent and ether as solvent. Melnick and Field adsorbed

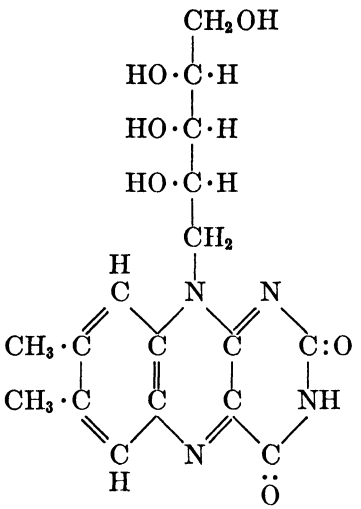


Vitamin B_1

the vitamin on a zeolite. After its elution, it was converted into a colored derivative with diazotized *p*-aminoacetophenone.

Vitamin B_2 (Lactoflavin) and Flavins (Lyochromes). Two modifications of the Tswett method have been used for preparation of the flavins. According to one procedure, these pigments are adsorbed directly from aqueous solution. According to the other procedure, the lyochromes are converted into esters that are soluble in organic solvents and that are adsorbable from these.

Kuhn and Kaltschmitt adsorbed the tetraacetyl-lactoflavin from 103 kg. of California alfalfa meal on alumina using alcohol-free ethyl acetate as solvent and for development of the chromatogram. The principal yellow zone was eluted with ethyl acetate and methanol (4:1), and the residue obtained by evaporation of the eluate was readsorbed from ethyl acetate. The pigment then separated into two bands. Elution of the upper band with water followed by partition of the extract between water and ethyl acetate provided some of the acetyl ester of the lyochrome in the ethyl acetate. This was com-



Vitamin B_2 (lactoflavin)

bined with the pigment eluted from the lower band with ethyl acetate and methanol. After recrystallization of the product twice from alcohol and twice from water, 17 mg. of the crystalline flavin acetate were obtained.

By a similar procedure, Heilbron, Parry and Phipers obtained acetylated lactoflavin from a fresh water alga. Flavin-like compounds have also been prepared from microorganisms (*Bacillus pyocyaneus*, etc.) by adsorption on carbon or Frankonit KL (Giral) and from *Corpus luteum* by adsorption on Frankonit from water (Euler and Brandt).

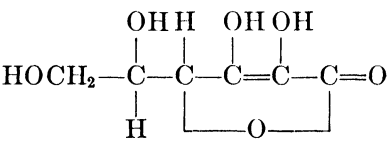
Flavins from 5,000 l. of human urine were isolated by Koschara (1–3). The urine was collected in batches of 200 l. in 3 l. of 25 per cent hydrochloric acid. The pigments were adsorbed on Floridin XXF (4 kg.) followed by elution with 10–15 l. of 20 per cent aqueous pyridine. After concentration of the eluate, removal of the purine fraction by filtration, and further purification by precipitation with basic lead acetate and by extraction with ether, a solution of 700 mg. of lyochrome in 4.2 l. of water was obtained. This solution was passed through a column of Floridin XXF. Aquoflavin passed through the column. Uroflavin, which was strongly adsorbed, was eluted with methanol, pyridine and water. A second adsorption, followed by intensive washing with pyridine and water, yielded a

percolate from which 220 mg. of recrystallized uroflavin were obtained.

Adsorption has been made the basis of a method for estimation of the flavins in urine (Koschara 3). These pigments were separated from other coloring matter both before and after treatment with permanganate. It is interesting that aquoflavin is converted into a strongly adsorbed lumi-aquoflavin by irradiation.

A number of synthetic flavins have been purified by adsorption on columns of alumina. 6,7-Dimethyl-9-*n*-amyl-flavin was slowly washed through the column whereas the contaminants were washed through rapidly. Solvent: xylol + methanol. (Kuhn and Weygand.) 6,7-Dimethyl-flavin acetic acid methyl ester and 6,7-tetramethylen-9-*l*-araboflavin-tetraacetate and 6,7-trimethylen-9-*l*-araboflavin-tetraacetate were adsorbed from chloroform solution (Kuhn and Rudy; Kuhn, Vetter and Rzeppa).

Preparation of flavins by adsorption of extracts of various plant and animal sources has been described by many authors notably



Vitamin C

Ellinger and Koschara; Kuhn, Gyorgy and Wagner-Jauregg; Kuhn, Wagner-Jauregg and Kaltschmitt (adsorbent, fuller's earth) Karrer, Salomon and Schopp (lead sulfide, Frankonit).

Vitamin C. Ascorbic acid or vitamin C contained in urine was isolated by chromatographic adsorption after conversion into the colored 2,4-dinitrophenylhydrazine derivative. A mixture of ethanol and acetone was used as solvent for the adsorption of the derivative on alumina; acetone was employed for development of the chromatogram, and acetic acid for elution of the resolved materials. Because the 2,4-dinitrophenylhydrazine derivative obtained from urine was contaminated with other similar compounds, it was necessary to repeat the adsorption and elution three times before a pure substance was prepared (Drumm, Scarborough and Stewart).

10. Hormones

Plant Hormones. Few applications of the chromatographic adsorption methods are found in the literature pertaining to preparation

of the plant hormones or auxins. Kögl, Haagen-Smit and Erxleben purified hetero-auxin (β-indoleacetic acid) by adsorption on calcium carbonate from benzene and alcohol. The column was sectioned empirically and the hetero-auxin was eluted with alcohol. Except for a small region containing impurities near the top, the column contained hetero-auxin through most of its length.

A hormone that stimulates movement of the sensitive *Mimosa pudica* has been purified by adsorption on alumina (Hesse). The extract of the plant material was first purified by clarification with lead acetate and by precipitation with mercuric acetate. Removal of the mercury with hydrogen sulfide provided a solution of the hormone that was passed through a column of alumina. The adsorbed hormone was eluted with absolute alcohol.

Animal Hormones. Adrenaline (epinephrine) and other basic substances have been concentrated by adsorption on columns of asbestos and kaolin (Fink; Whitehorn). Whitehorn has utilized this procedure for the estimation of adrenaline in blood. First, proteins were removed from the blood with trichloroacetic acid. The solution separated from the proteins was neutralized, buffered and passed through a column of silicic acid that had been washed previously with acid, with water and with sodium sulfite until neutral. The column with the adsorbed adrenaline was then washed with water in order to remove glutathione and other reducing substances. Adrenaline was finally eluted with acid and estimated with arsenomolybdic acid.

Adsorption methods have found extensive use in the preparation and purification of the sex hormones. These physiologically reactive and specific compounds, that are related to the sterols, are extracted from urine. For the preparation of estrone (folliculin), Duschinsky and Lederer extracted the acidified urine of pregnant mares with benzene. This extract was concentrated; the residue was dissolved in petroleum ether; and the hormone in the solution was extracted with aqueous alcohol. The hormone in the alcohol was transferred to benzene, and this benzene solution was passed through a column of lime. A violet band passed slowly down the column. This band and the region immediately above it contained most of the estrone which was eluted with acetone and crystallized from alcohol. The hormone was also recovered from the adsorbent by dissolution of the latter in hydrochloric acid followed by extraction of the solution with ether.

When the hormone mixture from the urine of mares was adsorbed on alumina from benzene, a red-violet band containing indirubin

(investigated subsequently by Musajo) appeared near the top of the column. Just below this band was found the hormone equilenin which was eluted and isolated as the picrate.

Chromatographic adsorption has been employed for preparation of estrone (Hofmann-LaRoche A.G.), (Zechmeister and Cholnoky 10) and for the resolution of mixtures of 17-dihydroequilenin, β-estradiol and other compounds (Hirschmann and Wintersteiner). In order to locate the colorless bands of adsorbed material, the adsorbent was extruded from the tube, and small portions of the adsorbent from various locations were eluted with ethanol. The eluates were permitted to react with *p*-nitro-diazobenzene to form colored products with the hormones. After the positions of the hormones on the column were established, the cylinder of adsorbent was cut into sections and the adsorbed materials were eluted from the respective portions.

Adsorption methods have found use in examination of the keto-steroids in human urine. This group of compounds (1.35 g) isolated from the urine of eunuchs, was dissolved in carbon tetrachloride and the solution was passed through a column of alumina (1 by 20 cm.). The chromatogram was developed first with carbon tetrachloride and then with carbon terrachloride containing 0.1 per cent ethanol. From the first 1,075 ml. of percolate collected in three fractions, only a small quantity of unidentified material was obtained. From the next 450 ml. of percolate of the ethanol carbon tetrachloride mixture, collected in two fractions, there was recovered 72 1 mg. of crude *trans*-dehydro-androsterone. The following 220 ml. of percolate contained 15.7 mg. androsterone. Following this, 190 ml. of solution containing only gum were collected. From the next 570 ml. of percolate, 22.7 mg. of crude aetio-cholan-3(α)-ol-17-one were obtained. These are the same keto-steroids found in the urine of normal persons; hence it was concluded that these compounds arose from the adrenal cortex rather than from the gonads (Callow and Callow).

As shown by the work of Steiger and Reichstein and by Reichstein and his co-workers, the adrenal cortex contains a group of hormones closely related to the sterols and sex hormones. These constituents of the adrenal glands have been purified by the use of methods similar to those employed for preparation of the sex hormones and sterols using alumina as adsorbent and petroleum ether and benzene as solvents.

In addition to their use for the isolation of sex hormones, Tswett

adsorption methods have found extensive application to purification of chemical alteration products of these and related compounds. Most of these compounds have been adsorbed on alumina from solution in petroleum ether, petroleum ether and benzene, benzene or acetone and benzene. For specific examples, reference should be made to the numerous papers by Scrini; Steiger; Reichstein; Butenandt; Ruzicka; Ehrenstein; Shoppee and their co-workers.

11. Enzymes, Coenzymes and Proteins

For many years, enzymes and related proteinaceous compounds have been purified by addition of adsorbent to the solution. Unadsorbed compounds were removed with the solvent and the adsorbed materials were eluted from the adsorbent in a greatly refined condition (Willstatter 2). But when attempts were made to separate mixtures of proteinaceous compounds by adsorption upon Tswett columns, considerable difficulty was encountered. Enzymes and other proteins adsorbed on columns often spread out into diffuse bands that separated slowly and incompletely from one another (Adler and Michaelis). Elution of the adsorbed compounds was slow. Changes in the hydrogen ion concentration at the surface of the adsorbent and variations in the concentration of the salts in solution exerted a profound effect upon the proteins themselves as well as upon their adsorbabilities. Advances in this field will probably require the development of more selective adsorbents and careful attention to the preliminary purification and buffering of the solutions.

Cozymase. For the purification of cozymase from yeast, Euler and Schlenk (2) passed a solution of the crude material through a column of Brockmann alumina about 10 cm. deep and washed the column with water. Successive 10 ml. portions of the percolate were collected separately and tested for the presence of cozymase by the fermentation method. The first 20 ml. of percolate contained no cozymase; but succeeding portions contained increasing quantities. The adsorption and washing of the column required about 1.5 hours. Traces of alumina in the percolate were removed with ammonia by bringing the solution to pH 7. Finally the cozymase was precipitated with alcohol.

Codehydrase II, another essential coenzyme of the fermentation system, was separated from cozymase by adsorption of the mixture on columns of alumina (Euler and Adler). Nearly all the cozymase

was carried into the percolate. The material remaining on the column was not eluted. Instead, the adsorbent was removed from the tube in successive portions and dried in vacuum. Each portion of adsorbent was then tested for the amount of enzyme that it contained by treatment with the substrate and a reducible dye in a closed tube. All the codehydrase was found in the upper portions of the column. A similar procedure has been used for the detection of codehydrase II in liver (Das).

Enzymes. Enzymes contained in a commercial preparation of emulsin were resolved by adsorption on a column of bauxite from an aqueous solution buffered with acetate at pH 4.7. The β-glucosidase was most strongly adsorbed and was thus separated from α-galactosidase and chitinase which passed through the column. Readsorption of this percolate on a fresh column of bauxite resulted in a separation of the two enzymes, the α-galactosidae remaining on the adsorbent, the chitinase passing into the percolate. For elution of the glucosidase and galactosidase, a 0.1 per cent solution of ammonia was used (Zechmeister, Tóth and Bálint).

Zechmeister with Tóth and with Tóth and Vajda resolved the mixture of α-glucosidase and chitinase found in extracts of the snail *Helix pomatia* by adsorption upon columns of bauxite. Apparently the separation was difficult because Neuberger and Rivers obtained only a partial separation of these enzymes under what they presumed to be the same conditions. It is important to note that in these experiments glass wool was used to support the adsorbent, because cotton might have been dissolved by the enzymes.

Young and Hartmann have recommended the use of soapstone as an adsorbent for lipase and amylase; bauxite for lipase and trypsin; graphite for lipase; carborundum for trypsin; and magnesium silicate for amylase and trypsin.

As demonstrated first by Agner and subsequently by Sumner, Dounce and Frampton, adsorption on a column of tricalcium phosphate increased the activity of catalase. Catalase prepared from horse liver was dissolved in water (0.2 mg. per ml.) and brought to pH 5.5 with potassium acid phosphate. This solution was filtered through a column of tricalcium phosphate. The enzyme formed a distinct band near the top. It was eluted with phosphate buffer at pH 8.

Previously purified tuberculin was not purified further by adsorption and elution (Gözsy and Vásárhelyi).

Allergen. An interesting procedure for purification of an active allergen from cottonseed meal has been described by Spies, Coulson, Bernton and Stevens. After partial purification, the protein component was converted into the picrate. This was dissolved in 50 per cent alcohol and adsorbed on a column of alumina. The adsorbed material was eluted with 0.05 N sodium hydroxide and reconverted to the picrate with a recovery of 64 per cent.

