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IN
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1938-1947

For the literature prior to 1938 the reader is referred to the following monograph :

PRINCIPLES AND PRACTICE OF CHROMATOGRAPHY

by

L. ZECHMEISTER and L. CHOLNOKY

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[*Frontispiece*

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PREFACE

ALMOST a decade has passed since L. Cholnoky and the undersigned attempted to discuss in a systematic manner the principles and practice of chromatography. Since 1938 adsorption procedures have been applied to the study of problems in many fields, and some new and very important methods have been introduced or developed, such as partition chromatography, paper chromatography, the Tiselius-Claesson method, exchange chromatography, etc. Recently, theoretical treatments of chromatographic processes have been presented by competent investigators, although they have had so far a limited influence only on experimentation which has still remained essentially empirical.

The author has felt that more assistance could be given to those interested in this field by offering the present progress report rather than by re-editing the original monograph. A glimpse at the Bibliography will show that the literature of chromatography has reached such a volume that completeness in survey and treatment cannot be claimed. Considerable difficulty was also caused by the heterogeneity of the widely-scattered material. It is, of course, never easy to survey a subject which is changing rapidly, and to compare critically the various data. However, even hints about methods, adsorbents or solvents which can be used within a certain class of compounds may be welcome and time-saving for the experimenter.

Inorganic Chromatography forms a separate chapter of the present report but the adsorption analysis of gases has not been treated.

During the preparation of the manuscript the author has enjoyed the valuable assistance of Dr. W. A. Schroeder (Pasadena) and Mr. H. J. Pinckard (Pasadena). Eventually

the text was kindly revised by Mr. A. L. Bacharach (London) and Mr. F. A. Robinson (Ware, Herts).

Professor A. Tiselius (Upsala) and Dr. A. J. P. Martin (London) kindly provided excellent photographs. The author wishes to express his thanks also to Mr. J. L. Bale and Mr. G. Parr of Chapman and Hall, Ltd.

Special thanks are due to Professor Ch. Dhéré (Geneva) for one of the rare portraits of the inventor of chromatography, Michael Tswett to whose memory this volume is respectfully dedicated.

PREFACE TO THE SECOND IMPRESSION

With the exception of a few minor corrections no changes have been made in the text of this progress report. The author wishes to take this opportunity to remind the reader that, as the title indicates, this volume is limited to information published between 1938 and 1947, a particularly fertile decade in the advance of chromatography. Since that time progress has been ever more rapid. Nevertheless, it is to be hoped that this book will continue to be of some use, especially to those who are experimentally active in the field.

L. Z.

CONTENTS

GENERAL SECTION

SPECIAL SECTION

LIST OF TABLES

LIST OF FIGURES

GENERAL SECTION

The term " Chromatography " designates those processes which allow the resolution of solute mixtures by selective fixation and liberation on a solid surface or support, with the aid of a fluid streaming in a definite direction.

This report demonstrates again the high efficiency of chromatographic adsorption analysis which still gives information when other analytical means fail. (Tswett.)

CHAPTER I

PRINCIPLES

History and Literature

The personality of Tswett and his early work on chromatography has been recently discussed by Dhéré (3) on the basis of extensive historical material. A short biographical sketch has been published by the author (8).

Tswett's achievements were closely connected with his investigations of the leaf pigments, but some experimental attempts made independently of him in America used crude petroleum as the starting material, and might in favourable circumstances have been successful in the elaboration of chromatographic procedures. Indeed, nine years before Tswett's fundamental paper appeared, Day used a long glass tube filled with fuller's earth through which he filtered crude green Pennsylvania oil. Light gasoline appeared first in the filtrate. In a reverse experiment the oil was allowed to rise in the column by capillary forces and the light hydrocarbons rose more rapidly. Subsequent investigations by Gilpin and Cram, Gilpin and Bransky, and Gilpin and Schneeberger between 1908 and 1913, which were evidently made without the knowledge of Tswett's method, showed that if the oil is sucked by a pump upward through the column, the light aliphatic hydrocarbons are accumulated in a top section ; aromatics and unsaturated compounds do not rise as high ; and, finally, the amount of nitrogen and sulphur containing compounds gradually decreases from bottom to top because of " selective adsorption " (cf. author 9).

Since 1938 a great number of more or less extensive general surveys of chromatography have been published, especially by Brockman (6–8) ; Cassidy (1) ; Clarke ; Cook ; Copley ; Fieser ; Fisk ; Griffiths, Gull and Whaley ; Hesse (4, 5) ; Lederer (17) ; Meyer ; Robinson and Bacharach ; Strain (12) ; Tananajew ; Vahrman ; Vetter ; and Willstaedt (12) ; and a

detailed monograph, " Chromatographic Adsorption Analysis " by Strain (15). The Deitz " Bibliography of Solid Adsorbents " includes a wealth of material in the form of pertinent abstracts. In a short survey by Martin (1) and in a recent laboratory manual by T. I. Williams, coloured reproductions of chromatograms can be found.

Theory

The present state of our knowledge in this field will here be only briefly outlined, since the theoretical efforts made so far have not influenced experimental work. While in practical chromatography several substances are present, most theoretical results that agree with experimental data refer to a single solute (or two solutes).

Wilson must be credited with having worked out the first mathematical theory of chromatography (1940). As he indicated, his theory mainly accounts qualitatively for the processes which take place on the Tswett column. Some of his basic assumptions, viz., that equilibrium between adsorbate and solution is established instantaneously and, furthermore, that diffusion effects can be neglected, led to results not in accordance, e.g., with the observed broadening of zones during development. In a simple experimental example reasonable agreement was found with Wilson's treatment by Cassidy and Wood—whose equations have been critically discussed by Martin and Synge (2) as well as by Weiss. By extending Wilson's simple theory, DeVault obtained certain significant results. In agreement with numerous observations his equations predict for a single solute whose adsorption isotherm is of the normal type that, upon development, a sharp bottom boundary and a gradual tailing in the top section of the broadening zone will appear.

Independently of DeVault, Weiss gave a theoretical treatment for the adsorption, development and elution processes that take place with a single compound, when Langmuir, Freundlich and linear isotherms are used. Trueblood has investigated experimentally the applicability of the Wilson-DeVault-Weiss theory ; he correlated the adsorption isotherms

with the rates of development, using *p*-nitroaniline, its N-ethyl-, N-diethyl-, N-phenyl- and N-diphenyl derivatives, and sym. diethyl urea, and a variety of developers on silicic acid-Celite. The development rates predicted by the theory from the (linear) adsorption isotherms agreed well with the experimental values. The profiles of developed zones had the general shape of an error-function curve, as predicted explicitly by Martin and Synge and expected on the basis of the theory mentioned above.

Weiss' conclusions were found on many points to be in agreement with an experimental study by Weil-Malherbe, who used benzpyrene. He investigated fractions of the chromatographic filtrate rather than the column itself and characterized the partial processes by plotting the quantity of the substance contained in the filtrate against the total volume of the filtrate. The shape of such " elution curves " depends on the Freundlich exponential coefficient α and some other factors. The " threshold volume ", V_f, i.e., the volume of solvent which had percolated through before the substance appeared in the filtrate, is influenced by the quantity of the adsorbent and of the substance contained in the initial solution. A study by Jacobs and Tompkins (3) has some features in common with Weil-Malherbe's paper and is based on the concentration distribution of Cu in filtrates (p. 273, 276); cf. also Le Rosen (3).

In connection with partition chromatography (p. 38) Martin and Synge (2) put forward a treatment in which they adapted some concepts gained in the study of fractionating columns. Their " height equivalent to one theoretical plate " is defined as " the thickness of the layer such that the solution issuing from it is in equilibrium with the main concentration of solute in the non-mobile phase throughout the layer." In the chromatograms discussed it has a value of about 0·002 cm. The non-mobile phase can be either a solid or, in partition chromatography, a second liquid phase. As pointed out by Martin and Synge, their assumption that the partition coefficient is a constant does not hold in practice, in which poorer separations may be obtained than the theory predicts.

A brief mathematical treatment for two solutes was given by Offord and Weiss, whose equations were (in part) criticized by Glueckauf. Walter (1, 2) published a detailed mathematical discussion for the behaviour of two solutes. He postulates a bimolecular reaction between solute and the solid phase and follows a kinetic approach to the problem as also does Thomas.

Recently, a broad theoretical investigation of chromatographic processes was published by Glueckauf (1–6), for the details of which reference must be made to the original papers. One of the results is that adsorption and exchange isotherms can be calculated from chromatographic data, in particular from elution curves. Glueckauf and Coates discuss the influence of an incomplete equilibrium on front boundaries. While a true local equilibrium was not obtainable, the equilibrium becomes more complete upon decrease of particle size and of flow rates. Experimental results were found to agree with the theory in many respects, and this was demonstrated by Coates and Glueckauf by a separation of Cu^{++} and Mn^{++} on Zeo-karb H-I.

A theory for the chromatographic boundary method (Tiselius-Claesson method) was developed by Claesson (3–8; p. 47).

Chromatographic Sequence

The chromatographic sequence, and therefore the separability of two compounds, cannot be deduced in any general way from their adsorption isotherms based upon pure solutions of each compound. The mutual influence on the adsorbabilities observed in mixed solutions may be an overriding factor. This was demonstrated, e.g., by Cassidy for some fatty acids, and similar observations for dilute aqueous solutions were made earlier by Jones, Hudson and Jones. Only in favourable instances is it possible to determine the adsorption isotherms from chromatographic data (Glueckauf 2).

Generally, in adsorption certain *anchoring groups* of the molecules will have a decisive function.

The *relative positions* of two or more compounds are reproducible provided that the adsorbent and solvent (developer)

remain the same. When we change from one chromatographic system to another, the adsorbability of each individual compound may be subject to alteration. If the direction and extent of these alterations are similar, the second chromatogram will be, at least qualitatively, similar to the first. This has been observed in a very great number of instances. However, it is likewise well known from practical experimenting, that satisfactory resolution of a mixture may be obtained on one adsorbent but not on another. Such differences may depend also on the developer. In an extreme case the change of the adsorbent or developer will effect an *inversion* of the originally observed sequence, that is, the two compounds have responded in an entirely different, perhaps opposite, manner to the change. Very enlightening is such a phenomenon when it is brought about by a change in the solvent only : as was pointed out by Strain, Manning and Hardin (2), there must then exist a mixture of the two solvents in which no resolution will take place. Even if the probability of such a situation is not great, it lays further emphasis on the need for repeating mixed chromatogram tests under various conditions.