Inhibitor of Blood Coagulation. A substance that inhibits coagulation of blood was freed of colored contaminants by filtration through a column of alumina. This material, obtained from the washed and extracted red, blood cells from sheep, was dissolved in methanol and petroleum ether (9:1) before the adsorption. Under the conditions employed, the inhibitor was not adsorbed and passed into the percolate. The colored contaminants remained on the adsorbent (Chargaff 3).

Proteins. When a solution of hemoglobin was passed through a column of alumina (Altschul, Sidwell and Hogness) or of Lloyd's reagent and siliceous earth (Schwerdt), two bands of hemoglobin separated. The nature of the two different compounds has not been determined.

Attempts to separate proteins in combination with fluorochromes such as coriphosphin O and thiochrom S were unsuccessful. The use of the biuret reagent to locate colorless proteins on the column also failed to give satisfactory results (Schwerdt).

12. Anthocyanins

Considerable difficulty has been encountered in the preparation of the anthocyanins and in the resolution of their mixtures by the use of chromatographic adsorption methods. Variation of the color of these compounds with changes in the hydrogen ion concentration produced by the adsorbent may lead to the formation of two bands when a pure compound is adsorbed on the column (Price and Robinson). Meager solubility of the anthocyanins in the organic solvents has necessitated the use of aqueous solutions (Tswett 8, 20; Karrer and Strong 1; Karrer and Weber).

Separation of a mixture of the chlorides of cyanin and paeonin was reported by Karrer and Weber. Paeonin, which is the monomethyl ether of cyanin, is not so strongly adsorbed as cyanin itself. For the separation, 1.5 g. of crude paeonin chloride was dissolved in

200 ml. of water and passed through a column of activated alumina. The chromatogram was developed with water which carried most of the paeonin chloride into the percolate. The upper band on the column was removed, and the pigment was eluted with dilute hydrochloric acid. Crystals obtained by concentration of the eluate were redissolved in water and readsorbed on alumina. The elution and adsorption was repeated a third time before pure crystals of cyanin chloride were obtained. Paeonin chloride was obtained by concentration of the percolates from the first and second adsorptions.

Althaein chloride, the pigment from black mallow, was found to be a mixture when it was adsorbed on a column of hydrated calcium sulfate. One band passed rapidly through the column. This pigment was purified by way of the picrate and reconverted into the chloride. It proved to be a monoglucoside of delphinidin-dimethyl-ether identical with oenin. The pigment remaining on the adsorption column was eluted with warm, dilute hydrochloric acid and, after concentration of the solution, readsorbed on another column of gypsum. Two bands of pigment were obtained, but because the separation was incomplete, the materials could not be identified with certainty (Karrer and Weber).

Separation of derivatives of flavanons by adsorption has been reported by Fujise and Nagasaki.

13. Pterins

A group of widely distributed compounds, notable for their high nitrogen content and for their occurrence in insects, are known as pterins (Wieland and Schopf; Schopf and Becker). These pigments, which are presumably related to the pyrimidines or purines, are strongly adsorbed on alumina from water, methanol or dilute acid. In the presence of even very dilute acids, the pterins are easily decomposed. According to the reports by Schopf and Becker and by Becker and Schopf, the four principal pterins of insects exhibit the following behavior on adsorption columns.

Erythropterin formed a red zone (dark brown in ultraviolet light) when adsorbed on alumina from its solution in water or in acidified methanol. The adsorbed pigment was eluted with 0.5 N ammonia or with 4 per cent pyridine.

Xanthopterin formed a yellow zone (yellow-green in ultraviolet light) when adsorbed on alumina from dilute aqueous acids. It was

not so strongly adsorbed as erythropterin from which it was separated by adsorption of a solution of the mixture in acidified methanol. Either ammonia or pyridine eluted the adsorbed xanthopterin.

Guanopterin, a colorless base, was adsorbed on Frankonit KL from a solution in dilute aqueous acid. Ammonia was employed for elution of the adsorbed base.

Chrysopterin was more strongly adsorbed than xanthopterin from dilute aqueous acids. It formed a yellow-green, fluorescent band on the alumina.

Leucopterin has been investigated by Wieland and his co-workers, and the relation of this pterin to other pterins has been established.

Allantoin has been found in *Catopsilia rurina* along with guanopterin. The former compound was isolated by adsorption on Carboraffin although rather large losses were entailed in the preparation (Schopf, Kottler and Reichert).

Uropterin, about 1 part of which occurs in 1 million parts of urine, is strongly adsorbed on Floridin XXF and on Frankonit KL (Koschara). For its isolation from human urine, 200 l. portions of the latter were collected in 3 l. of 25 per cent hydrochloric acid. The pigments in this mixture were adsorbed on 4 kg. of Floridin XXF and subsequently eluted with aqueous pyridine. This eluate was concentrated, and combined with similar concentrates from a total of 5,000 l. of urine. Crystals that had separated were removed by filtration and extracted with sodium hydroxide solution. Acidification of the alkaline extract provided another batch of crystals that were removed by filtration. Bases in the filtrate were adsorbed on Floridin that was then washed free of acid. The adsorbed bases were eluted with aqueous pyridine and the eluate was concentrated and treated with ammonia and ammonium chloride. A solution of silver nitrate was added and the precipitate removed. Addition of more silver nitrate to the solution precipitated the pigments. This precipitate was collected by centrifugation and decomposed with hydrochloric acid. Pigments in the solution were again adsorbed on Floridin and eluted with aqueous pyridine. Pyridine was removed from the solution in vacuum, and after the addition of phosphate buffer to the residue (pH 7.6), the solution was passed through a column 12.3 cm. in diameter containing 90 g. of Frankonit KL. The column was washed with secondary phosphate buffer (pH 8.3) and then with borate buffer (pH 9.2). In this way the uropterin fraction was carried into the percolate. Although it was difficult to effect a

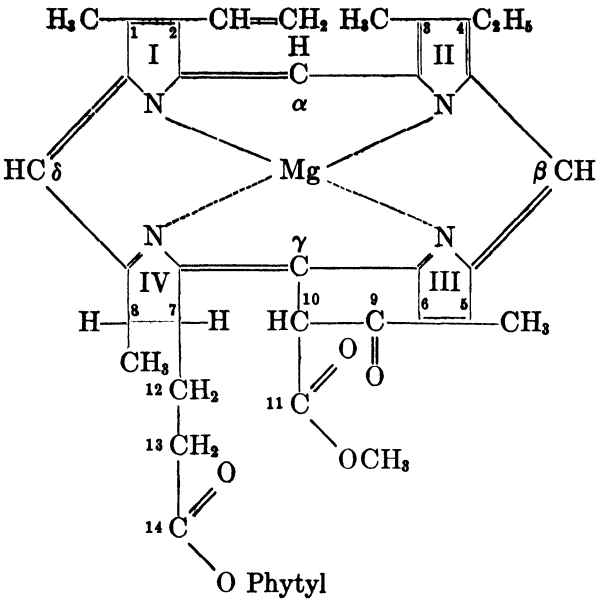
complete separation of the uropterin from other pigments, the band of adsorbed uropterin was readily observable by its intense red fluorescence in ultraviolet light. The uropterin in the percolate was subjected to further purification by precipitation with silver nitrate. Only 3.8 mg. of the pure uropterin were obtained from the 5,000 l. of urine.

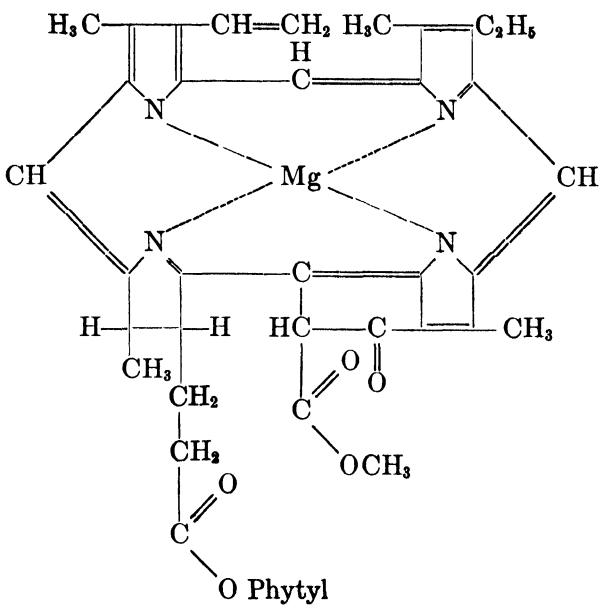
Separation of micro quantities of the pterins was investigated by Becker and Schopf with the micro columns illustrated on page 39. Erythropterin formed the most definite bands when adsorbed on columns of alumina from solution in 0.004 N hydrochloric acid. In order to obtain a band of adsorbed pigment about 1 mm. in depth, it was necessary to adsorb about 5 μg. of the pigment contained in about 5 ml. of solution. Xanthopterin, on the other hand, was observable when only 1 μg. in 5 ml. of a methanol solution containing 0.01 per cent hydrochloric acid was passed through the column. Erythropterin did not interfere with the detection of the xanthopterin.

These micro methods were applied to the detection of the pterins in insects. The pigmented portions of the wings from five male *Gonepteryx rhamni* were extracted with 0.5 and with 2 N ammonia. The extract was clarified by centrifugation and evaporated to dryness over sulfuric acid in vacuum. An extract of the residue with 1 ml. of 0.004 N hydrochloric acid was passed through a micro column of alumina that was then washed with 0.75 ml of the acid. A small grey band (3 mm.) at the top of the column was preceded by an orange-red band (1.5 mm.) of erythropterin. By a similar procedure with 0.004 N hydrochloric acid in methanol as solvent, pigments of the abdominal integument of the hornet, *Vespa crabro*, were found to be principally xanthopterin.

14. Chlorophylls

In spite of the early observations of Tswett on the separation of chlorophylls *a* and *b* by adsorption, these pigments were first prepared in solid form in sufficient quantity for subsequent analysis by the laborious and costly partition method (Willstätter and Isler). When it was finally discovered that the two chlorophylls could be conveniently prepared after separation by adsorption (Winterstein and Stein 2; Zscheile), a great impetus was given to investigations of the properties of the pure chlorophylls. This research led to the testing and utilization of a variety of adsorbents, several of which





Alternative structures for chlorophyll *a*
In chlorophyll *b* the methyl group at position 3 occurs as an aldehyde

have been found useful for the preparation of the chlorophylls in quantity.

Organic substances have been found to be the most satisfactory adsorbents for separation of the chlorophylls in relatively large quantities. Those found to give the best separations of the pigments were sucrose (Winterstein and Stein 2; Simonis; Seybold and Egle), starch (Strott) and inulin and magnesium citrate (Mackinney; Spoehr). With all these compounds, the adsorption capacity varied with the source of the material and with the methods of preparation. Many other substances, particularly inorganic compounds, adsorbed large quantities of the chlorophylls, but the pigments were frequently altered by the adsorption. Moreover, the possible existence of a third chlorophyll component, chlorophyll *c*, first observed by the resolution of chlorophyll on talc (Zscheile) could not be confirmed by use of the mild organic adsorbents (Winterstein and Schön; Mackinney).

Chlorophylls contained in petroleum ether or benzene extracts of plant material may be separated by passage of the untreated solutions through columns of organic adsorbents. However, by this procedure, it is difficult to separate the chlorophylls from other substances contained in the extracts. These contaminants also reduce the adsorbability of the chlorophylls; hence for preparative purposes, it is better to remove as many of these contaminants as possible before the adsorption. This may be accomplished by the well-established procedure of Willstätter and Stoll (2) or by precipitation of the chlorophyll from petroleum ether (Winterstein and Stein (2)).

Preparation of Chlorophyll *a* and *b*. For the separation of chlorophyll *a* and *b* by adsorption, Winterstein and Stein dissolved 3 g. of a crude chlorophyll precipitate in benzene (100 ml.) and diluted the solution with petroleum ether (1,500 ml.). This solution was passed through an adsorptive, powdered sugar that had been packed into the large apparatus shown on page 37. The sugar was first made into a slurry with petroleum ether and this was poured into the adsorption column. After all the chlorophyll had been adsorbed, the column was washed with petroleum ether and benzene (14:1, 150 ml.) and then with petroleum ether. Finally the column was sucked free of solvent and divided into three sections. The lowest blue-green portion contained pure chlorophyll *a*; the upper yellow-green section contained nearly pure chlorophyll *b* (90 per cent); and the middle portion contained a mixture of the two pigments. The pigments were

eluted with acetone and ether (1:1), and transferred to the ether by addition of water. Upon evaporation of the ether to dryness, followed by dissolution of the residual pigments in a little ether, a solution was obtained from which the chlorophyll was precipitated by addition of petroleum ether. After some time, the solid chlorophyll was collected by centrifugation, redissolved in ether and dried in vacuum.

The chlorophyll *b* was purified further by solution of 0.2 g. in 15 ml. of benzene which was then diluted to 150 ml. with petroleum ether and passed through a column of sugar (5.5 by 12 cm.). The column was washed and separated as just described. Only the pigment in the principal chlorophyll *b* zone was eluted. Material eluted from several adsorption columns was combined, dissolved in ether and precipitated with petroleum ether several times. It was then dried in vacuum.

Mackinney prepared chlorophyll *a* and *b* in the following way. A crude chlorophyll precipitate (0.5 g.) from alfalfa was dissolved in ether (75 ml.) and diluted with petroleum ether until the ether concentration was 20 per cent by volume. This solution was passed through a column (5 by 30 cm.) prepared from magnesium citrate $.6H_2O$ previously dried over calcium chloride in vacuum. When completely adsorbed, the chlorophylls were on the upper third of the column. A narrow zone of carotene was washed ahead without adsorption, and a small zone of pheophytin occurred between this and the chlorophyll. After the column had been washed with a total of 0.5 l. of fresh solvent (total elapsed time ca. 4.5 hours), the pheophytin was separated from the blue chlorophyll *a* zone by about 2 cm. The latter was contiguous with a light green zone and this in turn with a darker green zone. At the top of the column was a 2 cm. zone of nondescript greenish-grey that was removed and discarded. The three remaining green zones were removed separately with a spatula and washed with acetone on filters. The solution of chlorophyll *a* was centrifuged to insure removal of particles of the adsorbent. This pigment (in about 300 ml. of acetone) was transferred in 100 ml. portions to 75 ml. of petroleum ether by the addition of water. Because the chlorophyll solution contained some xanthophyll, the petroleum ether was washed with five 15 ml. portions of 85 per cent methanol. Finally the alcohol was removed by extraction with water whereupon the chlorophyll precipitated from the solution. This

precipitate was collected by centrifugation and dried over P_2O_5 in vacuum. About 0.1 g. of blue-black flakes was obtained.

The chlorophyll *b* fraction (containing some chlorophyll *a* and xanthophyll) in acetone was transferred to 100 ml. of 20 per cent ether in petroleum ether, and the acetone was removed by extraction with water. After dehydration with a little sodium sulfate, the pigment solution was decanted into a column of inulin (3 by 30 cm.) followed by about 300 ml. of the solvent mixture. The chlorophyll *b* zone was then about 4 cm. in width. It was approximately 4 cm. from the surface of the adsorbent and was clearly separated from the chlorophyll *a*. The chlorophyll *b* band was removed from the column and the pigment eluted with acetone, centrifuged and recovered as described under chlorophyll *a*. Separation on the inulin required some 3.5 hours. About 0.05 g. of chlorophyll *b* was recovered.

Chlorophyll was decomposed rapidly when adsorbed upon magnesium citrate that exhibited acidic reaction in aqueous solution. This deleterious action of the solid, acidic magnesium citrate may be eliminated by addition of dimethylaniline to the chlorophyll solutions and to the solvents that are to be passed through the columns. Dimethylaniline reduces the adsorbability of the leaf pigments; hence, about ten per cent of it should be used with petroleum ether as solvent when crude leaf extracts are to be adsorbed. With neutral magnesium citrate, there was no decomposition of the chlorophyll.

Chlorophylls *a* and *b* produced by aerobic oxidation of the leuco, hydrogenated chlorophylls were purified by adsorption on sucrose from solution in petroleum ether. The impurities remained near the top of the column (Kuhn and Winterstein 2).

Estimation of Chlorophyll *a* and *b*. For determination of the chlorophylls in leaves, Winterstein and Stein froze 1 or 2 leaves in liquid nitrogen, ground them in a mortar and extracted the pulp with methanol and a mixture of petroleum ether and benzene (9:1). The pigments in the extract were transferred to the petroleum ether and benzene by the addition of water, and the chlorophyll solution was washed free of methanol with water. This green solution was passed through an adsorption column containing sucrose in the upper two thirds, calcium carbonate in the next sixth and alumina in the lowest sixth (figure 31, page 40). The column was washed with petroleum ether and benzene (9:1) whereupon the carotene was adsorbed upon the alumina, the xanthophyll upon the calcium

carbonate, and the chlorophyll upon the sucrose. After the column was sucked dry in a current of carbon dioxide, each band of pigment was removed separately; the pigments were eluted with methanol and ether and transferred to ether by addition of water to the mixture. These ether solutions were brought to definite volumes and the pigments were estimated by colorimetric comparison with standard solutions of the same compounds.

Seybold and Egle, using the apparatus of Spohn shown on page 36, have estimated the chlorophylls and carotene of leaves after separation of the mixture by adsorption on sugar. Powdered sugar was freshly sieved and mixed with about an equal quantity of granular sugar. This mixture was tamped into a tube 2.4 by 40 cm. The leaves were ground with sand, then with methanol (90–95 per cent) and with petroleum ether. After removal of the pulp by filtration, the chlorophylls and carotene were transferred to the petroleum ether by dilution of the methanol with water until the methanol concentration was 80 per cent. Separation of the methanol layer was followed by repeated extraction of it with petroleum ether until all the chlorophyll was removed. Alcohol was removed from the combined petroleum ether extracts (100–200 ml.) with water, and the solution was concentrated to 5–10 ml. at a temperature below 35°.

This concentrate was passed through the sugar column which was washed with petroleum ether until the carotene was carried into the percolate. This percolate was evaporated; the residue was dissolved in benzene; and the carotene was estimated colorimetrically. Small quantities of xanthophyll remained with the chlorophyll on the adsorption column. These were removed by washing the column with benzene (about 5 ml.) followed by petroleum ether plus benzene (2:1 or 4:1). The same result was also accomplished by use of petroleum ether (50 ml.) with 5 or 6 drops of methanol. This washing resulted in a complete separation of the chlorophyll *a* and *b* zones, and it carried the small quantity of the xanthophyll into the percolate that was then combined with the methanol layers of the leaf extracts for colorimetric estimation. The column was washed with petroleum ether in order to remove the benzene; the two bands were removed separately; and the pigments were eluted with ether and methanol and transferred to ether for the colorimetric determination. Results obtained by washing the column with benzene or with petroleum ether and alcohol as described above were in close agreement.

It should be pointed out here that the leaf pigments including the

chlorophylls are susceptible to rapid decolorization through induced enzymatic oxidation when leaves of many plants are ground in air or frozen and thawed in air (Strain 10). In order to avoid these deleterious oxidation reactions, it is desirable to grind the leaves directly in alcohol or to heat them (Strain; Spohn). Further comments on the estimation of xanthophylls and carotenes are to be found on pages 131 and 147.

Other Chlorophylls. Chlorophyll-like substances from sources other than leaves are readily detectable and preparable by use of chromatographic adsorption methods. Bacteriochlorophyll from *Thiocystis* bacteria has been adsorbed on talcum from alcohol, acetone and ether (Gaffron 2). Protochlorophyll, an assumed precursor of the leaf chlorophylls, has been observed in etiolated barley leaves by adsorption on columns of magnesium carbonate (Strain 10); and it has been prepared from certain *Cucurbita* seeds by adsorption on sugar (Seybold). On the latter adsorbent with petroleum ether and petroleum ether plus benzene as solvent, the protochlorophyll was separated into two pigments that exhibited similar spectral absorption properties. Green pigments that were different from chlorophyll were obtained from the liver of the snail *Helix pomatia* by adsorption of the extracts on columns of calcium carbonate (Dhéré and Vegezzi; Vegezzi).

Alteration Products of Chlorophylls. Many of the chemical and biochemical alteration products of the chlorophylls have been prepared with the aid of adsorption columns. Pheophytin *a* and *b*, the magnesium-free chlorophylls, have been separated from their mixtures by adsorption on sugar and on mixtures of sugar and talcum. Petroleum ether plus benzene was used as the developing liquid (Winterstein and Stein 2). A pheophytin obtained from the bacteriochlorophyll of Thiocystis bacteria was examined by Gaffron (1) and by Fischer and Hasenkamp (1). The latter workers adsorbed the mother liquors of a pheophytin preparation on talcum using ether as solvent and acetone for development of the chromatogram. A small quantity of phorbide-like material was observed in the bacteriopheophytin.

Numerous examples of the purification of chlorophyll derivatives are found in the publications of Fischer and his co-workers. Most of the compounds were adsorbed on columns of talc using ether, ether plus acetone or methanol as solvents. In many instances, the materials to be purified were washed through the columns and were

recovered from the percolates, the impurities remaining on the adsorbent. The following are examples of the materials purified by adsorption;

Dihydro-pheophorbide *a* (Fischer and Hasenkamp 2),

Probophorbide from sheep dung (Fischer and Stadler),

Chlorophyllid from pheophorbide *b* (Fischer and Spielberger),

Pheophorbide *a* geranyl ester (Fischer and Schmidt),

2-α-Hydroxy-meso-cholorin e_6-trimethyl ester (Fischer, Lautsch and Lin),

Meso-pyro-pheophorbide (Fischer and Laubereau),

Methyl-pheophorbide *a* addition compound with diazoacetate (Fischer and Medick).

15. Derivatives of Hemoglobin

Hemin. A water-soluble *c*-hemin found in the blood of many species (Barkan and Schales) has been concentrated by adsorption (Schales). The crude *c*-hemin was obtained by evaporation of the mother liquors from a hemin preparation. It was dissolved in water containing pyridine and sodium hydrosulfite and passed through a column of alumina. Adsorbed pigments were washed with water containing hydrosulfite. The principal red zone that became brown on oxidation was removed and the pigment was eluted with 0.67 N hydrochloric acid. Upon neutralization with soda, this elutriate deposited flocculent crystals of the *c*-hemin.

Porphyrins. Coproporphyrin I, a normal minor constituent of urine, was concentrated by adsorption on cotton and asbestos at pH 3.6 (Fink). It was also adsorbable on alumina (Zeile and Rau; Waldenstrom; Turner). With the latter adsorbent, most of the colored constituents of urine passed through the column; the porphyrin remained on the adsorbent. This has been made the basis of a method for determination of the porphyrins in urine, especially in the urine from persons afflicted with lead poisoning and other pathological conditions (Waldenstrom). It has also been used for preparation of the porphyrins from feces. These pigments were extracted with ether and ethyl acetate and transferred to strong hydrochloric acid. The acid extract was partially neutralized with sodium acetate and passed through a column of alumina. Porphyrins were retained by the adsorbent while other pigments passed into the percolate. Elution of the porphyrins from the alumina with ether and acetic acid was followed by readsorption of the eluted material

from solution in ether. In this way, a lower green zone was separated from the principal porphyrin pigment (Waldenström).

Fischer and Hofmann adsorbed the urinary porphyrins obtained from a patient with congenital porphyrinuria on columns of talcum using chloroform and methanol as solvent. When the chromatogram was developed with chloroform, two bands of pigments were obtained. The lower rose band was washed into the percolate from which crystals of the pigment were obtained by evaporation of the solvent. The brown pigment remaining on the column was eluted with glacial acetic acid and crystallized by concentration of the extract.

For the preliminary purification of porphyrins from feces, Grotepass and Defalque passed the acidulated extracts (partially purified by repeated partition between ether and hydrochloric acid) through a short column (2 mm.) of talcum. The percolate was passed through another column containing infusorial earth which retained the porphyrins. These were finally washed through the column with a large volume of 5 per cent hydrochloric acid and recovered by the methods commonly employed.

A number of synthetic porphyrins have been purified by the use of adsorption columns. Among these were:

Coproporphyrin I and III (Waldenstrom),

Desoxo-phyllerythro-etioporphyrin (adsorbed on talcum in a conical glass tube using ether and chloroform (Fischer and Hofmann),

Phyllophorphyrin ester (from formic acid degradation of phytochlorin, adsorbed on talcum, eluted with pyridine and ether) (Fischer and Bauer),

Octaphenyl-porphyrazin and the magnesium and copper compounds (adsorbed on alumina from benzene, eluted with pyridine) (Cook and Linstead),

Tetrabenzo-porphin and the iron and magnesium compounds (adsorbed on alumina from ether and pyridine) (Helberger, Rebay and Hevér).

16. Bile Pigments

Urobilin in acidulated urine is only weakly bound by alumina. Bilirubin and biliverdin are more strongly adsorbed (Waldenström) Adsorption on alumina has also been employed for separation of the bilirubin of blood from the associated carotenes and xanthophylls (Süllmann, Szécényi-Nagy and Verzár). The serum (10 ml.) was

diluted with ethanol (75 ml.) and filtered after standing 0.5 hours in the dark. Pigments in the filtrate were transferred to petroleum ether by the addition of this solvent and water. When the petroleum ether solution was passed through a column of alumina, three bands of pigment were observed. The uppermost yellow band contained the bilirubin; the middle band contained the xanthophyll; and the lowest band contained the carotene.

Oxidation products of mesobilirubinogen, when adsorbed on talcum from chloroform plus ether, formed two bands. The small upper red zone contained mesobilirhodin; the larger, lower violet zone contained mesobiliviolin (Siedel). A bilirubin-like pigment, etioglaucobilin, and other unidentified substances were prepared by irradiation of the sodium ethylate addition product of etioporphyrin. The mixture of products was resolved by adsorption on alumina from ether (Fischer and Herrle). A synthetic hexapyrren was purified by adsorption on alumina from chloroform. Acetone was used for development of the chromatogram and methanol for elution of the adsorbed pigment (Fischer and Reinecke).