In the field of natural pigments the following examples illustrating the influence of polar solvents on adsorption sequence were given by Strain (13). The sequence, from top to bottom, on a sugar column with petroleum ether is : chlorophyll b, chlorophyll a, fucoxanthin a ; the addition of 0·5 per cent propyl alcohol to the petroleum ether brings about the following sequence : fucoxanthin a, chlorophyll b, chlorophyll a. Two other sequences observed in an analogous manner were : chlorophyll b, chlorophyll a, lutein ; and chlorophyll b, lutein, chlorophyll a. On magnesia, when dichloroethane was the developer, a sequence from top to bottom was : neoxanthin, violaxanthin, zeaxanthin, lutein ; with petroleum ether + 25 per cent acetone it was : neoxanthin + zeaxanthin, lutein, violaxanthin (see also Table 1). Further interesting cases were reported by Strain (16) who succeeded in resolving some ternary mixtures in four out of the six possible different sequences.

Without altering the polarity of the developer inversions were obtained solely by changing the adsorbent. LeRosen (1) reported that the top to bottom sequence on alumina or calcium carbonate of cryptoxanthin and lycopene is inverted when calcium hydroxide is used, although all the chromatograms were developed with benzene. Evidently, with the hydroxide the presence of a longer conjugated system is more decisive than that of a hydroxyl group. When developed with light petroleum, phytofluene is adsorbed somewhat below α-carotene on calcium hydroxide, alumina, magnesium oxide or magnesium hydroxide but above it on silicic acid (author and Sandoval 3). According to M. Kofler (2) carotene is more strongly retained than tocopherol on floridin but less strongly on alumina.

In the field of the lipoids Cassidy found that materials like his " Carbon I " adsorb some higher molecular weight fatty acids more strongly than the lower ones, while silica gel shows the opposite behaviour. Trappe reports that cholesterol is located above triglycerides on silicic acid or alumina columns but below it on acid earths.

TABLE 1

Adsorption Sequence of Some Xanthophylls : (I) when developed on Magnesia and Light Petroleum + 25 per cent Acetone, and (II) on Sugar with Light Petroleum + 0·5 to 1 per cent Propyl alcohol (Strain, Manning and Hardin).

I	II
Neoneoxanthin A	Neoxanthin
Neoneoxanthin B	Peridinin
Neoxanthin + zeaxanthin	Neofucoxanthin A
Fucoxanthin	Neofucoxanthin B
Peridinin	Violeoxanthin
Violeoxanthin + lutein	Violaxanthin
Dinoxanthin	Fucoxanthin
Tareoxanthin + violaxanthin	Tareoxanthin
Taraxanthin	Taraxanthin
Cryptoxanthin	Zeaxanthin + lutein
	Cryptoxanthin

While most of the above examples of inversion refer to complicated natural products, some simple carbon compounds show similar phenomena (Schroeder 2). On a silicic acid + Celite (2 : 1) column the first compound of each following pair

was found to be more strongly adsorbed if the chromatogram was developed with benzene or benzene + light petroleum. However, this sequence was inverted by the use of ether + light petroleum : 3-nitrophenol and 4-nitro-N-ethylaniline ; N-nitroso-diphenylamine and nitroglycerine ; N-methyl-N-phenyl-urethane and diethyl phthalate ; N, N-diethyl-N, N′-diphenyl-urea and N, N-diphenyl-formamide.

These examples involve an essential change in the developer. In contrast, in the following, remarkable case inversion was caused by slight variation in the proportion of the two components of the developing agent :

	2 *per cent ethyl acetate in light petroleum :*	5 *per cent ethyl acetate in light petroleum :*
(top)	N-ethyl-N,N'-diphenylurea	4-nitro-N-ethylaniline
(bottom)	4-nitro-N-ethylaniline	N-ethyl-N,N'-diphenylurea

The *dipoles* of the solutes may be of importance in determining the adsorption sequence on alumina or other polar solids. As was pointed out by J. T. Arnold, *p*-, *m*- and *o*-mononitrophenols are adsorbed in this order of decreasing strengths of the permanent dipoles. The rule mentioned seems to be valid more generally for such sets of isomers containing the same number and kind of functional groups. It becomes invalid, however, if the number of the isolated dipoles is different in the compounds ; e.g., picric acid is more strongly adsorbed on alumina than *o*-nitrophenol, although its dipole is weaker. If no permanent dipole is present, the compound with the greatest polarizability would be most strongly adsorbed on an adequate polar medium (Arnold ; cf. Tamamushi ; Gupta and De).

A chromatographic sequence may also be affected by the *temperature* (Strain 16). It was found for decalin + 0·5 per cent. propyl alcohol solutions, on sugar, that the top to bottom sequence of lutein, chlorophyll *a* was inverted at 95°.

Inversion of cation sequences, caused by pH changes : cf. in Inorganic Chromatography, p. 274.

In discussions of the relative adsorption affinities in homologous series, the possibly low adsorption rates of some high molecular substances should also be considered. Thus

Baum and Broda found that acetyl carbohydrates in dioxane did not establish adsorption equilibria on charcoal or alumina even after several months. They assume that this observation could possibly explain the finding of Mark and Saito that some acetyl cellulose fractions of lower viscosity were retained near the top of the column in contrast to those with higher viscosity. It seems, however, that such an explanation is not required since an increase in adsorbability with the lengthening of the chain is by no means a general postulate. Thus, Claesson and Claesson found, on active carbon, that the adsorption affinities increase with decreasing molecular weights within the classes of nitrocellulose, polyvinyl acetates, and synthetic rubber, respectively. These adsorption equilibria were not reached instantaneously, and the time required for attaining equilibrium was found longer for lower molecular weight fractions in this instance.

Separation of Stereoisomers

As was shown recently, chromatographic methods have acquired an increasing importance in experimental stereochemistry. Indeed, in many instances configuration differences can be of stronger influence on the relative adsorption affinities than certain structural features. Comparatively small progress has been achieved in the separation of optical isomes, with the exception of some sugars and their derivatives (see Carbohydrates, pp. 116–138); general and efficient chromatographic methods for the resolution of racemates are not yet available. Greater progress has been made in the field of *cis-trans* isomers (cf. author 10).

Separation of Optical Isomers.—Few data are available for separations by the Tiselius method (p. 47). According to Tiselius (9) the difference in the retardation volumes of *dl*-tartaric and mesotartaric acids is great enough to make a separation on active carbon possible.

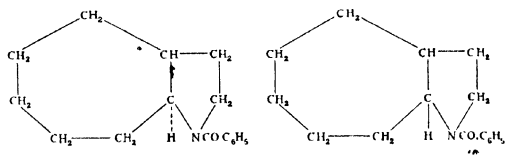
In the following column experiments only minor effects were obtained (Jamison and Turner). When *l*-menthyl-*dl*-mandelate was developed with light petroleum on alumina (120 × 1·2 cm.), the material contained in the top 15-cm.

section yielded upon saponification an acid with $[\alpha]_{5461} = -18.2°$, while the rotation for the fourth 15-cm. section was $+ 64°$. A partial resolution of brucine-*dl*-mandelate on dextrose was carried out by Hass, de Vries and Jaffé (benzene, liquid chromatogram). According to Fischgold and Ammon, when *dl*-mandelic acid and an optically active alkaloid were adsorbed simultaneously on charcoal, the unadsorbed portion of the acid showed some rotation.

The experiments of Henderson and Rule (2) on the resolution of *p*-phenylene-bis-imino-camphor have now been described in more detail.

Lactose, the adsorbent, was activated by boiling for 5 minutes with chloroform which removed some oil. The sugar was filtered, washed with hot chloroform and dried in a warm air current and then in vacuo in order to eliminate chloroform traces, which diminish the adsorptive power. 0·03 g. of material in 5 l. of benzene + light petroleum (8 : 1) required as much as 6 kg. of the lactose (tube, 130 × 10 cm.) and the development took 60 hours. The upper half contained 0·0023 g. of material, $[\alpha]_{5461} = + 485°$, and the lower fourth, 0·0018 g., with a rotation of $- 728°$. Four treatments and a large amount of adsorbent are needed for a complete resolution.

A clear separation of the two diastereomeric N-benzoyl-cyclopheptano-2 : 3-pyrrolidines was achieved by Prelog and Geyer (1) by means of a liquid chromatogram method.



N-Benzoyl-cycloheptano-2 : 3-pyrrolidines.

Resolution of Racemates containing Asymmetric Trivalent Nitrogen.—It had earlier not been possible to resolve compounds containing an asymmetric trivalent nitrogen-atom into their optically active components. With Tröger's base, however, Prelog and P. Wieland recently succeeded in carrying out such a resolution (for details, see p. 206). While a fractionation of the *d*-camphor-sulphonate salts gave only partial and not reliably reproducible resolutions, the preparation of a liquid

chromatogram on *d*-tartaric acid or *d*-potassium hydrogen tartarate, and especially on lactose, can be carried out with success in light petroleum solutions. The first two compounds adsorb the *d*-base more strongly, whereas *d*-lactose-hydrate prefers the *l*-form. The efficiency of the method is indicated by the fact that when 450 parts of adsorbent and 1 part of the racemic compound were used, 5·5 per cent was resolved in a single operation, the fractions ranging from $[\alpha]_D = +75°$ to $-52°$. This experiment involved considerably smaller quantities of the adsorbent and produced much greater effects than those reported, e.g., by Henderson and Rule.

A partial resolution of racemic *triethylene-diamine chromichloride* $(Cr\ en_3)Cl_3 + 3\ 1/2\ H_2O$, was obtained by Karagunis and Coumoulos by adsorption on a column prepared by pulverising *d*- or *l*-quartz, followed by fractional elutions with 85 per cent alcohol.