17. Carotenoids

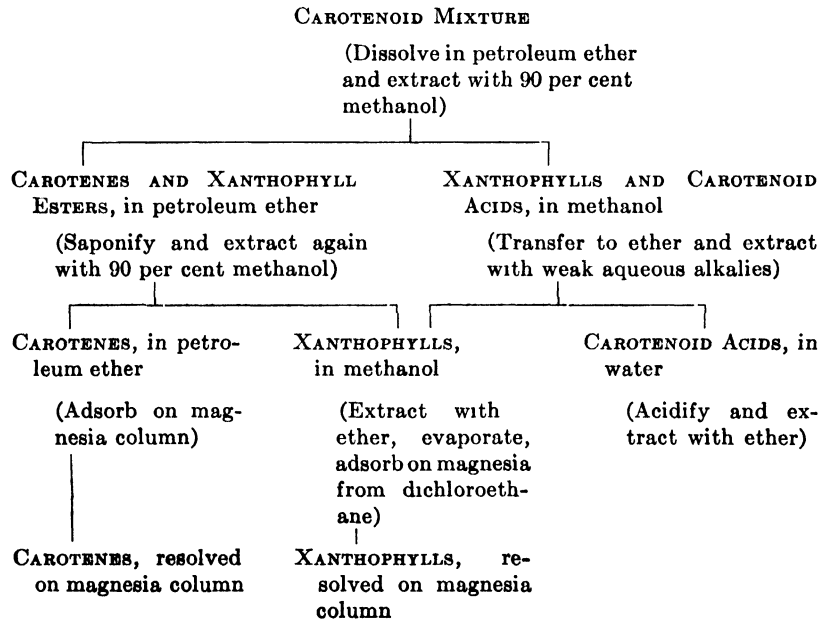
Classification and Properties. Some of the best examples of applications of the Tswett adsorption method are found in the highly specialized investigations of the carotenoid pigments. It was for the preparation of these, yellow, widely distributed, unsaturated compounds in quantities sufficient for chemical analysis that the columnar adsorption procedure was reintroduced into chemical practice. It was through studies of these fat-soluble pigments, some of which have been found to play the role of vitamins and hormones, that the relation between unsaturation and adsorbability of organic compounds was first established. For the preparation of these labile pigments some of the most active and selective adsorbents such as alumina and magnesia were developed. These methods are still the only satisfactory means for preparation of many of the carotenoids from the complex mixtures in which they occur.

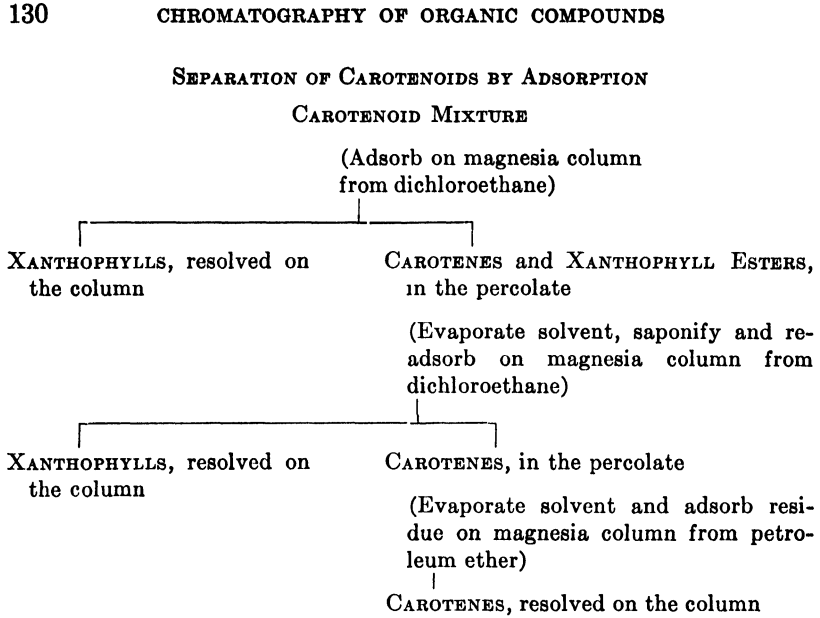
The molecules of carotenoid pigments contain a long aliphatic chain with attached methyl groups. In some of the pigments the ends of this chain are looped into rings like those of the ionones. All these molecules owe their yellow or red color to the presence of a conjugated system of double bonds in the central portion of the atom chain. Differences between the adsorbability of many of the carotenoids depend upon the number and arrangement of these

double bonds as has been pointed out in section 8 of chapter II. On the basis of composition and physical properties, the carotenoids are divided into four principal groups: namely, 1, carotenes, hydrocarbons usually of the formula $C_{40}H_{56}$; 2, xanthophylls, oxy and hydroxy derivatives of the carotenes; 3, carotenoid acids, carboxyl derivatives of the carotenes; and 4, xanthophyll esters, esters of the xanthophylls with fatty acids. Some five dozen of these compounds have been described, and chromatographic adsorption has been employed for the preparation, purification and identification of nearly every one of them.

Because of the great number of carotenoid pigments and because of the similarity between them, it is impossible to resolve a mixture of all these compounds on a single adsorption column. Consequently, it is necessary to make a preliminary separation of the different groups of carotenoids from one another before adsorption of the several mixtures. This is commonly done by partition of the pigments between immiscible solvents both before and after saponification. It may also be accomplished by crystallization of the mixtures and by adsorption itself. These procedures are illustrated by the following outlines.

SEPARATION OF CAROTENOIDS BY PARTITION AND ADSORPTION





Carotenoids partitioned between petroleum ether and 90 per cent methanol separate as follows: *epiphasic*, those in the upper layer, the carotenes and xanthophyll esters; and *hypophasic*, those in the lower layer, the free xanthophylls and the carotenoid acids. Following saponification of the pigments in the upper layer with alcoholic potassium hydroxide, the xanthophylls liberated from the esters are extractable with methanol. After the mixture of xanthophylls and carotenoid acids obtained in the first partition is transferred to ether, the acidic compounds are extractable with aqueous alkalies.

Separation of xanthophylls from carotenes and xanthophyll esters occurs when the mixture is crystallized from petroleum ether. Under these conditions, the xanthophylls crystallize; the carotenes and xanthophyll esters remain in solution. This procedure has been utilized for preparation of the leaf xanthophylls for adsorption (page 143).

Carotenes and xanthophyll esters are weakly adsorbed on alumina and magnesia from solutions in benzene or dichloroethane; hence they may be separated from the more strongly adsorbed xanthophylls by passage of the mixtures through columns of these adsorbents. Under these conditions the xanthophylls are simultaneously separated from one another on the column. These same separations may be effected by passage of petroleum ether solutions of the mixture through columns of sucrose or of the alkaline earth carbonates or soda ash. The

percolate containing the carotenes may then be passed through a column of magnesia or alumina.

Colorless fatty substances that decrease the adsorbability of the carotenoids frequently occur with these pigments in plants and animals. For the preparation of the carotenoids by adsorption it is necessary to determine whether or not the fatty contaminants are present in sufficient quantities to affect the adsorbability of the mixtures. This is done by adsorbing a portion of the mixture on a small column. If the carotenoids are weakly adsorbed, the fatty substances must be removed by saponification, by partition between immiscible solvents or by crystallization. Examples of these procedures are to be found on pages 132 and 147. Care must be exercised to prevent losses of some of the pigments during these preliminary purifications.

The relative positions of the carotenoids on the columns depends upon the number of double bonds and hydroxyl groups in the molecule as has been pointed out already in tables 9, 10 and 11 on pages 24, 25 and 26. Carotenoids removed from the adsorption column by elution with solvents containing a little alcohol are identified by comparison with pure pigments from other sources. Comparison may be made by use of the three tube method or mixed chromatogram, by determination of the spectral absorption coefficients, through determination of the wave lengths of light most strongly absorbed (λ max.), and by estimation of the chemical composition and number of double bonds. For comprehensive discussions of the methods of investigations of the carotenoids reference should be made to the books on these pigments (Palmer; Zechmeister; Willstaedt; Lederer, Willstatter and Stoll; Mayer; Walker; Strain).

Adsorption and elution of the carotenoids may be accompanied by a considerable loss of the pigments. Such losses vary with the solvents, with the adsorbents, with the time the carotenoids remain on the adsorbent, with substances that may be adsorbed simultaneously, and with other conditions. These changes commonly result in the destruction of only a few per cent of the adsorbed material, but occasionally most of the pigment may be lost. Thus far, a thorough study of the factors that cause alteration of the adsorbed carotenoids has not been made.

Carotenoids are easily changed into isomeric compounds that are resolvable upon the adsorption columns and that may be confused with the native pigments. Isomerization may be produced by the action of heat, acids, halogens and some adsorbents upon solutions of

the pigments (Cf. references quoted above; Quackenbush, Steenbock and Peterson; and recent papers by Zechmeister and co-workers). Carotenes are also rapidly isomerized by potassium amide in liquid ammonia (Strain 8).

Owing to the importance of carotenoids in the nutrition of plants and animals, many carotenoid assays of various organs and specialized tissues have been made by means of the chromatographic methods. Carotenes and xanthophylls of such diverse materials as bacteria and some hundred species of the lower and higher sea and land plants have been separated and identified. The fat-soluble, yellow pigments of worms, snails, larvae, reptiles and crustacae have been isolated by the adsorption procedure. The method has been utilized for examination of the carotenoids in the bills, legs, skin, feathers, fat, liver and eggs of birds. It has also found application to the determination of carotenoids contained in the fat, milk, blood, liver, placenta, *corpus luteum* and other organs of man and many mammals including whales. It played a role in elucidation of the relation between carotenoids and vitamin A and of the function of these compounds in the phenomenon of vision. Numbers of these applications of the chromatographic method contain little to interest the student of adsorption technique. For this reason, it has not seemed desirable to review each of these many papers. Instead, the carotenoids have been recorded in tables 18 and 19 with references to the methods by which they have been conveniently prepared. Only the best sources of the pigments are recorded. All these sources contained other pigments that were separated by the method of preparation.

References to the unquoted papers may be obtained through the index and the bibliography and through the review articles appearing at intervals in *Annual Review of Biochemistry.* Examples of adsorption methods that illustrate particular and significant procedures have been described in detail following the tables.

Separation of Carotenes From Xanthophylls and Chlorophylls. Assays of the vitamin A activity of most foods and fodders composed of green plants are based upon colorimetric estimation of the carotene content. This depends in turn upon the separation of the carotenes from other colored fat-soluble pigments, principally the xanthophylls and chlorophylls. For this separation Tswett recommended the use of columns of sugar or alkaline earth carbonates (page 59). Recently soda ash has been used as adsorbent for the separation of the carotenes from other colored constituents of leaves (Kernohan). Certain

TABLE 18

Carotenes: Their principal sources; adsorbents and solvents for their preparation

		Lycopene	
Tomatoes	magnesia	petroleum ether	(page 138)
Rose hips	alumina	petroleum ether and benzene	Willstaedt 5
Diospyros costata fruits	alumina	petroleum ether	Schön
Solanum dulcamara fruits	lime	benzene	Zechmeister and Cholnoky 6
Citrus grandis pink fruits	fibrous alumina	benzene	Matlack
		GAMMA-*Carotene*	
Carrot roots	alumina	petroleum ether and benzene	Kuhn and Brockmann 7, 8
Carrot roots	magnesia	petroleum ether	Strain
Gonocaryum pyriforme fruits	alumina and fibrous alumina	petroleum ether	Winterstein 2, 3
Cuscuta salina (dodder)	magnesia	petroleum ether	Mackinney 2
Allomyces	magnesia	petroleum ether	Emerson and Fox
		DELTA-*Carotene*	
Gonocaryum pyriforme fruits	alumina and fibrous alumina	petroleum ether	Winterstein 2, 3
Carrot roots	magnesia	petroleum ether	Strain
		BETA-*Carotene*	
Carrot roots	fibrous alumina	petroleum ether	Kuhn and Lederer 1
Leaves of many plants	magnesia	petroleum ether	Mackinney 1; Strain 3
Cucurbita maxima fruit	lime	petroleum ether	Zechmeister and Tuzson 3
Capsicum annuum (paprika)	lime	petroleum ether	Zechmeister and Cholnoky 3, 4
Butter	magnesia	petroleum ether carbon tetrachloride	Strain
		ALPHA-*Carotene*	
Carrot roots	fibrous alumina	petroleum ether	Kuhn and Lederer 1
	lime	petroleum ether	Karrer and Walker
	magnesia	petroleum ether	Strain
Palm oil	magnesia	petroleum ether	Strain
Libocedrus decurrens	magnesia	petroleum ether	Mackinney

TABLE 18—*Concluded*

		Flavoxanthin-like Carotene	
Carrot roots	alumina	petroleum ether	Karrer, Schöpp and Morf
	magnesia	petroleum ether	Strain
		Torulene	
Torula rubra	alumina	petroleum ether	Lederer
		Rhodopurpurin	
Rhodovibrio bacteria	lime	petroleum ether	Karrer and Solmssen
		Sarcinin	
Sarcina lutea	fibrous alumina	petroleum ether	Chargaff 1
		Flavorhodin	
Rhodovibrio bacteria	lime	petroleum ether	Karrer and Solmssen
		Leprotin	
Acid-fast bacterium	alumina	petroleum ether	Grundmann and Takeda

preparations of magnesia (Fraps and co-workers) and dicalcium phosphate (Moore), when activated under specified conditions, are also effective adsorbents. With all these adsorbents, petroleum ether was employed as solvent. With Micron Brand magnesia as adsorbent, dichloroethane was utilized as solvent (Strain 10). For a large scale separation, about 1 kg. of dried and powdered carrot leaves was extracted with 5.5 l. dichloroethane. The extract was concentrated under reduced pressure to a volume of about 100 ml., and the concentrate was passed through a column 6.6 by 22 cm. filled with a 1:1 mixture of magnesium oxide and Hyflo Super Cel. When the column was washed with fresh dichloroethane, all the carotene was carried into the percolate.

For separation of the carotenes from 1 g. of leaf material a column 2 by 14 cm. was used, and the leaf powder was placed in the column above the adsorbent. Extraction and separation of the carotene was thus accomplished in one operation.