Although *dyeing* and adsorption are not congruent processes, unequal dyeing rates of optical isomers are good indications of differences in the adsorption process. Willstätter's earlier idea, that it would be well worth while to test the possibly selective dyeing of wool and silk with asymmetric pigments, was eventually carried out by Ingersoll and Adams as well as by Porter and Ihrig. The latter authors used azo dyes which had been obtained by diazotizing and coupling of *dl*-aminomandelic acids. In favourable cases the two antipodes were taken up by the fibre at very different rates, for the fraction which remained in the solution was found to be levorotatory (not confirmed by Brode and Adams or Henderson and Rule 2).

A comparable principle was tested experimentally by H. Martin and W. Kuhn who passed an endless wool strip (optically active) through a solution of rac. mandelic acid. A temperature gradient was maintained by keeping one surface at 100° and the other at 20°. The remaining solution became optically active.

Separation of cis-trans Isomers. The shape of a molecule as determined by a *cis* or *trans* configuration must

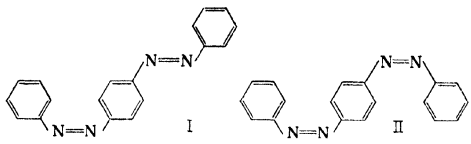
influence the adsorbability of the compound, which can be demonstrated, e.g., on the simple pairs of maleic-fumaric or mesaconic-citraconic acids, from aqueous solutions of which the *trans* form is more strongly adsorbed on charcoal than the *cis* form (Freundlich and Schikorr ; Schilow and Nekrassow). However, the relative adsorption affinities of such *cis-trans* isomers cannot be predicted in any general manner, that is, it cannot be postulated that either isomer will be more strongly adsorbed. The relative positions on the Tswett column will be influenced also by other factors, especially by the choice of the solvent and adsorbent.

Freundlich and Heller state concerning the adsorbabilities of *cis-* and *trans*-azobenzene : " The *cis* isomer is adsorbed more strongly by aluminum oxide, particularly in light petroleum, less in methyl alcohol ; the *trans* isomer is adsorbed more strongly by charcoal, particularly in methyl alcohol, less in light petroleum. . . . The more hydrophylic solute, here the *cis* isomer, is adsorbed more strongly by a hydrophilic adsorbent, here aluminum oxide, in a more hydrophobic medium, here light petroleum, than by a more hydrophobic adsorbent, here charcoal, in a more hydrophilic medium, here methyl alcohol." As pointed out by R. T. Arnold, the stronger adsorbability of *cis*-azobenzene is in accordance with the concept of the interaction between the solute dipoles and the fixed dipoles of the polar adsorbent.

New experimental data refer mainly to the stereochemistry of azo compounds and polyenes. For practical directions, see pp. 84, 193 and 195.

Separation of cis-trans Azo Compounds. The separation of the two stereoisomeric azobenzenes and a number of analogous pairs was carried out by A. H. Cook (2 ; Cook and Jones). He found that the *cis* compounds, which can be obtained by irradiation of the *trans* forms, separate easily when the solution is chromatographed on alumina. So far all observations show that the *cis* isomers possess a considerably stronger adsorbability than the corresponding *trans* compound. The same is valid for some more complicated structures. For

example, the sequence of the three possible stereoisomeric bis-benzeneazobenzenes from top to bottom is : *cis-trans* (I) ; *cis-cis* (II) ; and *trans-trans* (p. 193).



p-Nitrobenzylidene-*p*-nitroanaline was assigned *trans* configuration by Jensen and Hofman-Bang,) on the basis of its chromatographic behaviour.

Separation of cis-trans Diphenylpolyenes, $C_6H_5(CH = CH)_n \cdot C_6H_5$. For the lowest member of this series, *stilbene* (and its *p*-methyl or *p*-methoxy derivative) it was found that the two stereoisomers separate when their light petroleum solution is chromatographed on alumina. They can be located by brushing the column with permanganate (author and McNeely). In each instance the *trans* form showed the stronger adsorption affinity (p. 195).

Concerning higher members of this class, Cook (2) made the following brief remark : " Although analogous independence of the double bonds in α,ω-diphenylpolyenes has not been confirmed by the isolation of stereoisomerides, the behaviour of diphenylhexatriene and diphenyloctatetraene on a chromatographic column after irradiation in benzene-petroleum ether solution . . . indicates the formation of labile (*cis*-) isomerides." Stereoisomeric *diphenyloctatraenes* were investigated by the author and LeRosen (1, 2) (cf. also author 7.) By refluxing, melting, illumination or under the catalytic influence of iodine, this compound is partially converted into its stereoisomers. When adsorbed from a mixture of benzene and light petroleum on calcium hydroxide, ordinary or all-*trans*-diphenyloctatetraene shows the strongest adsorption affinity while some stereoisomers are located lower on the column. The very probable sequence is, *trans-trans-trans-trans* ; *trans-cis-trans-trans*, and *trans-cis-cis-trans* (some configurations are sterically hindered).

The three stereoisomeric *diphenylbutadienes* when chromatographed in light petroleum solution, on alumina, show the sequence : *trans-trans* (top), *cis-trans*, and *cis-cis*. The *trans-trans* zone can be easily separated because of its bluish fluorescence in ultraviolet light. The two *cis* compounds do not fluoresce but, when present in appreciable quantity, may be located by their moderate quenching of the weak fluorescence of alumina. A sharper separation can be accomplished, however, by the liquid chromatogram procedure as follows. The detection of either *cis* isomer in the filtrate fractions is carried out by treating a few drops of each fraction with a drop of dilute iodine (in light petroleum), followed by brief illumination under a daylight lamp. In this way, because of catalytic conversion into the *trans-trans* form, the solution acquires fluorescence, and its diagnosis can be made under the quartz lamp. The limit of detection is 0·5—1 mg. per litre (Pinckard, Wille and author ; Sandoval and author).

Separation of cis-trans Carotenoids. Without varying the solvent or adsorbent and by working with a complicated polyene system which offers many steric possibilities, recent experiments show that *trans→cis* rotations may either decrease or increase adsorption affinity. Thus, if all-*trans*-α- or β-carotene (with 10 and 11 conjugated double bonds) is converted, for example, by a catalytic treatment with iodine in light, into a mixture of stereoisomers, a subsequent chromatographic resolution shows *cis* compounds located both below and above the unchanged all-*trans* form which constitutes the main portion (Polgár and the author 1). Evidently a wide field is open for theoretical investigations which will have to explain this great dependence of the adsorbability on the number and distribution of those points in the conjugated system around which the molecule has been bent. A review of the present experimental knowledge was contributed by the author (7). The data reported seem to indicate that *trans→cis* rotations around peripherally rather than centrally located double bonds of carotenoids are necessary for an increase in adsorption affinity. Such a statement, however, does not cover hydroxy

compounds like zeaxanthin or lutein, the main isomerization products of which are generally located above the all-*trans* form on the Tswett column (pp. 84–88).

The existence of a great number of stereoisomeric carotenoids is responsible for many complicated chromatograms. While a mixture of ordinary, all-*trans* carotenoids yields a comparatively simple sequence, the presence of many stereoisomers may cause even an overlapping of two or more such column sections which include members of a certain stereoisomeric set.

For example, the following sequence appears on calcium hydroxide, upon development with light petroleum + 2 per cent acetone: neo-β-carotene U (top), neo-β-carotene V, neo-α-carotene U, all-*trans*-β-carotene, neo-α-carotene V, neo-β-carotene B, neo-β-carotene E, neo-α-carotene W, neo-β-carotene F, all-*trans*-α-carotene, neo-α-carotene B, and neo-α-carotenes C, D, etc. (bottom) (author and Polgár 4).

Cis-trans isomeric vitamins A: p. 224.

Interaction Between Adsorbent and Adsorbed Substance

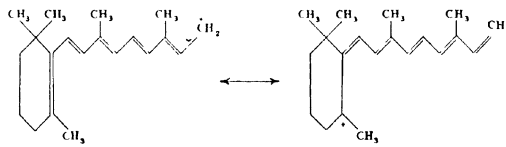
As is well known, the manifold possibilities for chromatographic separations are based on the fact that in a surprisingly large number of instances no chemical interaction occurs during the adsorption process or, to be more exact, the starting material can be recovered in an unaltered state by simple elution. Even if a pigment shows various colours on different adsorbents, as a rule the respective eluates are identical. However, to deny that chemical interactions are possible in such a system would be equivalent to the statement that no solute is able to react with the surface layer of a solid phase.

We may assume that in a great many instances adsorption takes place without causing any essential change in the electronic structure of the adsorbed molecule; for example, already existing dipoles may play a decisive part. Sometimes, however, under the influence of the active surface, a more or less far-reaching *polarization* which facilitates the adsorption process must be postulated. In an extreme case such a "polarizing adsorption" will induce a fundamental change in the initial state of the molecule and create a system with highly increased resonance. Then the process may become manifest

by the appearance of colour or by deepening of the initial colour. The existence of such mechanisms was clearly recognized and extensively studied by Weitz and Schmidt (1, 2; Weitz, Schmidt, and Singer 1, 2). They found that triphenylcarbinol $(C_6H_5)_3C.OH$, for example, is adsorbed from its colourless benzene or chloroform solution to give a yellow colour on alumina or a brownish yellow one on silica gel. Elution with alcohol or acetone involves complete bleaching.

A well known colour change takes place when some colourless polyenes, like vitamin A or phytofluene as well as coloured polyenes like carotenoids or diphenylpolyenes are adsorbed from non-polar solvents on acid earths. Such zones which are usually located at the top are deep blue or greenish-blue while some steroids produce a more orange colour. According to the interpretation of Meunier (cf. Meunier, Dulou and Vinet 1, 2; Meunier and Raoul) the polyene donates unshared electrons to the adsorbent which had possessed incomplete electronic octets. In this manner the compound undergoes polarization and forms a strongly resonating structure. Washing the column with alcohol or acetone causes bleaching and elution.