Estimation of carotene contained in petroleum ether extracts of

TABLE 19

Xanthophylls: Their principal sources; adsorbents and solvents for their preparation

	Sulcatoxanthin, $C_{40}H_{52}O_8$		
Anemonia sulcata	alumina	benzene	Heilbron, Jackson and Jones
	Myxoxanthophyll, $C_{40}H_{56\pm2}O_7$		
Oscillatoria rubrescens	calcium carbonate, alumina	petroleum ether and benzene	Heilbron and Lythgoe
	Fucoxanthin, $C_{40}H_{54-60}O_6$		
Fucus vesiculosus	alumina	petroleum ether	Heilbron and Phipers
	Pentaxanthin, $C_{40}H_{56\pm2}O_5$		
Echinus esculentus	alumina, calcium carbonate	benzene	Lederer 8, 10
	Euglenarhodon, $C_{40}H_{48}O_4$		
Euglena helioru-brescens	alumina	ether	Tischer 1
	Astacin, $C_{40}H_{48}O_4$		
Nephrops norvegicus	alumina	petroleum ether	Burkhardt, Heilbron, Jackson, Parry and Lovern
Retinas of chickens	lime, alumina	petroleum ether	Wald and Zussman
	Astaxanthin, $C_{40}H_{52}O_4$		
Lobsters and other sources	sugar	petroleum ether and benzene	Kuhn, Stene and Sørensen
	Neoxanthin, $C_{40}H_{56}O_4$		
Green leaves	magnesia	dichloroethane	Strain
	Isolutein, $C_{42}H_{60}O_4$		
Green leaves	magnesia	dichloroethane	Strain
	Capsorubin, $C_{40}H_{58\pm2}O_4$		
Capsicum annuum (paprika)	calcium carbonate lime	carbon disulfide petroleum ether	Zechmeister and Cholnoky 3, 4

TABLE 19—*Continued*

Taraxanthin, $C_{40}H_{56}O_4$			
Dandelions	calcium carbonate	petroleum ether	Kuhn and Lederer 2
Ranunculus acer flowers	calcium carbonate	petroleum ether	Kuhn and Brockmann 5
Violaxanthin, $C_{40}H_{56}O_4$			
Leaves	calcium carbonate	petroleum ether	Kuhn and Brockmann 3
Arbutus unedo	alumina	petroleum ether	Schon
Pectenoxanthin, $C_{40}H_{52}O_3$			
Pecten maximus *Botryllus Schlosseri*	calcium carbonate	petroleum ether and benzene	Lederer 7, 8
Capsanthin, $C_{40}H_{58}O_3$			
Capsicum annuum (paprika)	calcium carbonate lime	carbon disulfide petroleum ether	Zechmeister and Cholnoky 3, 4
Lilium tigrinum anthers	lime	benzene	Karrer and Oswald 1
Antheraxanthin, $C_{40}H_{56-58}O_3$			
Lilium tigrinum anthers	lime	benzene	Karrer and Oswald 1
Eloxanthin, $C_{40}H_{56}O_3$			
Elodea canadensis	alumina	petroleum ether and benzene	Hey
Petaloxanthin, $C_{40}H_{56-58}O_3$			
Cucurbita Pepo flowers	calcium carbonate	carbon disulfide	Zechmeister, Béres and Ujhelyi
Flavoxanthin, $C_{40}H_{56}O_3$			
Ranunculus acer flowers	calcium carbonate	petroleum ether	Kuhn and Brockmann 5
Green leaves	magnesia	dichloroethane	Strain
Rhodopin and *Spirilloxanthin* or *Rhodoviolascin*, $C_{42}H_{60}O_2$ or $C_{48}H_{66}O_3$			
Spirillum rubrum	magnesia	dichloroethane	van Niel and Smith
Rhodovibrio bacteria	lime	petroleum ether	Karrer and Solmssen

TABLE 19—*Continued*

	Eschscholtzxanthin, $C_{40}H_{54\pm2}O_2$		
Eschscholtzia californica flowers	magnesia	dichloroethane	Strain 7
	Lycophyll, $C_{40}H_{56}O_2$		
Solanum dulcamara fruits	lime	benzene	Zechmeister and Cholnoky 6
	Rhodoxanthin, $C_{40}H_{50}O_2$		
Taxus baccata arils	alumina	petroleum ether and benzene	Kuhn and Brockmann 9
	Zeaxanthin, $C_{40}H_{50}O_2$		
Physalis Alkekengi fruit and calyx	alumina	petroleum ether and benzene	Kuhn and Grundmann 3
	magnesia	dichloroethane	Strain
Zea mays	alumina	petroleum ether and benzene	Kuhn and Grundmann 5
Leaves	magnesia	dichloroethane	Strain
Eggyolks	magnesia	dichloroethane	Strain
	magnesium carbonate		Euler and Gard
	Lutein, (xanthophyll) $C_{40}H_{56}O_2$		
Green leaves	magnesia	dichloroethane	Strain
	calcium carbonate	carbon disulfide	Kuhn, Winterstein and Lederer
Helianthus annuus petals	calcium carbonate	petroleum ether	Zechmeister and Tuzson 2
Wheat germ oil	alumina	petroleum ether	Drummond, Singer and MacWalter
	magnesia	dichloroethane	Strain 10
	Echinenone, $C_{40}H_{58\pm2}O$		
Echinus esculentus	alumina	petroleum ether	Lederer 10
	Lycoxanthin, $C_{40}H_{56}O$		
Solanum dulcamara	lime	benzene	Zechmeister and Cholnoky 6

TABLE 19—*Concluded*

Rubixanthin, $C_{40}H_{56}O$			
Rosa rubinosa	alumina	petroleum ether and benzene	Kuhn and Grundmann 4 Willstaedt and With
Cryptoxanthin, $C_{40}H_{56}O$			
Physalis Alkekengi	alumina	petroleum ether and benzene	Kuhn and Grundmann 3
	magnesia	dichloroethane	Strain 10
Zea mays	alumina	petroleum ether and benzene	Kuhn and Grundmann 5
Myxoxanthin, $C_{40}H_{54}O$			
Oscillatoria rubrescens	alumina	petroleum ether and benzene	Heilbron, Jackson and Jones

Other xanthophylls examined by adsorption were: *alpha*- and *beta*-bacterioruberin from *Bacterium halobium* (Petter), aphanin, aphanicin, flavicin and aphanizophyll from *Aphanizomenon flos-aquae* (Tischer 4), cynthiaxanthin from *Halocynthia papillosa* (Lederer 7), citraurin from *Citrus aurantium* (Zechmeister and Tuzson 14, 15 Tuzson 2), viol-erythrin from *Actinia equina* (Heilbron, Jackson and Jones; Fabre and Lederer; Lederer 5), glycymerine from *Pectunculus glycymeris* (Fabre and Lederer 2), mytiloxanthin from *Mytilus californianus* (Scheer).

dried and powdered leaf material by passage of these solutions through columns of technical soda ash followed by colorimetric determination of the carotene in the percolate has several advantages. The adsorbent is cheap and selective. Under the conditions employed, the carotene is not adsorbed so that there is little or no decomposition of this pigment, and it is not contaminated with colored alteration products of the other leaf pigments.

Preparation of Lycopene. Lycopene can be prepared quickly from tomatoes by the following hitherto undescribed procedure. Fresh or canned tomatoes (about 125 g.) are rubbed with the hands in about a liter of alcohol. The pulp is collected, and ground with sand and with absolute alcohol and petroleum ether. The ground mass is collected on a filter, washed again with absolute alcohol and petroleum ether. All the extracts are combined, treated with sodium hydroxide (about 10 g.) and agitated gently for 1 hour. After the addition of

some water to cause separation of the petroleum ether, the upper petroleum layer is separated and washed with 80 per cent methanol and then with water which removes the last traces of alcohol. This washed petroleum ether is passed through a column of magnesia (Micron Brand No. 2641 and Hyflo Super Cel, 1:1) 2.5 by 15 cm. The strongly adsorbed pigments are washed with benzene that carries the carotene rapidly through the column. A narrow red-orange band near the top of the column is removed and discarded. A wide, red-orange band of lycopene in the upper portion of the column is removed, and the pigment is eluted with benzene and alcohol. This extract is concentrated at reduced pressure and at a temperature below 25°. When absolute ethanol is added to the concentrated residue, crystals of lycopene separate rapidly. These are collected, washed with alcohol and dried in vacuum.

α- and β-Carotene From Leaves. Carotenes from 1 kg. of the dried leaves of the yellow carrot were separated from the xanthophylls as just described on page 134. The carotene solution was evaporated to dryness at reduced pressure and the residue was dissolved in petroleum ether which was passed through a column 3 by 22 cm. of magnesia and Hyflo Super Cel (1:1). Adsorbed pigments were washed with petroleum ether until the α-carotene had been carried into the percolate. Upon evaporation of this solution followed by crystallization of the residue from *n*-heptane, 9.7 mg. of α-carotene were obtained. The β-carotene remaining on the column was eluted with petroleum ether containing a little ethanol. After crystallization it weighed 90.5 mg. (Strain 3).

Flavoxanthin-Like Carotene, γ-, δ-, β- and α-Carotene From Carrots. Carrot roots (27.3 kg.) were sliced, extracted with warm water, dried, powdered and extracted with petroleum ether (5 l.) (Strain 8). After concentration of the extract to about 750 ml., fats associated with the carotenes were saponified with potassium hydroxide in methanol. In order to remove the resultant soaps, the petroleum ether solution was extracted repeatedly with 90 per cent methanol. Methanol was finally removed by extraction with water and the petroleum ether was concentrated to about 250 ml. This concentrated solution was passed through a column (I) of magnesia and Hyflo Super Cel (1:1) 6.7 by 37 cm. which was then washed with petroleum ether until the α- and β-carotene were carried through. In this way the following chromatogram was obtained:

Column I

3 cm. orange (mixture)
15 cm. colorless
3 cm. red-orange (δ-carotene)
1 cm. colorless
2 cm. orange (γ-carotene)
1 cm. colorless
12 cm. lemon yellow (flavoxanthin-like carotene)

The percolate from column I was collected in portions of approximately 150 ml. and these were passed successively through a second column (II) identical with column I. Under these conditions, β-carotene formed a wide orange band in the upper third of the column, and below it, the α-carotene formed a narrower yellow-orange band. Below the α-carotene there appeared two colorless bands that were strongly fluorescent (white) in ultraviolet light and that resembled those obtained by adsorption of extracts of leaves (Strain 6).

Pigments contained in the bands on the two columns were eluted with petroleum ether and ethanol. After the elutriates had been concentrated at reduced pressure, the pigments were caused to separate by addition of methanol to the concentrates.

Micro Separation of Carotene Mixtures. Willstaedt and With adsorbed a mixture of 33 μg. of xanthophyll and 36 μg. of lycopene on a column of alumina 1 by 10 cm. using the apparatus illustrated on page 38. After the chromatogram had been developed with a mixture of petroleum ether and benzene (1:4), the xanthophyll formed a band 1 cm. deep in the upper portion of the column; the lycopene formed a zone about 0.5 cm. deep some 5 cm. below that of the xanthophyll. Elution of the pigments with solvent containing alcohol followed by colorimetric estimation of their concentration revealed that about 47 per cent of the xanthophyll and 78 per cent of the lycopene had been recovered.

Separation of similar quantities of β-carotene and lycopene on alumina with petroleum ether as solvent, resulted in the recovery of 86 per cent of the lycopene and 94 per cent of the carotene. Other analogous applications of small adsorption columns are described by van Veen and Lanzing. By the use of magnesia columns of very small diameter (about 1 mm.), it has been possible to separate as little as 1.5 μg. of carotene from carrot roots into α- and β-carotene. Separations of equally small portions of xanthophylls have been

made and the pigments identified by readsorption with known xanthophylls. This micro adsorption method has proved extremely useful for the identification of the pigments contained in single organs of plants (Strain 10) (figure 30, page 40).

Separation of a Mixture of β-Carotene, Cryptoxanthin, Lutein Zeaxanthin and Neoxanthin. The efficiency of magnesia as an adsorbent for the separation of xanthophylls by the Tswett adsorption method is illustrated by the following experiment described by Strain (10). β-Carotene (100 mg.) cryptoxanthin (100 mg.), lutein (100 mg.), zeaxanthin (100 mg.), and neoxanthin (10 mg.) were dissolved in dichloroethane (14 ml.) and passed through an adsorption column 3 by 25 cm. which contained 99 g. of a mixture of equal parts by weight of magnesia (Micron Brand Magnesium Oxide No. 2641) and heat-treated siliceous earth (Hyflo Super Cel F.A. 501). The column was then washed with fresh portions of dichloroethane, whereupon five colored bands of adsorbed pigments separated. As shown by subsequent determinations of the spectral absorption properties of the pigments isolated from each of the bands, these pigments occurred in the following order, beginning at the top of the column: neoxanthin, zeaxanthin, lutein, cryptoxanthin, and β-carotene. The column was washed with dichloroethane until the carotene, the cryptoxanthin, and the lutein had been collected separately in the percolate. These pigments were then isolated by concentration of the respective portions of the percolate followed by crystallization of the pigments from methanol. The bands of adsorbed neoxanthin and zeaxanthin were dug from the column with a spatula, and the pigments were eluted with ethanol and isolated by crystallization from methanol after concentration of the elutriate. The yields of the recovered pigments were: β-carotene 90 mg., cryptoxanthin 90 mg., lutein 85 mg., zeaxanthin 85 mg, and neoxanthin 5 mg. The separation of the pigments on the column required 6 hours and 20 minutes.

Capsorubin, Capsanthin, Zeaxanthin, Cryptoxanthin and Carotene From Paprika. (Zechmeister and Cholnoky 2). Paprika skins (200 g.) were extracted with 1.5 l. of petroleum ether which was then evaporated. Xanthophyll esters in the residue were dissolved in ether and saponified with potassium hydroxide in methanol. After removal of the soaps and methanol with water, the ether was evaporated and the xanthophylls in the residue were dissolved in carbon disulfide. Portions of this solution were passed through ten columns

of calcium carbonate. After development of the chromatograms with carbon disulfide, the following series of bands was observed (dimensions in mm.).