The colour obtained on adsorbents that have been commercially pretreated with acids can well be compared with some colour reactions of dissolved carotenoids (with sulphuric acid or antimony trichloride). These and a number of connected phenomena have been recently discussed by Meunier and Vinet (3) in their stimulating new book. For example, for vitamin A ion they postulate the following limiting resonance formulas in the adsorbed state (some other ionic structures with free-radical character can also be formulated).



The practical chromatographer should be aware of the fact that such polarizing adsorptions are in many instances irreversible in the sense that upon elution with a polar solvent a

chemically changed substance is recovered; for example, anhydro-vitamin A instead of the vitamin. This parallels to some extent the earlier observation that β-carotene when regenerated from its iodide (Karrer and Schwab 1) or from its Carr-Price reaction product (Gillam, Heilbron, Morton and Drummond) appears in the form of a dehydro-carotene (cf. Meunier and Vinet 3). Furthermore, the colourless polyene, phytofluene (probably $C_{40}H_{64}$), upon elution from its azure blue filtrol-adsorbate, does not show its typical extinction curve any more (author and Sandoval 1). Starting from vitamin D_2, Thibaudet obtained tachysterol by adsorption on an acid earth and elution with a polar solvent; in this case a rearrangement of the double bond system took place.

For further chemical changes in the Tswett column the following examples may suffice.

The formation of small quantities of a polyene pigment when vitamin A solutions are chromatographed on alumina (Holmes and Corbet) was found by Meunier and Vinet (2) to be a dehydration process which gives rise to a symmetrical ether oxide. According to Holmes and Corbet ionone also develops a colouration on activated alumina.

The hydrolysis of triglycerides on alumina is discussed on p. 112.

In the field of the heterocyclics it was observed by Brockmann and Junge (1–3, p. 99) that alcohol was split off from an anthocyanidine-like pigment during chromatography on alumina—a reaction which also took place when the solution was refluxed. According to H. Fischer and Conrad, when rhodin g_1-trimethylester was developed with methanol on a talc column, an acetal was formed because of the acidity of the adsorbent. This, however, did not occur if the talc was neutral. While neo-rhodinporphyrin g_3 is stable on Brockmann's alumina (standardized, Merck), on other alumina samples an addition of water to the vinyl group occurred. A similar addition of methanol seems to be responsible for a marked shift of the spectral bands of vinyl-porphyrine a_5 towards shorter wave-lengths upon development with methyl alcohol on alumina columns.

It should be stressed that not all conversions which occur on the Tswett column are necessarily undesirable. Sometimes they can be made use of for preparative purposes. For example, a straightforward method for the regeneration of terpenes, steroids, etc., from their picrates and especially their trinitrobenzolates consists of filtration through an alumina column. While trinitrobenzene remains adsorbed, the other component passes through (Plattner and Pfau). The conversion of *p*-anthraquinone sulphonates of some curare alkaloids into the corresponding hydrochlorides can be carried out in a similar manner, provided that the alumina has been pre-treated with HCl. The hydrochloride can then be crystallized from the filtrate while anthraquinone sulphonic acid remains adsorbed near the top (Wieland and Pistor). Using " basic " alumina the free alkaloid can be obtained (Wieland, Pistor and Bähr).

As an example of an analogous cleavage of a chromoproteid, the observation of Weygand and Birkofer may be mentioned that the prosthetic component of the " old " yellow enzyme is retained on a frankonite column while the protein reaches the filtrate.

While in the examples just given it was possible to define the conversions that take place on the column, in others no such formulation is yet possible. For example, chlorophyll suffers irreversible changes on certain adsorbents but not on others (p. 60). Vitamin K_1 loses its bio-activity on alumina or magnesia, while it is stable on sugar (Dam and Lewis ; p. 242).

In another group of phenomena chemical alterations during chromatography are not caused by an interaction of the solute with the column material itself but by its sensitivity in the adsorbed state. Thus, some unsaturated fatty acids or amino acids undergo autoxidation on charcoal or alumina. Such results of heavy metal catalysis may be prevented by pretreating the adsorbent with KCN or HCN (Tiselius 7 ; Turba, Richter and Kuchar ; Schramm and Primosigh 1, etc.). In one

instance a preextraction of floridin with $SnCl_2$ and HCl prevented subsequent autoxidation of tocopherol (Glavind, Kjölhede and Prange).

Some processes in adsorbates may be promoted by light. Thus, Levy and Campbell observed when chromatographing 2 : 3-benzanthracene that a zone turned orange on the side facing the window due to a quinoid compound formed by photo-oxidation. For photo-stereoisomerization, cf. p. 35.

Finally, mention should be made of Tswett's following prediction : " For special purposes . . . one will deliberately use chemically effective adsorbents (hydrolyzers, reducers, oxidizers)."

Exchange Adsorption

As is well known, the practical difference in the function of an adsorbent proper and an exchange adsorbent is that with the latter the process necessarily involves the release of some constituent from the solid surface to the liquid phase. Mostly, this will be an ionic exchange, the importance of which is increasing in the field of chromatography. Evidently such processes which take place in an exchanger column have much in common with " ordinary " chromatographic operations. For example, those ions for which the " exchange affinity " is strongest are retained near the top while others penetrate lower sections of the column, where their fixation by exchange takes place. The displacement principle is also valid for exchangers; and the final regeneration of the exchanger finds its parallel in the release by (fractional) elution.

For pertinent discussions in this field see the studies by Walton ; Applezweig (3) ; Cannan ; T. I. Taylor and Urey (2) ; Bray ; Le Rosen (3). A theoretical analysis for a system in which the distribution coefficient of the solute between the ion exchange column and the liquid remains constant throughout the column was given by Mayer and Tompkins. The behaviour of deep adsorbent beds under non-equilibrium conditions was investigated by Boyd, Myers, Jr., and Adamson who made use of radioactive tracer techniques in the continuous recording of concentration changes in the effluent. In

some examples, investigated by Austerweil, the ionic exchange included an initial adsorption followed by a chemical reaction.

Some theoretical results will be mentioned in Chapter XXIII (Inorganic Chromatography), p. 273.

The use of mineral precipitates which still adsorb some excess ions of the lattices has been demonstrated by Hamoir (1, 2) with amino acid mixtures (p. 151). Silver sulphide adsorbs silver ions irreversibly. The latter will then bind such anions as are able to form sparingly soluble silver salts in a manner analogous to " acid " alumina columns. With some amino acids the good agreement shown between the adsorbability and insolubility of the corresponding silver salt should allow some predictions concerning relative adsorbabilities. A related observation was made by Karlovitz according to whom indicators like amino- or dimethylamino-azobenzene, methyl red, alizarine and crystal violet are adsorbed from benzene on effloresced crystal surfaces with their " acid " colour, since some activated spots cause a change on the solid surface similar to that of hydrogen ions in solution.

Exchange Adsorptions on Synthetic Resins. A great stimulus to exchange chromatography has been given by the expanding industrial application of synthetic resins. These insoluble, high molecular materials, e.g., formaldehyde-polyphenolic resins, as was discovered by Adams and Holmes in 1935, have selective adsorptive properties, especially as ion exchangers. At present the most widely used materials seem to be the Amberlites (Resinous Products and Chemical Co., Philadelphia, Pa.) and the Wofatits (I. G. Farben). From the extensive literature we note only that general surveys were given by R. J. Myers ; Th. Wieland ; and Applezweig (3). For a diaminobenzene-formaldehyde resin cf. Smit ; adsorption of K-salts of organic acids : Bhatnagar, Kapur and Bhatnagar ; two-step exchange processes : Tiger and Sussman. The behaviour of certain amino acids on exchangers was studied by Englis and Fiess, as well as by Cleaver, Hardy and Cassidy.

The following features have been treated : influence of type, particle size and quantity of the resin ; pH effect ; concentration effect ; and the flow rate, cf. also Amino Acids, p. 139.

If an insoluble, macromolecular resin with acid character is designated as H*R*, it will form salts with the common bases, e.g., Na*R*. It is a true cation-exchange resin since reactions like the following may easily take place under suitable conditions :

$$\text{Na}R + \text{Cation}^+ \longrightarrow \text{Cation}R + \text{Na}^+$$
$$\text{or } \text{H}R + \text{Cation}^+ \longrightarrow \text{Cation}R + \text{H}^+.$$

Most acid-binding resins seem, however, to take over whole acid molecules from solutions. The adsorbed acids can then be replaced by dissolved molecules of another acid :

$$X + \text{Acid} \longrightarrow X\text{Acid}$$
$$X\text{Acid} + \text{Acid}' \longrightarrow X\text{Acid}' + \text{Acid}.$$

Sometimes two successive exchange operations of a different kind can be carried out. For example, Platt and Glock (1, 2) purified some muscle extracts first on a carbon containing zeolite (strong cation exchanger) and then by means of a naphthylene-diamine resin (anion exchanger).

The process of exchange adsorptions is much influenced by the pH of the solution, by dissociation constants and other factors. If an exchange resin is employed in column form, according to Myers, Eastes and Urquhart, such processes can be theoretically treated as a special case of ordinary chromatography in which a " zone " of exchanged or adsorbed cation (or anion) migrates downward the column. The point during the filtration at which the exchanging ion first appears in the filtrate designates the " break-through capacity."

For the purpose of chromatography a commercial exchange resin must be " conditioned " by taking it repeatedly through a cycle of reactions whereby some impurities are eliminated. This can be carried out in different ways. We give below the procedure as described recently by Cleaver, Hardy and Cassidy.

The cation-exchanger Amberlite IR-100 was treated in a vertical tube : **1**, with water until the filtrate was colourless ; **2**, with 5 per cent HCl until strong acidity was reached in the effluent liquid ; **3**, with

water until near neutrality to litmus and negative Ag-reaction were attained ; 4, with 5 per cent sodium carbonate or ammonia until the effluent liquid became strongly basic ; and 5, with water to neutrality. This cycle (beginning with the acid treatment) was repeated one to three times, until no coloured substances were noticed during a whole cycle. The operations can be stopped with an exchangeable cation, viz. H^+ or NH_4^+ or Na^+. The resin is then dried on filter paper or in an oven.

The acid-binding Amberlite IR - 4 was conditioned in a similar manner (water, 5 per cent HCl, water, 5 per cent sodium carbonate or 2 per cent NaOH, water). After five or six cycles the resin was neutral, colourless and free from formaldehyde odour. It was left activated either by sodium carbonate or hydroxide.

The physico-chemical properties of the cation exchange resin, *Dowex* 50, an aromatic hydrocarbonsulphonic acid polymer, were thoroughly described by Bauman and Eichhorn. This material is advantageous for theoretical studies since it shows high chemical resistance, and, furthermore, the sulphonic acid residue is the only exchange-active group at any pH.

It was found that at high dilutions the mass action reaction rate between the ions at the surface is decisive (product of the activity of one ion in the solid phase and of another in the liquid phase).

A semi-micro exchange column has been described by Applezweig.

CHAPTER II

METHODS

Adsorbents

In the extensive collection of abstracts by Deitz (1) many data can be found about adsorbents. A number of commercial materials as used in Germany have been discussed by Kainer. Monographs on activated alumina have been published by Krczil and by the Aluminium Ore Co., and on alumina " wool " (fibrous alumina) by Wislicenus. For surface areas cf. Deitz (2).

A satisfactory development in the handling of the adsorbent problem is that at the present time stress is not being laid on the increase in number of such materials but rather on a deeper study of the differences in their properties, especially when these are affected by planned pretreatment. This includes activation or de-activation processes that do not involve other essential changes in selectivity. Activation can be frequently achieved by heating, sometimes to red heat; de-activation may be caused by washing the column with some solvent. That methods of a very different kind are, in principle, also available is illustrated by the observation of Hedvall and Cohn that irradiation of cadmium sulphate with white light causes a decrease of its adsorbing power for some organic dyes like phenolphthalein.

Repeated treatment of the heated adsorbent with nitrogen and degassing in high vacuum may in rare cases be required for the elimination of oxygen. That some oxidations during a chromatographic experiment can be prevented in a convenient manner was mentioned above (p. 19).

Alumina is undoubtedly the most thoroughly investigated and most widely used adsorbent. It has been extensively employed in the study of colourless substances. Its activity depends on the dispersion, on the surface, on the water content and on the nature of the impurities, especially those which are markedly basic or acidic. Activated alumina can be prepared, e.g., by heating alumina hydrate (Brit. Aluminium Co.)

to 360° for 5 hours (Baddiley, Kenner, Lythgoe and Todd) or by intense heating of alumina itself. According to Jacobs and Tompkins (1), the adsorption capacity of alumina (for cupric ions) shows a distinct optimum after heating at 400–500°.

For the preparation of a series of alumina samples with well defined but different adsorptive powers a procedure proposed by Müller (1, 2) proved to be useful in the author's laboratory. Alumina (" Alorco ") is first brought to its maximum possible activity by strong heating. After cooling, a certain volume of water is added from a pipette : this is then evenly distributed by 1–2 hours mechanical shaking. Before use the material is kept standing for several hours in a closed bottle with occasional shaking. The addition of as little as 0·5 to 1 per cent water causes a marked decrease in activity ; this dependence becomes, however, much less sharp in the region of several up to 20 per cent water. The final products should not come into contact with air, since they have a tendency to weaken by attracting moisture. Well defined samples can also be obtained by thorough mixing of strongly active and partially de-activated alumina samples on the shaker.

It now seems that the alkaline reaction of alumina can have various causes. In some of the purest samples made in Germany (Kahlbaum or Merck), sodium carbonate and bicarbonate were responsible for it, while practically no sodium aluminate was found (Siewert and Jungnickel, cf. Venturello ; Kubli). Alkali-free alumina can be prepared from Brockmann's alumina or from a material obtained by heating the hydroxide at 400° for 2 hours, by washing several times with dist. water and methanol, drying in vacuo and re-activating at 200–210° (Euw, Lardon and Reichstein ; Christensen 2). The alkalinity of alumina can also be reduced by washing with phenol in light petroleum (Powell, Salmon, Bembry and Todd).

For the purpose of exchange adsorption of amino acids, etc., distinctly " basic " or " acid " alumina columns are required. The former are prepared, for example, by washing the adsorbent with CO_2-free water but the latter by a treatment with *N*-HCl and washing until a weakly acid reaction toward litmus

is reached (Th. Wieland). The washing of alumina with buffer solutions as a pre-treatment will be considered in the chapter Amino Acids, p. 139.

Acid or basic character can also be given to a column by building it up from an inert carrier on which either an acid or base is adsorbed or precipitated before the chromatographic experiment proper. This method was recently suggested by Catch, Cook and Heilbron, who precipitated alkali earth carbonates on the surface of silica gel. A suitable ratio of carrier to precipitate is, e.g., 40 : 1. Such a column was used for the purification of penicillin, i.e., for the resolution of a mixture whose individual components differed in the strength of their acidity. While a very great number of adsorptions and elutions is an essential postulate for the success of a separation on an ordinary Tswett column, in the adsorbent system mentioned " differences in acid strength are magnified by repeated neutralizations and acidifications." There is, of course, no sharp border-line between the above technique and the ordinary use of adsorbents, many of which have a clearly basic character. The method of Catch, Cook and Heilbron has some features in common also with Flood's procedure of loading paper strips with adsorbing precipitates (p. 279).

For the separation of alkaloids silica gel loaded with sodium hydrogen sulphate has been recommended.

Standardization of Adsorbents

One of the main factors at present preventing the more rapid progress of chromatography is the lack of a large number of reliably standardized commercial adsorbents of very different composition and grade. In this respect industrial production lags far behind the requirements of the chromatographer, and as a result it is sometimes impossible to reproduce experiments of reliable authors. Indeed, different batches of the " same " material may vary several fold in their adsorptive power. While such deviations may be caused occasionally by unsatisfactory milling or sifting, and may be eliminated by a mechanical treatment, generally it is the activity of the surface itself that must be dealt with. Evidently the adsorptive

power of each material should, if possible, be expressed by some characteristic figure obtained experimentally. In principle, several ways are open for this purpose. In what follows there are described some methods of standardization that make use of the behaviour of the adsorbent toward certain dyes or of the relative rates of migration of particular compounds. Another method, the equipment for which will not be available in some chemical laboratories, involves a calorimetric procedure.

Standardization of Alumina on the Basis of the Behaviour toward some Dyes. The best available information can be obtained from a paper published by Brockmann and Schodder. They designated the activity of strongest adsorbing alumina as "I," and that of the weakest as "V." The different aluminas are characterized by their behaviour towards binary mixtures of the following azo dyes: azobenzene, *p*-methoxyazobenzene, Sudan yellow, Sudan red, *p*-aminoazobenzene, and *p*-hydroxyazobenzene. In the following scheme the horizontal lines designate the top and bottom of the column. If the name of a dye is printed between these lines, it forms a zone in the approximate position indicated. A dye under both lines has passed into the filtrate (Table 2). The gradations in the activity are selected so that if a certain alumina retains both components of a dye mixture on the column, the next weaker grade adsorbent will not retain the less strongly adsorbed dye.

TABLE 2

STANDARDIZATION OF ALUMINA BY USING BINARY MIXTURES OF SOME AZO DYES (BROCKMANN AND SCHODDER; b. = BENZENE)

Activity: I	II	III	IV	V
Methoxy-azob.	Sudan yellow	Sudan red	Sudan red / Amino-azob.	Hydroxy-azob.
Azob.	Methoxy-azob. / Methoxy-azob.	Sudan yellow / Sudan yellow	Sudan red	Amino-azob.
	Azob.	Methoxy-azob.	Sudan yellow	

In order to test a given alumina sample, it is filled into a 10 × 1·5 cm. tube (widths in the clear) to a height of 5 cm., and a filter paper disc is placed on the top. The solution of

4 mg. of the binary pigment mixture in 2 ml. of purest benzene (distilled over KOH) and 8 ml. of light petroleum is then introduced and the chromatogram is developed with 20 ml. of benzene + light petroleum 4:1 (rate of flow, 20–30 drops per min.).

In the Special Section Roman numerals are sometimes used in the above sense to designate the quality of the alumina.

Preparation of Standardized Alumina Samples from " Aluminium hydroxide, Merck " (the latter passes through a sieve with 0·12 mm. mesh distance while half of it is retained if the mesh is 0·06 mm.) :

Activity I. Small portions of the aluminium hydroxide are heated red hot while stirring in an iron crucible and cooled in a desiccator. Test : *p*-methoxyazobenzene must be adsorbed as a sharp, 4–5 mm. zone near the top, separated from the azobenzene zone (2–3 cm.) by a 3–4 mm. nearly colourless interzone. (The sample is too weak if the azobenzene reaches the bottom of the column on development as indicated above.)

Activity II. The bottom of a larger pot which contains a 3-cm. layer of aluminium hydroxide is heated by a large burner for 4–6 hours. After cooling, the material is spread out in air in a thin layer and its activity is tested after ½ hour. Azobenzene must be washed into the filtrate while methoxyazobenzene remains 1 cm. below the top.

Activities III, IV, and V. These grades are prepared by shaking II in moist air (cellar) for such lengths of time as are determined by testing the material from time to time. Activity III is reached when Sudan red is adsorbed in a 1-cm. zone near the top while Sudan yellow forms a well separated, somewhat broader zone under it. Activity IV : Sudan yellow in the filtrate, Sudan red 1–2 cm. from the top. Activity V : *p*-hydroxyazobenzene near the top while *p*-aminoazobenzene forms a well separated 2-cm. zone below it.