5	brown
20	red-brown
0.25	yellow
5	violet, capsorubin
5	canary yellow
20	violet, capsanthin
	five very thin stripes
5	orange-yellow, zeaxanthin
0.2	violet
10	orange-yellow, cryptoxanthin
	filtrate, carotene

Pigments in the ten respective bands were eluted with ether containing a little alcohol; the extracts were concentrated; and the pigments were recovered by crystallization. In this way, 150 mg. of capsanthin and 18 mg. of zeaxanthin were obtained. The crude capsorubin was dissolved in carbon disulfide and readsorbed on calcium carbonate. Only small quantities were recovered. The cryptoxanthin was readsorbed on calcium carbonate from solution in petroleum ether, and the pigment from the middle portion of the band was adsorbed again from solution in petroleum ether. After elution from this third adsorption, the cryptoxanthin was crystallized from methanol. It weighed 20 mg.

Lycoxanthin, Lycophyll and Lycopene From Solanum Dulcamara (Zechmeister and Cholnoky 6). Fresh berries of the bittersweet (17 kg.) were divided into 200 g. portions and rubbed into a paste with sand. This paste was placed on a suction filter, washed twice with alcohol and then with peroxide-free ether (about 15 l.) until the percolate was nearly colorless. Alcohol was removed from the combined percolates with water and the ether was removed in vacuum. Benzene was added and evaporated in order to remove the last traces of other solvents. The residue was finally dissolved in 3 l. of warm benzene. Tar that separated when the solution was cooled was removed by filtration, and the filtrate was passed through 30 columns 5.5 by 20 cm. containing calcium hydroxide. Benzene was used for development of the chromatogram which appeared as follows (dimensions in mm.):

3 brown, oxidation products
2 deep brown, oxidation products
5 red, lycophyll (λ max. 444, 473, 503 mμ in hexane)
2 brown
8 red, lycoxanthin (λ max. 444, 473, 503 mμ)
2 yellow-red
10 yellow
100 red, lycopene (λ max. 444, 473, 503 mμ)
10 yellow-red
filtrate, bright yellow

Pigments contained in the three red bands were eluted with benzene and methanol. The crude lycophyll was purified by three successive adsorptions on columns of lime; the lycoxanthin was readsorbed twice and the lycopene once. The eluted pigments were crystallized by addition of methanol to their concentrated solutions in benzene. There were thus obtained 920 mg. of lycopene, 125 mg. of lycoxanthin and 9 mg. of lycophyll.

Preparation of Leaf Xanthophylls. (Strain 10). Dried coarsely ground barley leaves (1 kg.) were placed on a 25-cm. Buchner filter and extracted with warm methanol (99 per cent, 5 l.). The methanol was drawn slowly through the filter with gentle suction. Finally about 1 l. of water was added to the pulp and most of the residual methanol was removed with strong suction. The methanol extract (about 4 l.) was concentrated to about 500 ml. at reduced pressure. The concentrated extracts from two 1-kg. portions of leaves were then combined and diluted with an equal volume of ether. This caused the separation of large quantities of viscous material. After the mixture had stood from 2 to 16 hours at room temperature, the liquid was decanted from the precipitate and treated with a solution of potassium hydroxide (75 g.) in methanol (99 per cent, 300 ml.). The solution was shaken thoroughly and permitted to stand several hours. It was filtered through a pad of cotton on a Buchner funnel and the residue was washed with ether (1 l.), which was added to the filtrate. Water (2 to 3 l.) was added to the filtrate and the ether was separated. The water layer was extracted once with ether (about 400 ml.). The combined ether layers were washed with water, dried over sodium sulfate, and concentrated to about 100 ml. This solution, which frequently contained xanthophyll crystals, was poured into petroleum ether (750 ml.). After several hours, the xanthophyll

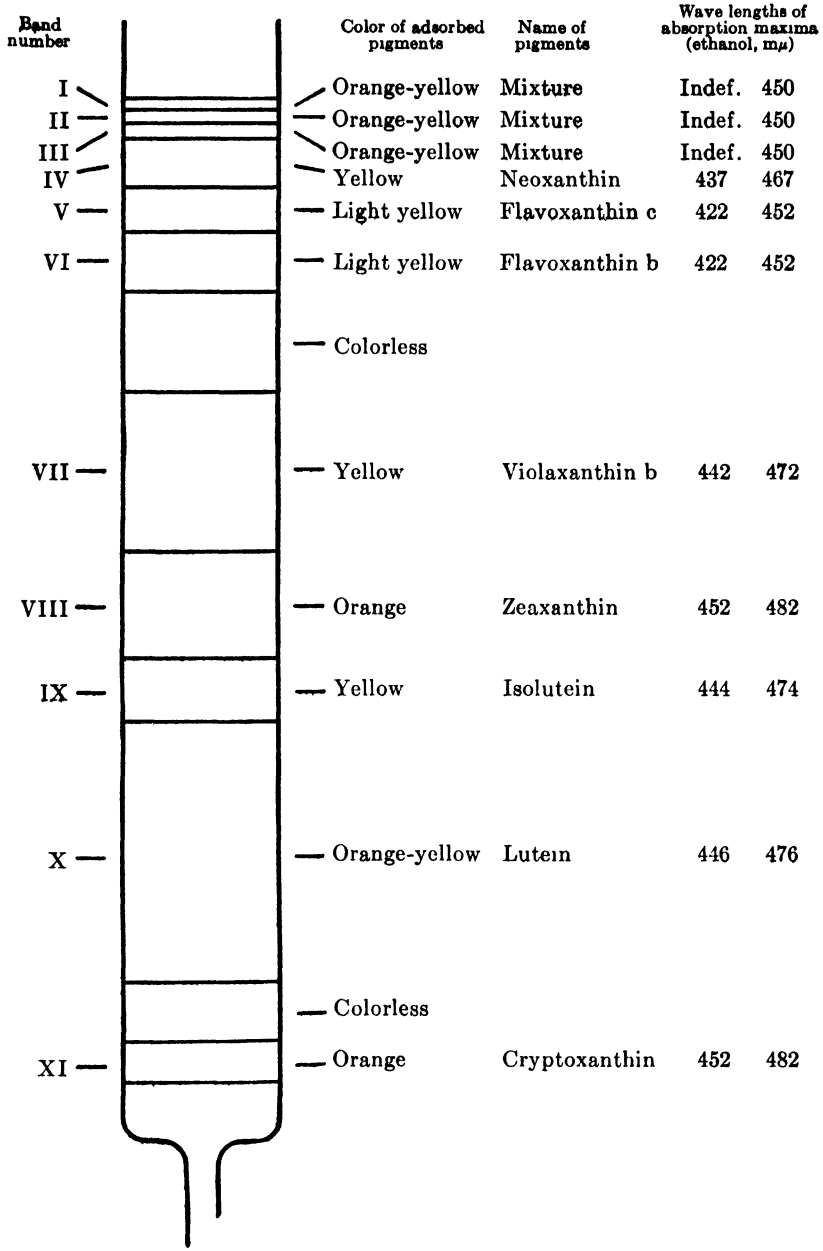


Fig. 35—Leaf xanthophylls separated by adsorption upon a magnesia column (one-third natural size).

crystals which had separated from the solution were isolated by filtration. They were contaminated with colorless crystals. This mixture was dissolved in dioxane (35 to 40 ml.) and recrystallized by the addition of petroleum ether (700 ml.). The recrystallized xanthophyll was separated and dried over calcium chloride and potassium hydroxide in vacuum. It weighed about 1.2 g.

One g. of leaf xanthophyll mixture, isolated as just described, was dissolved in dichloroethane (30–40 ml.) and the solution was passed through a column of magnesia and siliceous earth (780 g., 1:1) 6.5 by 42 cm. The column was washed with dichloroethane for 12 to 14

TABLE 20

Yields of xanthophylls obtained by adsorption of one-gram portions of crystalline leaf xanthophyll mixture

BAND*	SUBSTANCE	YIELD# (MG)	YIELD# (MG)
I	Mixture	53	
II	Mixture	20	
III	Mixture	4	
IV	Neoxanthin	80	100
V	Flavoxanthin c	22	8
VI	Flavoxanthin b	19	20
VII	Violaxanthin b	26	111
VIII	Zeaxanthin	8	25
IX	Isolutein	5	10
X	Lutein	250	280
XI	Cryptoxanthin	Trace	Trace
	Colorless crystals from percolate	80	60

* Band numbers refer to the bands shown in figure 35.

\# The results of two different separations are recorded.

hours, using a vacuum (about 26 cm. of mercury pressure) to draw the developer through the adsorbent. This caused the pigments to separate into bands as shown in the accompanying illustration (figure 35). After removal of the bands separately from the column and elution of the xanthophylls with ethanol, the resultant solutions were evaporated to dryness at reduced pressure. Pigments obtained from bands I to VII inclusive were crystallized from methanol by the addition of water. Zeaxanthin from band VIII was recrystallized from methanol (99 per cent). Xanthophylls from bands IX and X were crystallized from methanol or from dioxane by the addition of

petroleum ether. Incomplete separation of the zeaxanthin and violaxanthin b bands on the column necessitated purification of the former pigment by crystallization from methanol and resulted in loss of the associated violaxanthin b.

The yields of the pigments obtained from the different bands of the adsorption column in two typical experiments are shown in table 20. It may be observed that the total yield of the separated pigments accounts for only about half of the original mixture. This was not unexpected, because it was found that some colorless materials were present in the xanthophyll mixture, and it has been found by experience that quantitative recoveries of separated xanthophylls are never obtained. The loss of pigments in the mother liquors was, of course, very great.

All these xanthophylls differed in respect to color, composition and solubility. In the original report, precise spectral absorption curves of each pigment are recorded.

Rapid Separation of Leaf Xanthophylls. The following method for demonstration of the complex nature of leaf xanthophyll was devised by Strain. Fresh leaves (about 25 g.) were cut into small pieces with a pair of shears and placed in tall beakers (400 or 500 ml.) to which methanol (270 ml.), 99 per cent was added. The mixture was permitted to stand at 15–20° for 2 hours and the extract was then separated from the leaf material by decantation through cotton. The leaves were washed with methanol (50 ml.) which was combined with the first extract. In order to saponify the chlorophyll the combined extracts were treated with potassium hydroxide (15 g.) in methanol (35 ml.). After 0.5 hour, ether (150 ml.) was added followed by saturated salt solution (500 ml.) and water (100 to 200 ml.). The ether layer was separated and washed with water to insure complete removal of the saponified chlorophyll. Traces of xanthophylls in the combined aqueous layers were re-extracted with ether (50 ml.) and the combined ether layers were washed with water. This ether solution was dried with a little sodium sulfate and evaporated to dryness. The residue was dissolved in dichloroethane (5 to 7 ml.) and adsorbed upon a column of a 1:1 mixture of magnesia and siliceous earth (2 by 11 cm.). Under these conditions the xanthophylls were not very strongly adsorbed due apparently to the presence of colorless contaminants in the mixture. As a result, they separated rapidly into a series of bands identical with that described in the previous section.

Estimation of Xanthophylls. Because of the possible physiological importance of the yellow leaf pigments, many workers have attempted to separate the leaf xanthophylls from the associated, fat-soluble pigments by means of the Tswett adsorption method. Usually petroleum ether or petroleum ether and benzene extracts of leaf material were passed through columns of sugar or starch (Tswett; Winterstein and Stein; Seybold and Egle; Strott; Harder, Simonis and Bode; Simonis; Bode).

Bode extracted 100 mg. of dried and ground leaf material with small portions of a mixture of petroleum ether (25 ml.), methanol (7 ml.) and benzene (3 ml.). The extract soon separated into two layers. The lower methanol layer was removed, diluted with water and extracted with ether. This extract was combined with the upper petroleum ether layer, and the mixture was washed with water and concentrated to a few ml. This concentrate was passed through a column 1 by 9 cm. prepared by tamping powdered sugar into the tube while suction was applied at the base. The adsorbed pigments were washed with petroleum ether until all the carotene, which was not adsorbed, had been carried into the percolate and until the green chlorophyll bands were separated from each other. Chlorophyll *b* appeared near the top of the column, then chlorophyll *a* and below this a mixture of xanthophylls. The colored zones were separated from one another with a spatula, and the adsorbed pigments were eluted with methanol and ether. The concentrations of the pigments in the eluates were estimated colorimetrically by comparison with solutions of standard preparations of the chlorophylls and crystalline leaf xanthophyll. The probable error was said to be about 2 per cent.

In this and in most other adsorption methods for estimation of the xanthophylls, no control experiments demonstrating the per cent recovery of the adsorbed xanthophylls have been reported. Moreover, the experiments described by Strain have demonstrated that some of the xanthophylls of leaves are adsorbed with the chlorophylls, and these are frequently lost. Care must also be taken in the preparation and extraction of the leaf material if destructive oxidation reactions are to be prevented. Other complications are involved when one attempts to calculate the xanthophyll concentration from the intensity of the color of the solutions. Variations in the proportions and in the spectral absorption properties of the several xanthophylls make it impossible to calculate exactly the pigment concentration from colorimetric measurements.

Xanthophyll Esters. Most of the xanthophylls, particularly those found in seeds, fruits and flower petals, occur partly in the form of esters. These esters separate on adsorption columns in the same order as the xanthophylls themselves. They are adsorbed but slightly more strongly than the carotenes. This also applies to the synthetic esters as well as to the native ones.