Standardization Based on Relative Rates of Flow. Such a method was developed by LeRosen (1, 2) who made use of the following terms. S = length of column containing 1 unit volume of solvent/length of unfilled tube required to contain the same volume of solvent. S also gives the percentage of the tube volume which is occupied by the adsorbent, viz., $\% = 100\ (S - 1)/S$. V_c = rate of flow (mm./min.) of developer when a constant flow has been reached. R = rate of movement of a zone (mm./min.)/rate of flow of developer (V_c) ; usually R refers to the bottom edge of a zone. The approximate strength of the adsorption affinity can be characterized by

determining R. (R is equivalent to R_F of Martin and Synge; cf. "Partition chromatography.") T_{50} = seconds required for a solvent to penetrate 50 mm. into a 75 × 9 mm. column under vacuum of a water pump. Satisfactory values in many cases were found to be: V_c = 10 to 50 mm./min.; T_{50} = 20 to 100 sec.; R = 0·10 to 0·30.

For the determinations the tubes were filled under vacuum. Whether or not the top is pressed may cause considerable divergence in the results. Generally vigorous tapping on the walls of the tubes is adequate. In order to determine the number of mm. of column length equivalent to 1 ml. of solution, three successive 5 ml.-portions were pipetted on to the column; each was introduced as the preceding portion disappeared. Simultaneously, the bottom edge of the solvent was noted. From a burette attached to the top of the tube by a stopper provided with an outlet which could be closed when the air space was filled with liquid from the burette, the velocity of solvent (V_c) was determined from the flow (ml./min.) as shown by the burette; the estimation of R was carried out in a similar manner. For the standardization of calcium hydroxide, carotenoids were used which can be conveniently introduced in petroleum + benzene solution. They form a concentrated zone and are then developed with pure benzene.

LeRosen (3) also showed on the basis of flow experiments, using benzene or light petroleum on silicic acid and hydrated lime, that if a standard packing is postulated, the rate of flow can be calculated for different values of the pressure, solvent viscosity and length of the column.

A successful application of his method to different silicic acid-celite mixtures was recently made by LeRosen (2) who used *p*-nitroaniline in benzene.

In the system, *o*-nitroaniline, benzene, silicic acid, the same author (4) studied the movements (relative to that of the solvent) of the leading and trailing zone boundaries respectively. Both rates were practically independent of the position on the column; the movement of the trailing edge (which is difficult to follow) also seems to be independent of the initial

concentration or volume. In contrast, the front boundary was found to vary with the initial volume and still more with the initial concentration.

Weil-Malherbe suggests the standardization of adsorbents on the basis of the "threshold volumes" per unit weight of the adsorbent, k. The threshold volume is defined as the volume which had passed through a column before the substance appears in the filtrate. If s = quantity of adsorbent and a = the exponential coefficient of the Freundlich adsorption isotherm, then for different batches of the same adsorbent $V_t = k.s^{1/a}$ and $k = V_t/s^{1/a}$. For two different adsorbents $k_1/k_2 = (s_2/s_1)^{1/a}$ or $s_2 = s_1\ (k_1/k_2)^{\alpha}$ and where s_1 and s_2 are the respective quantities of the adsorbent.

For connected problems in inorganic chromatography (p. 273) cf. Jacobs and Tompkins (1–3).

Calorimetric Standardization of Alumina (Müller 1–3). As a measure of activity of a given adsorbent, heat production during the adsorption process can be used. For alumina different grades were prepared by the gradual addition of moisture to a sample which had been brought to a maximum activity by heating (cf. p. 25). Each sample of the adsorbent was brought into contact with comparatively small volumes of the solvents and the heat production was measured in a small, well isolated calorimeter. The results are only valid with a well defined solvent of a known degree of purity. In most instances the value $Q' = \triangle T.\ W_k$(cal.) was determined (W_k= heat capacity of the calorimeter). That the increase of the water content causes a decrease in the adsorptive power is shown by a lowered heat production. Examples :

g. water per 100 g. alumina :	0	0·5	1	2	4	16	24
$\triangle T.W_k$ (cal.) :		19·3	16·2	14·6	12·3	9·2	1·9	0·55

The influence of the purity of light petroleum can be demonstrated as follows. Under the applied conditions a system with a heat production of 10·0 cal. was needed for the optimum chromatographic resolution of crude β-carotene, if the petroleum had been treated with oleum, but 12·0 cal. if only sulphuric acid was used for this purification.

Solvents

In general no purification of the usual organic solvents is necessary for chromatographic purposes. However, if ultraviolet spectroscopy of the eluted zones or of the filtrate is intended, it may be advisable to eliminate disturbing contaminants of the column material by an appropriate washing prior to the introduction of the substance. A number of hydrocarbon solvents can be highly purified by filtration through activated silica gel (cf. e.g., Graff, O'Connor and Skau). When passed through an alumina column, ether loses its peroxide, aldehyde and acid contents (Fichler).

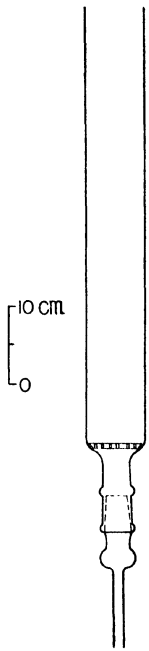


Fig. 1.—Large-size chromatographic tube.

Apparatus

The chromatographic tubes suggested by Cholnoky and the author and manufactured by the Scientific Glass Apparatus Co., Bloomfield, N.J., have been found satisfactory for many purposes. A modification of these tubes, devised by A. Sandoval in the author's laboratory, allows the use of a relatively small ground joint in spite of great capacity of the cylindrical part (Fig. 1). The clearance of the joint is sufficient for the introduction of a wooden rod and extrusion of the column plus the porcelain filter plate, which is wrapped in cloth. In favourable circumstances the column can be pushed out by gentle blowing. Using such tubes of about 30–60 cm. length, it may be advantageous to press out the column stepwise and to cut off the respective extruded sections while the rest is still in the tube. In order to prevent extruded portions from cracking because of their weight, it is recommended that a glass plate, as thick as the wall of the tube, be placed directly in front of the tube on the table.

A demountable chromatographic "tube" was constructed by Békésy (Fig. 2). It is composed of two parallel glass plates, 5 mm. thick, with wedge-shaped cork strips between, on which a veneer strip is glued. The whole device must be held together by screws. A stopcock may be attached at a convenient place for testing solution samples during the experiment. On a micro scale use is made of a carved glass plate and a glass slide which have been pasted together with a 4 per cent agar solution.

In order to carry out brushing or spotting tests during the development, Crowell and König recommend a lucite tube ($24 \times 1{\cdot}5$ cm.) in which along the entire length two symmetrical cuts converging at an angle of 90° were made and thus produced an opening 5 mm. and 13 mm. wide on the inside and outside circumferences respectively. The joints of the two parts of the tube were made tight with scotch tape and were held together by metal lids. The use of cellophane tubes was advocated by Ochiai and Takeuti. For a microtube cf. Lecoq.

The extrusion of columns by means of a compressed gas is recommended by Turkevich.

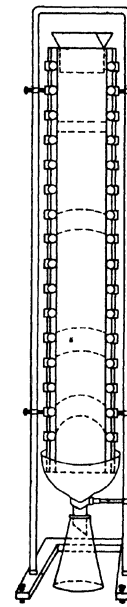


FIG. 2
Demountable chromatographic "tube" (Békésy).

Permanent replicas of pigment chromatograms can be made by using "Crayola" crayons of the desired colour which have been absorbed from benzene solution on talc (Stimmel).

LIQUID CHROMATOGRAMS

The application of this technique, which consists of fractional washing of individual compounds into the chromatographic filtrate, is being extended rapidly. Fractionation can thus be obtained even where the compounds do not form geometrically separated zones but are eluted fractionally from a system that can roughly be described as a mixed adsorbate. If several

developers with gradually increased eluting power are applied, a relatively small column is sufficient, although the solvent requirement might be considerable.

Especially in the field of terpenes and steroids, including sex hormones, many such separations were carried out with success, among others, in Reichstein's and Ruzicka's laboratories. A few examples will be given later (p. 166).

In most cases no specific equipment will be needed for collecting the fractions. However, for special purposes, automatic devices are welcome. A reliably operating fraction collector was described by S. Moore and Stein (p. 140).

Drake recommends the following arrangement for recording spot tests. At regular intervals a drop of the flow is deposited on filter paper which covers a rotating drum (12 × 7·5 cm.); the paper may be impregnated with some reagent beforehand or it can be sprayed at the end of the experiment.

In some investigations of the exchange behaviour of inorganic salts Glueckauf (5) determined concentrations by means of a recording micro-ammeter. (The arrangement of the circuit is given in the original.)

Further Variations of the Procedure

Semicircular chromatograms were prepared by Brown, who introduced the solution through the hole of one of two glass plates between which a sheet of blotting paper had been placed. This method can be demonstrated with CS_2 extracts of green leaves. A semicircular chromatogram is also obtained when the adsorbent is placed in a tilted Petri dish and the solution is dropped into the centre of the edge (Crowe). Similarly, for a preliminary test of galenicals, etc., Izmailov and Shraiber recommend putting a drop of the solution on a thin layer of the adsorbent, on an object glass, and developing the circular chromatogram with a few drops of a solvent. An

attempt has been made to carry out separations on a thin strip of adsorbent (8 cm. long) which was placed on a slightly inclined aluminium sheet (Lapp and Erali). Lowman (1) recommends a chromatographic analysis " in reverse," i.e., he drops portions of the adsorbent into the solution ; the pile shows an inverted sequence of the zones.

It seems probable that a small scale chromatogram or a micro-column will generally give more information than the techniques just mentioned.

Craig, Columbic, Mighton and Titus showed that their " Countercurrent Distribution " method can well be extended by the replacement of one of the liquid phases by a suitably chosen solid adsorbent.

Fractionation by adsorption on foam : Schütz ; Bader and Schütz ; Shedlovsky.

Location of Invisible Zones. In exceptional instances this may be made possible by simple inspection of the column material. For example, where silicic acid is moistened with some pure solvent, it becomes more or less translucent. Trappe detected the presence of some colourless zones by decreased translucence.

The two prevalent procedures at the present time are, the brush method and the observation of the column (and the chromatographic filtrate) during development with the help of a portable ultra-violet lamp. Some examples of both techniques are given in the Special Section.