Examples of xanthophyll esters investigated by adsorption are; zeaxanthin-dipalmitate or physalien and cryptoxanthin-palmitate from *Physalis Alkekengi* by adsorption on alumina (Kuhn and Brockmann 3), lutein-dipalmitate or helenien from flower petals of sunflower (Zechmeister and Tuzson 2) and *Tagetes aurea* (Kuhn, Winterstein and Lederer), synthetic di-*p*-nitrobenzoates of lutein and zeaxanthin adsorbed strongly on magnesia from carbon tetrachloride and adsorbed weakly from dichloroethane (Strain 10), actinioerythrin from *Actinia equina* by adsorption on calcium carbonate or alumina from solution in petroleum ether (Fabre and Lederer; Heilbron, Jackson and Jones), esters of eschscholtzxanthin (Strain 7), esters of capsanthin from paprika by adsorption on calcium carbonate from solution in petroleum ether (Zechmeister and Cholnoky 2), esters of astaxanthin (Kuhn, Stene and Sørensen). Other examples may be found by reference to the papers on the preparation of the xanthophylls themselves.

Carotenoid Acids. Saffron (*Crocus sativus*) contains the digentiobiose ester of a dicarboxylic acid known as crocetin, $C_{20}H_{24}O_4$. This acid and its methyl esters occur in a labile or *cis* form and a stable or *trans* form. According to Winterstein (1) and Winterstein and Stein (2), these two forms which are separable by crystallization are readily separable by adsorption. These investigators also report that norbixin, a carotenoid dicarboxylic acid of the formula $C_{24}H_{28}O_4$, and its methyl ester, found in seedpods of *Bixa orellana*, can be separated into *cis* and *trans* forms by adsorption.

Azafrin, a dihydroxy-monocarboxylic acid of the formula $C_{27}H_{38}O_4$, has been isolated from the roots of *Escobedia scabrifolia* by adsorption on calcium carbonate from solution in petroleum ether and benzene (Kuhn and Deutsch).

Isomerization Products of Carotenoids. Zechmeister and Tuzson (16, 17) separated the isomerization products of lycopene by adsorption on calcium hydroxide. Chromatographically homogeneous lycopene (35 mg.) was dissolved in 50 ml. of benzene and heated under reflux for 0.5 hour. The solution was cooled, petroleum ether (25

ml.) was added and the solution was passed through a column. When the adsorbed pigment was washed with petroleum ether and benzene (1:3) a band of neo-lycopene appeared below the main lycopene band.

Similar methods were used for preparation of the isomers formed from the xanthophylls (Zechmeister and co-workers; Strain 10). In the case of isomers formed from carotenoids by the action of iodine (Kuhn and Lederer 4), the new compounds were adsorbed below the original ones if the latter were hydrocarbons or contained only one hydroxyl group. If two or more hydroxyl groups were present in the molecule, the isomers were increased in number and were usually adsorbed above the original xanthophyll (Zechmeister and Tuzson).

Degradation Products of Carotenoids. In the course of chemical studies of the carotenoids, a great many new compounds have been synthesized. Most of these have been prepared or purified by adsorption on alumina, lime or calcium carbonate from solution in petroleum ether and benzene. Among these compounds were β-hydroxy-carotene, β-carotenone and β-carotenone-aldehyde from β-carotene (Kuhn and Grundmann 1,2); various oxidation products of capsanthin (Zechmeister and Cholnoky 4,7); azafrinone and its methyl ester, amide, nitrile and oxime (Kuhn and Deutsch; Kuhn and Brockmann 14); norbixin-aldehyde methyl esters from the permanganate oxidation of bixin (Karrer and Solmssen 8).

18. Coal Tar Dyes

Ruggli and Jensen, Ruggli and Stauble, and Jensen have separated mixtures of a number of coal tar dyes by adsorption on columns of alumina or calcium carbonate. Columns were prepared in tubes 1.7 by 13 cm. (or 6.5 by 21 cm.) from a slurry of the adsorbent and water. The adsorptive properties of the alumina were improved by washing it with distilled water containing a little lime. About 2 ml. of a 0.1 per cent solution of the dye in distilled water were then passed through the column followed by about 10 ml. of water.

The following nine basic dyes were adsorbed in the order given below.

Victoria blue B,
Methylene blue D, Patentphosphin G,
Crystal violet 5 BO, Fuchsin G, Safranin 00,
Brilliant green, Malachite green,
Auramine.

Of 36 mixtures containing two of these nine dyes all but four were resolved by adsorption. The four inseparable mixtures were composed of auramine and malachite green, brilliant green and malachite green, patentphosphin and methylene blue, and fuchsin G and safranin. Mixtures containing three dyes victoria blue, methylene blue or fuchsin and auramine were also separable.

Eosin or substituted phthalein dyes were found to be contaminated with other pigments that were removable by adsorption. The dyes themselves were adsorbed in the following order. Each dye contained the number of halogens indicated in the parentheses.

Rose bengale (4I, 2Cl)
Erythrosin (4I), Phloxin (4Br, 4Cl),
Eosin (4Br), Spirit-eosin (4Br)
Fluorescein

Some common azo compounds were adsorbed in the following order.

Diamine green (triazo)
Congo red (diazo)
Diamine rose (monoazo)

Congo red was often found to be contaminated with other pigments. After purification by crystallization from water and alcohol it was chromatographically homogeneous. As just indicated, mixtures of azo dyes were readily separable if the components contained different numbers of azo groups. For example, the so-called J-acid, 2-amino-5-naphthol-7-sulfonic acid, when diazotized and coupled with itself or with the condensation product, can be built into successively larger molecules each containing an additional diazotized J-acid. The adsorbability of these compounds is directly related to the number of J-acid groups in the molecule.

Ruggli and Jensen have studied the effect of the position of the substituents in amino-naphthol-sulfonic acid upon the adsorbability of the compounds formed by condensation of two molecules of these acids with diazotized benzidine. The adsorption order of the condensation products was

2-Amino-8-naphthol-6-sulfonic acid derivative
2-Amino-5-naphthol-7-sulfonic acid derivative
6-Amino-2-naphthol-4-sulfonic acid derivative
1-Amino-8-naphthol-4-sulfonic acid derivative
1-Amino-5-naphthol-7-sulfonic acid derivative.

It was also found that the adsorbability of sodium 2-naphthol-4-sulfonic acid was greater than that of sodium 1-naphthol-4-sulfonic acid. Because the former substance corresponds to a meta isomer in the benzene series and the latter compound to a para isomer, one is forced to the conclusion that the relation between stereoisomerism and adsorbability is different in these two series of compounds (table 13 page 27).

Ruggli and his co-workers determined the adsorbability of some acid dyes and indigo sols. The latter compounds were very weakly adsorbed, and their adsorbability varied with the temperature.

Franck found a number of medicinal dyes to be contaminated with other substances as shown by the formation of several bands upon columns of alumina. The products examined were; chrysarobin (3-methyl-1,8-dihydroxy-anthranol), cignolin (1,8-dihydroxy- anthranol), pellidol (di-acetyl-amino-azo-toluene), rivanol (2-ethoxy-6,9-diamino-acridine) and trypaflavin (3,6-diamino-acridine).

For student practice, Rieman recommended that a mixture of victoria blue B, crystal violet and auramine be separated on a column composed of 14 parts Merck's Reagent ignited aluminum oxide mixed with one part of Hyflo Super Cel. The dyes were adsorbed from 0.1 ml. of 95 per cent ethanol containing about 0.2 mg. of each pigment on a column 1.5 by 15 cm. This column was wet with an aqueous buffer, 0.002 M with respect to both primary and secondary potassium phosphates. The buffer, which was also used for development of the chromatogram, gave better results than other buffers of the same pH. In most experiments recovery of the resolved dyes eluted with ethanol was about 87 per cent of the quantity adsorbed, but with very small quantities of dyes, the recovery was considerably less.

Mixtures of dyes adsorbed upon Tswett columns have been separated by application of electrical potential to columns prepared in the following way (Strain 9). An electrode of coiled wire covered with cotton was placed in the constricted portion of an adsorption tube (2 by 13 to 3 by 23 cm.) and the tube was filled with an adsorbent (Hyflo Super Cel or occasionally cotton or mixtures of Super Cel and talcum). This column was attached to a suction flask and filled with water. A little of the solution of the mixture to be separated was then drawn into the upper portion of the adsorbent where it usually formed a rather homogeneous band. Water was added to the top of the column, and a second electrode was placed in this liquid as illustrated in figure 32 on page 40. In order to prevent liquid from flowing through the column, the suction was released and if

necessary slight pressure was applied to the base of the column. Application of electrical potential (175–200 volts) to the electrodes caused the columns to conduct a current of 0.5 to 2 mA. It also resulted in movement of the charged or ionized particles to the electrode of opposite charge with the simultaneous formation of zones or bands each of which contained a single pigment. Pigments separated in this way were recovered by the usual elution techniques.

A pronounced electro-osmotic effect was observed in all the experiments. Because the water usually moved toward the cathode, this electrode was placed at the top of the column so that the head of water could be adjusted to balance the electro-osmotic pressure. Passage of the water through the adsorbent could also be regulated, as customary in the Tswett method by application of suction to the base or of pressure to the top of the column.

In striking contrast to the effect obtained in the usual electrophoretic experiments where the bands of colored substances migrate with little or no widening, the materials on Tswett columns spread out rapidly until rather homogeneous colored zones were obtained. The pigments in these regions appeared to migrate along the surface of the adsorbent. This latter phenomenon was not observed in columns filled with weakly adsorptive, impervious materials such as sand and small glass beads. The bands of migrating pigments always exhibited well-defined boundaries throughout the mass of the adsorbent. In this connection it is interesting to note that Coolidge has found that a nonadsorptive filler also serves to prevent mixing of the migrating pigments in the usual electrophoresis apparatus.

Thus far, compounds separable by electrophoresis but inseparable by chromatographic adsorption have not been found. However, with columns of the same size and with equal quantities of pigments, electrophoresis often produced greater separation of the pigments than percolation alone.

The mixtures separated on Tswett columns by electrophoresis (the first compound named in each group remaining nearest the cathode) were: aminoazobenzene and indigo carmine, 3-nitro-4-aminoanisole and indigo carmine, methyl orange and 2,6-dichlorophenol indophenol, methyl orange and methyl red and indigo carmine, picric acid and methyl orange.

From weakly acid solutions, two forms of the indicators were separated both by chromatographic adsorption and by electrophoresis. The forms present in acid solutions were much more

strongly adsorbed than those present in weakly alkaline solution. Indicators adsorbed from acid solutions were gradually converted into the isomers observed in alkaline solutions upon prolonged washing of the columns with water or upon extended passage of the electric current. These methods provide another means for investigation of the tautomerism of indicators.

19. Various Natural Substances

Osajin. An ether solution of the pigment from the Osage orange (*Maclura pomifera*) was purified by passage through a column of wood charcoal and fuller's earth. Adsorption on alumina followed by elution with acetic acid did not yield a better product than that obtained by filtration of the solution through charcoal (Walter, Wolfrom and Hess).

Dracorubin. The red pigment of dragon's blood (from the palm *Sanguis draconis*) was partially purified by way of the picrate, dissolved in chloroform and adsorbed on a column of aluminum hydroxide. Dracorubin appeared as a bright red zone near the lowest pigment on the column. It was eluted with chloroform and methanol (Brockmann and Haase). The same pigment, under the name of "Dracocarmine," was also prepared by adsorption of the crude pigment on Brockmann alumina (Hesse).

Lactaro-violin. A pigment extracted from the mold *Lactarius deliciosus* was purified by adsorption on alumina from solution in petroleum ether. Petroleum ether and benzene were used for development of the chromatogram. The adsorbed pigment was eluted with petroleum ether and methanol (Willstaedt 6,7).

Azulenes. A number of hydrocarbons known as azulenes have been purified by chromatographic adsorption. Lactar-azulene, $C_{15}H_{18}$) extracted from *Lactarius deliciosus* after removal of the lactar-violin, was adsorbed on alumina forming a bright blue band. It was eluted with petroleum ether and alcohol (Willstaedt 6,7).

A synthetic azulene, prepared by dehydrogenation of the mixture obtained from cyclopentano-cycloheptanon, bromobenzene and magnesium, was purified by adsorption on alumina (Willstaedt 6,7).

A series of synthetic azulenes was studied by Plattner and Pfau who purified these unsaturated compounds by formation of their crystalline addition products with trinitrobenzene and with picric acid. Regeneration of the hydrocarbon from the addition compound by distillation or with alkalies proved to be incomplete. However,

by filtration of a solution of the mixture in cyclohexane and benzene through a column of alumina, the last traces of the nitro compounds were removed and the pure azulenes passed into the percolate.

Rottlerin. The yellow pigment from the fruit epidermis of *Rottlera tinctora* was purified by adsorption on calcium carbonate from solution in benzene and petroleum ether (1:1) (Brockmann and Maier).

Hypericin. Pigments of *Hypericum perforatum* causing photosensitization in animals have been purified by chromatographic adsorption and compared with similar pigments prepared in other ways (Pace and Mackinney).

Lignite. Zechmeister and Frehden passed a petroleum ether extract of lignite through a column of lime. In ultraviolet light, a number of fluorescent bands were observed. One of the compounds isolated in the form of a potassium salt, exhibited strongly reducing properties akin to those of ascorbic acid. Another constituent appeared to be a triterpene.

IX. INDUSTRIAL USES

1. Uses of Alumina

The Aluminum Ore Company has described a number of the industrial applications of activated alumina. These relate to the use of adsorptive alumina in towers or columns on a large scale and often at high pressures. They include the dehydration of natural gas, chlorine and sulfur dioxide and the removal of ammonia from cracked ammonia. Activated alumina is also utilized to remove oil vapor from compressed gases.

Many liquids are readily dehydrated with alumina (Derr and Willmore). These include benzene, toluene, ethyl acetate and pyridine. Others, such as carbon tetrachloride and animal and vegetable oils may be decolorized during the dehydration.

An interesting purification procedure is the decolorization of phthalic anhydride by its vaporization through a tube filled with alumina. Oil in electrical transformers is kept dry and acids are removed by continual passage over activated alumina. Special apparatus for this purpose employing the principle of the thermal syphon is illustrated in figure 36. Operation of electrical transformers is also maintained at a higher efficiency if the breather openings are connected with the atmosphere through tubes of alumina.

Equipment employed for dehydration of natural gas is illustrated in figure 37. The spent alumina is revivified by passage of hot gas through one tower while the other is in use. This cycle requires about 1 hour.

Activated magnesia has also been used for purification of solvents. Liquids employed in the so-called dry cleaning of clothes are passed continuously through a tower of magnesia that removes the colored contaminants (Seaton).

2. Purification of Water

Removal of calcium and magnesium ions from water is usually accomplished by passage of the water through a tower filled with a sodium zeolite. These zeolites exchange sodium ion for those of the

alkaline earths. When they have been saturated with the latter ions, the spent zeolites are regenerated by treatment with a strong solution of sodium chloride followed by removal of the excess sodium chloride with water. In practice, this is accomplished by use of two towers similar to those shown in Figure 37. One tower is used for softening the water while the zeolite in the other is being regenerated.

Natural zeolites are known, but those most commonly used are synthetic, hydrated sodium aluminum silicates. They are usually called permutites. Two such commercial products are Permutit and Decalso (page 60).

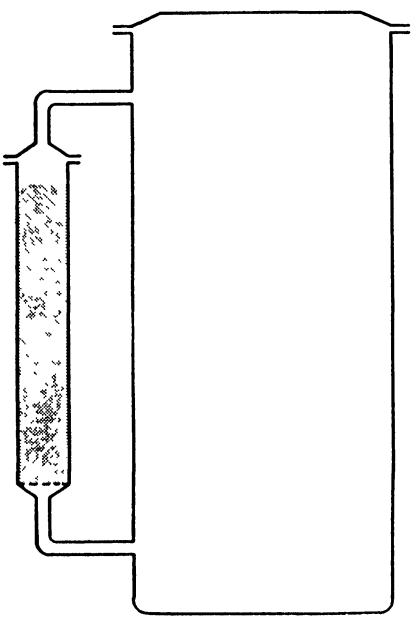


Fig. 36. Electrical transformer with adsorption column operated by thermal siphon of the insulating oil. (After Aluminum Ore Company)

In certain regions, flourine contained in the drinking water leads to discoloration or mottling of the enamel of growing teeth. In order to avoid this condition, attempts have been made to remove the flourine from the water by adsorption. One of the few adsorbents that shows promise for the accomplishment of this is bone ash.

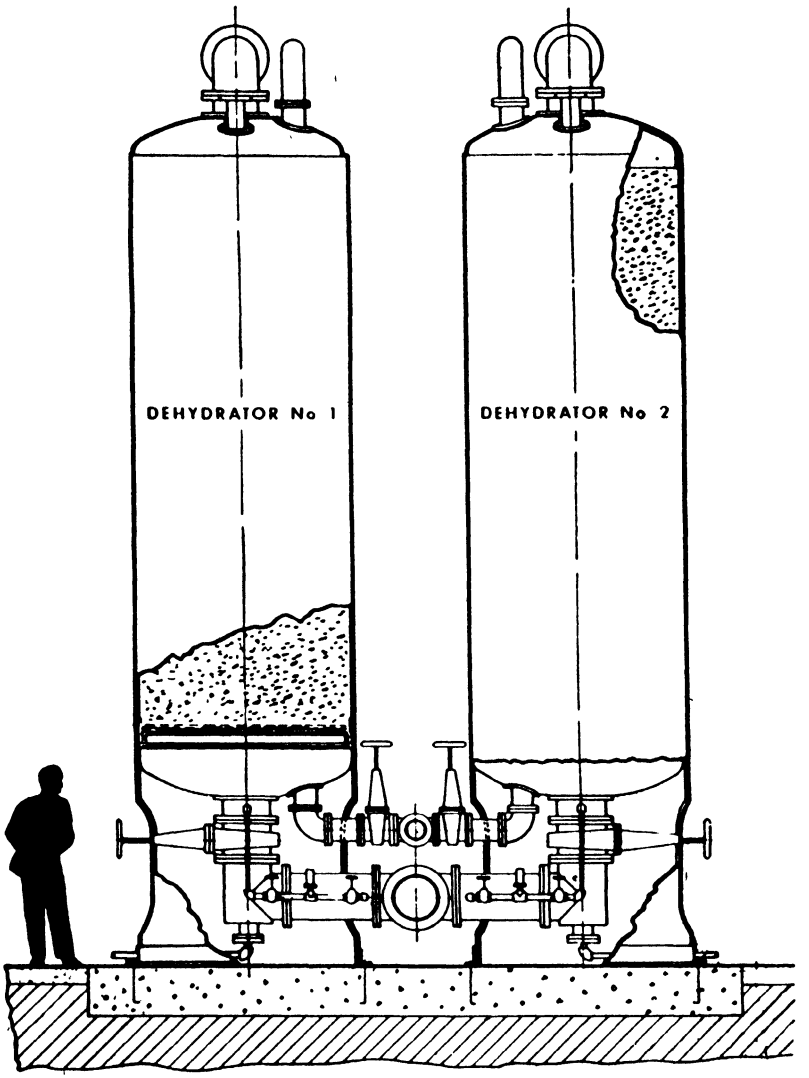


Fig. 37. Equipment designed to dry natural gas continuously at the rate of 25,000,000 cu. ft. per day. Pressure, 250 pounds per sq. inch; adsorbent, activated alumina. Original moisture content, saturation at 80°F; final dew point, −60°F. (Aluminum Ore Company).

3. Oils and Fats

For the control of raw materials, for the determination of their sources and for the detection of impurities, chromatographic adsorp-

tion shows promise of extensive application in the technology of oils and fats. Thaler has investigated the detection of various coloring matters in almond oil and in coconut oil. These fats were dissolved in petroleum ether, and the resultant solutions were passed through columns of alumina and of Clarit, an adsorptive earth. In this way very small quantities of carotene, carrot extracts, xanthophyll, anatto, bixin, and calendula and curcuma extracts were detected as yellow to orange bands at the top of the alumina columns. Green to red colors were observed on the Clarit columns. Dimethyl yellow and *p*-aminoazobenzene were weakly adsorbed on alumina, but strongly adsorbed on Clarit forming blood red bands. Martius yellow was not adsorbed on Clarit columns. Sudan III, scarlet R, and fat ponceau formed red to red-brown bands on alumina and blue to violet bands on Clarit. The quantities of dyes just detectable by this procedure were very small; namely, about 0.01 mg. per 10 g. of fat.

By procedures similar to those just described, Thaler compared the chromatogram of normal butter with that of adulterated butter. In this way indications of adulteration were obtained even when the adulterants were the same as those occurring in the unadulterated butter. For example, butter adulterated with carrot root carotene would show an increase in the relative proportions of α-carotene. In this connection, however, one may recall that the pigment composition of butter depends upon the nature of the rations given to the cows (Strain 8). As a consequence the presence of α-carotene or of certain fluorescent substances may be indicative of changes in the rations of the animals as well as of adulteration of the butter itself.

Chromatograms of various natural oils adsorbed on alumina from petroleum ether have been reported by Boekenoogen. The method served to indicate the presence of sesame, peanut, palm, olive, sunflower and other oils. If, however, the oils had been purified by adsorption, their chromatograms were altered greatly and their identification was difficult or impossible.

Preparation of medicinal or edible mineral oils usually involves the removal of colored contaminants by adsorption. Previously distilled and partially purified oils are allowed to percolate through towers of adsorptive earth. In order to facilitate percolation of the viscous oil through the adsorbent, the tower, often many feet in diameter, is placed in an insulated building that is heated to about 40°.

By this procedure, a colorless, tasteless, nontoxic, nonfluorescent percolate is obtained.

Lubricating oil is sometimes purified by filtration through adsorptive clays. The spent clay is reactivated by combustion of the contaminants in special kilns that prevent sintering of the clay itself [Ind. Eng. Chem. News Ed., **19**, 160 (1941)].

4. Sugar

One of the oldest applications of adsorption methods in industry is the decolorization of sucrose solutions obtained by extraction of plant material. The plant extract or crystals of crude sugar are dissolved in water and this solution is passed through large towers filled with activated charcoal. Channeling or uneven percolation is prevented by uniform distribution of the adsorbent in the tower. Usually the dry charcoal is distributed layer upon layer with a rotating mechanical spreader. Revivification of the spent charcoal by burning makes possible its reuse.

In laboratory tests, activated bauxite has been found as efficient as bone char for the purification of sugar solutions (La Lande). The experiments were performed in steel columns with hot water jackets. At 68° the alumina removed both invert sugar and the inorganic contaminants. Spent alumina was revivified by elution with water followed by dehydration at 315-485° for 0.5 hour. On repeated use and reactivation, the capacity for adsorption of inorganic compounds was scarcely impaired.

5. Tannins

The source and the nature of tannins used for production of leather from hides have been determined by adsorption of the tannins themselves or by adsorption of extracts of the leather. Grassmann and also Grassmann and Lang added two parts by volume of methanol to concentrated aqueous solutions of tannins and adsorbed the mixture on alumina or magnesia. The columns were washed with ethyl acetate or with methanol and ethyl acetate (1:3), and observed in ultraviolet light.

Extracts of pine bark, oak wood and quebracho wood were adsorbed and compared. All of these yielded complex chromatograms with many fluorescent zones. The fluorescence varied with the pH of the solution, being more intense in acid than in alkaline solutions.

Extracts of oak wood and of chestnut wood could be differentiated after oxidation with bromine water. The oxidized tannins were diluted with methanol and adsorbed whereupon the oak extract produced a blue-green fluorescent band, the chestnut extract a blue fluorescent band.

Adsorption of tannin and catechin preparations on columns of alumina gave single blue and green bands respectively. On the other hand, a synthetic tanning material (Tanigan O) gave a series of black, yellow, brown, red-brown, yellow, yellow-green and violet bands.

6. Drugs

Considerable information regarding the origin of drugs and tinctures has come from examination of their chromatograms. Valentin (1) differentiated between natural and artificial Peru balsam by adsorption of alcohol and petroleum ether extracts on alumina. In daylight, alcohol extracts of the natural compound formed a more complex chromatogram than extracts of the synthetic material. Petroleum ether extracts of the two materials exhibited just the opposite effect. In ultraviolet light, the chromatogram of the petroleum ether extract of artificial balsam was exceedingly complex.

Digitalis tincture yielded slightly different chromatograms depending upon whether absolute or dilute alcohol was used for the extraction (Valentin). Safran adulterated with *Flores calendulae* yielded a more complex chromatogram than safran alone (Franck).

Other drugs examined by adsorption upon alumina were:

Cantharidin tincture (Valentin and Franck)
Oleum hyocyami (Franck)
Vinum Condurango (Franck)
Absinthii tincture (Franck)
Strophanthii tincture (Franck)
Digitalis tincture (Franck; Valentin)
Coffee (aqueous extracts) (Valentin 2)
Hemlock (aqueous extracts) (Schneider and Willert)

Chromatographic adsorption has been employed for determination of the alkaloid content of a series of German medicinal preparations (Merz and Franck). Pure, water-free alumina (Merck) was used in the apparatus described by Valentin (page 35). An extract of the

drug in 70 per cent alcohol (10 ml.) was passed through the column which was then washed with fresh alcohol until about 50 ml. of percolate were obtained. Alkaloids contained in this percolate were usually determined by titration with alkali after addition of a known quantity of acid. In the case of belladonna tincture, the alkaloid content was estimated from the weight of material extracted with ether. The preparations examined were; Extractum Chinae spir.; Extractum Chinae fluidum; Tinctura Strychni; Tinctura Ipecacuanhe; and Tinctura Belladonnae.

Ernst and Weiner compared the chromatograms formed by adsorption of various drugs on columns of magnesia. Pulverized plant material (4 g.) was allowed to stand 24 hours with 40 ml. of 96 per cent, cold alcohol. Then 10 ml. of the extract was passed through the column (1.3 by 25 cm.) which had first been wet with water (1 ml.). The columns were washed with alcohol (10 ml.) and with water (10 ml.). In the original paper, the colors of the chromatograms, both in daylight and in ultraviolet light were described by reference to a standard color chart. Preparations from the following plants were examined: Aloe, Araraba, Cascara, Cassia, Frangulae, Rhamnus, Rhei, and Sennae. With Frangulae, differences were found between freshly dried and stored preparations.

Tests for identification of the principal constituents of the drugs of the plants listed in the preceding paragraph were also reported. Hydroxymethyl-anthraquinone, which forms a red-brown band on the magnesia columns, forms a red band when the adsorbed material is treated with potassium hydroxide. It is eluted with ether. Adsorbed chrysophanic acid, 1,8-dihydroxy-3-methyl-anthraquinone, is eluted with soda and extracted from the eluate with petroleum ether. When the yellow petroleum ether solution is treated with ammonia, the latter turns violet. Frangulae emodin, 1,6,8-trihydroxy-3-methyl-anthraquinone, gives a similar test after the adsorbed compound is eluted with potassium hydroxide and transferred to benzene.

7. Wine

Red wine, diluted with alcohol until the concentration of the latter is nearly 50 per cent and then adsorbed on alumina, yielded a chromatogram of two red bands with an adjoining lower greenish blue zone (Mohler and Hammerle). The filtrate was nearly colorless. Pigments contained in the bands were eluted with 50 per cent alcohol

containing 2 per cent tartaric acid. Wine that had been colored with Bordeaux red, a coal tar dye, and adsorbed yielded colored percolates, the dye passing through the column without adsorption. If much tannin (2.5 to 4 per cent) and tartaric acid (5 to 10 per cent) were present in artificially colored wine, these were strongly adsorbed and retained the Bordeaux red below the red bands of the wine itself.

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