For observations of fluorescence glass tubes of normal thickness will allow a correct location of the zones, and the use of expensive quartz equipment is thus unnecessary. In order to avoid darkening of the laboratory, the experimenter may use a black cloth.

The moistening of pigment adsorbates with solvents has a definite effect on the intensity of fluorescence, as was reported

by Bandow (1, 2); in certain instances the presence of 0·2 g. of benzene per gram alumina was found to be the optimum ratio.

It should be mentioned that a prolonged inspection of the column in ultraviolet light may cause some steric changes Thus, if a developed chromatogram of diphenyl-octatetraene and some of its stereoisomers was illuminated for a minute, an isomer (located directly below the all-*trans* compound) showed on further development a clear division into two fluorescent sections, of which the upper one was newly formed all-*trans*-diphenyloctatetraene. This resolution was restricted to the cylindrical surface while the inside of the column contained the original zoning (author and Le Rosen 2). Such disturbances can be reduced to a minimum if the chromatographic tube is wrapped in black paper which leaves a narrow vertical strip open for observation (author and Sandoval 3).

A promising new technique is the use of *fluorescent columns* which was recommended, independently, by Brockmann and Volpers as well as by Sease. On these adsorbents such colourless substances as are able to quench locally the fluorescence of the column material appear as dark zones ("negative chromatogram"). While in Brockmann and Volpers' experiments the columns were prepared by impregnating them with some fluorescent organic compound (alumina + morin; silicic acid + berberin), Sease mixes his adsorbents with solid inorganic fluorescent substances. His method will generally be preferable, since it excludes contamination by the added organic material. Using silicic acid and zinc Sulfide (Patterson Screen Division of E. I. Du Pont de Nemours and Co., No. 62) about 2·5 per cent of the latter was needed. Strongly fluorescent zinc silicates may also be used.

Examples for binary mixtures which can be easily separated on 9—15 × 1·8 cm. silica + ZnS columns, using light petroleum as a developer: cinnamaldehyde (top; 4·9 mg.) and salicylaldehyde (2·6 mg.); salicylaldehyde (2·6 mg.) and nitrobenzene (6 mg.); xanthone (3·6 mg.) and *p*-nitrobenzyl bromide (10·9 mg.); nitrobenzene (6 mg.) and iodoform (4·6 mg.).

In exceptional instances some compounds quench the slight fluorescence of commercial alumina itself. (Example, p. 15.)

A rather generally applicable new method, which is based on a visual observation of the changes in the *refractive index* in the flow, was recently devised by Claesson (5). A rectangular chromatographic tube is used, which can be built by clamping against a U-shaped metal plate two glass windows, one of which is thick and has polished edges. The colourless chromatogram should be observed from 1 m. distance, at the proper angle, preferably in monochromatic light. Then the boundary of the total reflection area becomes visible and its position will depend on the refractive index of the solution that wets the glass wall. In a well differentiated chromatogram a notched line will appear. (Similar observations can also be made on paper strips by placing the glass on the strip.)

The possibility of locating zones by measuring *dielectric constants* was demonstrated by Troitskii, who used sound changes as heard in telephone equipment.

In a *radiometric* adsorption analysis a solution containing yttrium earths was irradiated with neutrons and investigated by measuring the radioactivity along the column (Lindner and Peter). A microanalysis of radioactive derivatives of amino acid mixtures on filter paper strips was described by Keston, Udenfriend and Levy.

The mixture was treated with iodophenyl-sulphonyl chloride containing ^{131}I. The purified mixture (1 to 7 μg., in ammoniacal alcohol) was then placed as a transverse line on a 57 × 2 cm. Whatman No. 1 strip and developed with *n*-pentanol which had been saturated with ammonia. The counts were taken using successive 5-mm. sections.

Likewise, ^{131}I was used by Fink, Dent and Fink who investigated a neutral hydrolyzate from the thyroids of rats that had been given radio-iodine. After a phenol-collidine development

a radio-autograph was taken with the two-dimensional paper-chromatogram (cf. p. 42).

A Combination of Chromatography and Electrophoresis for water soluble substances was suggested by Strain (10) (Fig. 3) who found that a better resolution can sometimes be obtained with this combination than by simple chromatography. For 2 to 15 mg. quantities and using 23 × 3 to 13 × 2 cm. columns (soaked with water) the appropriate potentials were 175–200 volts (0·5 to 2 m.a.). Hyflo Super Cel alone or mixed with talcum, or in some instances cotton, can be used as adsorbent. Because of electro-osmotic effects, it is advisable to place the cathode at the top. Under these conditions, the first mentioned of each of the following groups was the most strongly adsorbed : amino-azobenzene and indigo carmine ; 3-nitro-4-aminoanisole and indigo carmine ; methyl orange and 2:6-dichlorophenol-indophenol ; methyl orange and methyl red ; indigo carmine, picric acid, and methyl orange. (In weak acid solutions, both forms of the indicators were observed.) Cf. also Lecoq (3) ; p. 273.

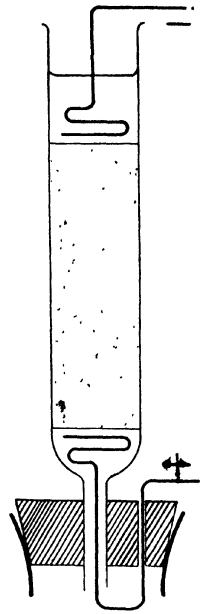


FIG. 3. — Apparatus for chromatography and electrophoresis (Strain).

Spectra of Chromatographic Zones. A simple and practical method for the detailed spectroscopic characterization of coloured zones in reflected light has not yet been given. An approximate characterization is possible, according to Schwab and Issidoridis, by a consecutive use of the different light filters of the Pulfrich photometer. For this purpose the chromatogram is developed in a quadrangular cell which is pasted together from glass plates. A similar cell is filled with the pure adsorbent and both are placed in a cooling trough

which fits into the Pulfrich apparatus (Figs. 4–5). The two surfaces which are behind the circular windows are illuminated by a 150-watt lamp and the diffusely reflected light is led to the observer.

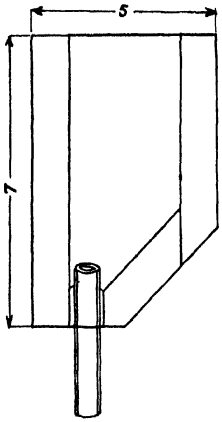


FIG. 4.—Cell for spectroscopic observation of zones (Schwab and Issidoridis).

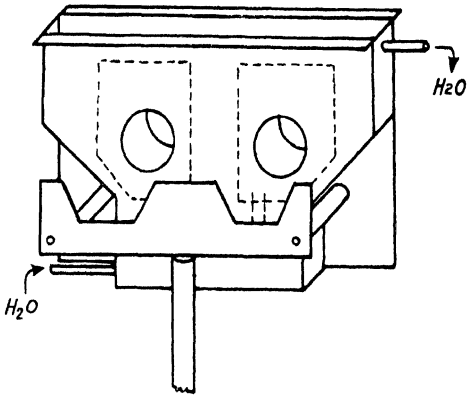


FIG. 5.—Cooling system for two cells (cf. Fig. 4).

PARTITION CHROMATOGRAPHY

While during the development of a typical Tswett chromatogram the locally changing distribution of the solutes between a solid and a liquid phase constitutes the essential feature of differentiation, the use of *two liquid phases* was recently introduced with success by Martin and Synge (1, 2). These investigators keep the column form but make use of the adsorbent, for example, silica gel, mainly as a support for one liquid phase (water) while the other liquid, immiscible with the first, travels down the column. In the resolution of mixtures the individual partition coefficients are the determining factors in this method. So far it has been applied mainly to the separation of amino acids (cf. the chapter, Amino Acids, p. 139); and then it is possible to add an indicator to the silica gel (during its preparation) and to gain all of the advantages of the presence of visible zones. Recent survey: Martin (2).

A precursor of this important method was an investigation by Martin and Synge (1). The original name, " liquid-liquid chromatography," has now been abandoned, because of the accepted use of the term " liquid chromatogram " in another sense ; and the name " partition chromatography " was introduced by Gordon, Martin and Synge at the suggestion of E. Lester Smith. A sharp differentiation in nomenclature between " partition chromatography " and " adsorption chromatography " does not seem to be advantageous because of possible further developments (cf. Wachtel and Cassidy). It is suggested that " partition chromatography " in its present form should be considered as a part of "chromatography " in general and that the latter term should include all similar operations.

Two fundamental conditions must be fulfilled in partition chromatography : (1) that the solid column material should be able to bind a substantial quantity of the non-mobile liquid phase, and (2) that it should not disturb the partition between the two liquids by acting as an ordinary adsorbent for the solute. These postulates are fulfilled, e.g., for amino acid separations in the silica gel-water or silica gel-butanol column. That a well selected solid phase acts only as a support and does not take part in the separation was claimed by Synge (1) on the basis that antipodes or racemates travel at identical rates in the asymmetrical starch or cellulose column. (This proof, however, is in the negative.)

The mobile phases studied by Gordon, Martin and Synge were chloroform-*n*-butanol, cyclohexane-*n*-propanol and ethyl acetate. An expression like " 1 per cent butanol-chloroform " means that 1 per cent butanol (v/v) was dissolved in the chloroform and the mixture was then saturated with water. The choice of the solvent may affect the sequence and the separability, e.g., of acetamino acids. For example, acetyl-phenylalanine precedes acetyl-leucine in the butanol-chloroform-water system, but these two compounds are inseparable in propanol-cyclohexane-water. The polarity of the solvent

seems to play an important part in the separation of different types of amino acids; however, no simple quantitative rule can be given on this matter at the present time.

The *theory* of partition chromatography was worked out by Martin and Synge (2). The relative rate of movement of a certain zone is defined as R = movement of position of maximum concentration of solute/simultaneous movement of surface of developer above the column. The connection between R and the partition coefficient a (g. solute per ml. of non-mobile phase/g. solute per ml. of mobile phase) is expressed by the terms,

$$R = \frac{A}{A_L + aA_S} = \frac{A_L + A_S + A_I}{A_L + aA_S}$$

from which the partition coefficient, $a = \frac{A}{RA_S} - \frac{A_L}{A_S}$

In these expressions A = the area of cross-section of the column; A_S, A_L and A_I = the respective areas of cross-section of the non-mobile phase, mobile phase and inert solid; and $A = A_S + A_L + A_I$.

Separations based on different partition coefficients of solutes are, therefore, to be expected in spite of the fact that a is not a constant but depends on the absolute concentration (Martin and Synge 2; E. Lester Smith). Furthermore, the interaction of the respective solutes alters the theoretically forecast picture. Nevertheless, in many instances calculated and determined partition coefficients are in good agreement.

Example: A mixture of 2 mg. of acetyl-*l*-proline hydrate and 2 mg. of acetyl-*dl*-phenylalanine was developed on a 20 × 1 cm. column with chloroform + 1 per cent (v/v) *n*-butyl alcohol until good separation occurred. The movement of the centre of each zone for a given drop of the liquid surface above the column was measured. Since the column contained 5 g. of dry silica gel (density, 2·3 g./ml.), 3·5 ml. of water

and 10 ml. of chloroform phase, $A_I = 0{\cdot}11$ cm.^{2}, $A_S = 0{\cdot}175$ cm.^{2}, and $A_L = 0{\cdot}5$ cm.^{2}. From the above equation the partition coefficient, $a = 9{\cdot}4$ and $1{\cdot}4$, for acetylproline and acetylphenylalanine respectively, in good agreement with direct estimations.

Materials : Variations in the properties of *silica gel* may make it difficult to obtain reproducible results by means of partition chromatography. This complication was recently discussed in detail by Gordon, Martin and Synge (7) whose two batches of water glass yielded gels with very different characteristics. With water glass " I " the presence of methyl orange during the precipitation was found to be necessary, lest a " tailing " gel be formed, and " aging " was also required. Neither of these conditions was necessary with batch " II." For the purpose of the separation of acetylated amino acids the " background " colour may offer some indication. This colour is flesh-pink in good silica specimens but yellow or orange-yellow in " tailing " ones.

The following *standard test* was suggested for the silica gel. A mixture of 3 mg. of acetylphenylalanine and 3 mg. of acetylleucine is chromatographed on 3 g. of the sample (diameter of column, 1 cm.) with 1–3 per cent butanol-chloroform as a developer. The sum of the titration values of the two fractions obtained should not differ by more than 2 per cent from the corresponding value of the starting material (*cf.* also Harris and Wick).

Preparation of starch for partition chromatography (Synge 1). The crude material was washed by decantation with 6 × 10 parts of water (ninhydrin reaction, with heating, became negative), dried in air, extracted with *n*-butanol (saturated with water ; 20 ml. per 5 g.) at room temperature for 24 hours, and dried at 37°. It was washed under gravity with one column length of wet butanol.

The use of *cellulose acetate* ($\frac{1}{4}$" staple filament, pretreated with butanol at 50°) as the stationary phase was described by Boscott.

Indicators : Gordon, Martin and Synge (7) pointed out that methyl orange has the disadvantage of leaching from the columns under the influence of solvent mixtures of higher alcohol content. This is so, e.g., in the separation of acetylated amino acids. An ideal indicator should be sensitive on 1–3

per cent butanol-chloroform columns but not leached in the presence of 17 per cent. It seems that this requirement can be closely met by a dye which is obtained by coupling diazotized amino-R acid with N-phenyl-α-naphthylamine (used as an ammonium salt) as suggested by Liddel and Rydon. Unfortunately, it gives satisfactory results on some silica gel specimens only. Some anthocyanins, e.g., peonin or pelargonidin chloride were used with advantage by Gordon, Martin and Synge (7).

Two-dimensional Chromatograms on Paper

Separation experiments on filter paper strips have their early precursors in those reported by Schoenbein. He stressed that the migration of compounds at different rates on the paper " may assist the analyst as a qualitative tool in such cases in which other reagents are invalid, for example, for the separation of dissolved organic dyes." The field of " capillary analysis " was developed later by Goppelsroeder and reported in his well-known monograph. Mention should be made of a remarkable, early contribution by E. Fischer and Schmidmer. A newer survey has been written by Rheinboldt. The development of zones on paper strips with pure solvent was also applied by Liesegang (1, 2).

A kind of a transition between the column and paper strip technique is recommended by Flood.

A new and important method is conveniently termed the *two-dimensional paper chromatography*, (p. 279).

Two-dimensional chromatograms may constitute a combination of Goppelsroeder capillary analysis with partition chromatography. The method was devised by Consden, Gordon and Martin (1, 2) and applied to the qualitative microanalysis of protein hydrolysates or other amino acid mixtures. In this technique the separation depends on the differences in partition coefficient between cellulose (which is saturated with water) and the mobile solvent. In order to obtain a two-dimensional chromatogram, for example, a wool

FIG. 7.—Two-dimensional chromatogram of a wool hydrolysate (Consden, Gordon and Martin).

To face page 43

hydrolysate was applied at the point "W" on Fig. 6 and developed first with collidine along the line AB and then with phenol in the direction AC. Fig. 7 is the reproduction of an original photograph published by Consden, Gordon and Martin.

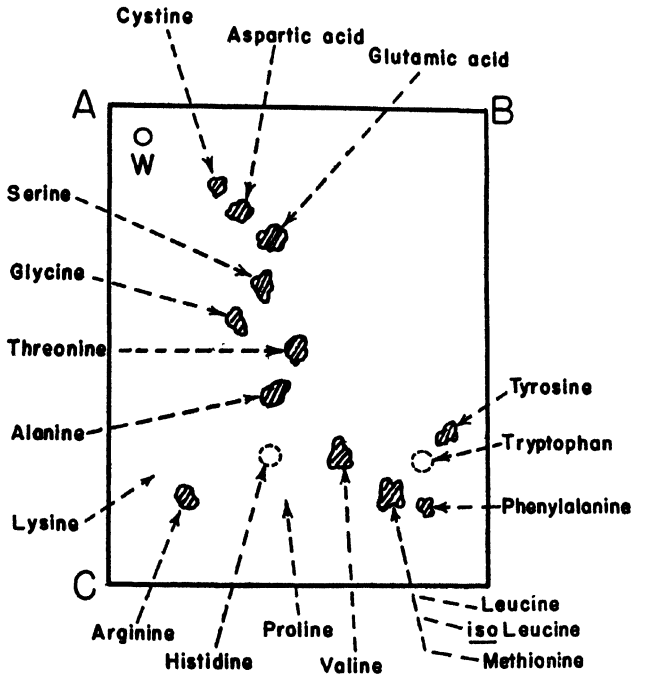


FIG. 6.—Two-dimensional chromatogram : schematic picture of a wool hydrolysate (Consden, Gordon and Martin).

The relation between the partition coefficient and the rate of movement is expressed by the term R_F which is essentially equivalent to R as used by Le Rosen (p. 28).

$$R_F = \frac{\text{movement of band}}{\text{movement of advancing front of liquid}} = \frac{RA_L}{A} = \frac{A_L}{A_L + \alpha A_S}$$

where A = cross-sectional area of paper + water + solvent · A_L = cross-sectional area of solvent phase ; A_S = cross-

sectional area of water phase ; and a = partition coefficient = conc. in water phase/conc. in solvent phase (cf. Martin and Synge).

The R_F values of a number of amino acids in different solvents at room temperature were given by Consden, Gordon and Martin. An extract follows in Table 3 in which the colour with ninhydrin is also indicated, viz., B = blue, G = grey, O = orange, P = purple, R = red, and Y = yellow.

TABLE 3

R_F VALUES ON WHATMAN NO. 1 FILTER PAPER

	Phenol (3 per cent NH_3 in the tray)	Collidine	Benzyl alcohol
Glycine	0·40 RP	0·25 P	0·02
Alanine	0·54 P	0·32 P	0·03
Norvaline	0·79 P	0·48 P	0·12
Valine	0·76 P	0·45 P	0·11
Norleucine	0·85 P	0·60 P	0·27
Isoleucine	0·81 P	0·54 P	0·18
Leucine	0·83 P	0·58 P	0·21
Phenylalanine	0·87 P	0·59 G	0·36
Tyrosine	0·63 B	0·64 G	0·14
Serine	0·33 P	0·28 G	0·01
Threonine	0·41 P	0·32 P	0·02
Hydroxyproline	0·50 O	0·34 OY	0·04
Proline	0·85 Y	0·35 Y	0·12
Tryptophane	—	0·62 P	—
Histidine	0·68 B	0·28 G	0·02
Arginine	0·89 P	0·16 P	0·01
Ornithine	0·73 P	0·13 BG	0·00
Lysine	0·82 P	0·14 BG	0·00
Aspartic acid	0·12 B	0·22 B	0·00
Glutamic acid	0·13 P	0·25 P	0·00
Lanthionine	0·19 G	0·12 G	0·00
Cystine	0·24 YG	0·14 G	0·00
Methionine	0·76 P	0·57 GP	0·17

Tyrosine, histidine, arginine, ornithine, lysine, lanthionine, and cystine were put on as the hydrochlorides and neutralized with ammonia before development.

The initial colour of ninhydrin and aspartic acid colour is green.

In order to decide which two solvents should be used in a given case, the R_F values in one solvent are plotted as ordinates

against the corresponding values in the other solvent as abscissæ. In this manner the expected approximate positions of the individual amino acids can be determined in a diagram.

Experience has shown that two amino acids may be resolved if the difference in R_F values is more than 10 per cent, e.g., leucine and isoleucine are not separable in benzyl alcohol. The difference in R_F values of strict duplicates does not exceed 4 per cent but runs carried out at long intervals show much greater variation.

Materials and equipment: Whatman No. 1 filter paper was used (standard sheet, 18 × 22·5 in.). The advancing front of liquid is yellowish-brown but this contaminant of the paper moves so rapidly that it does not usually interfere. The troughs for two-dimensional experiments are represented in Fig. 8. For the same experiments the chamber consists

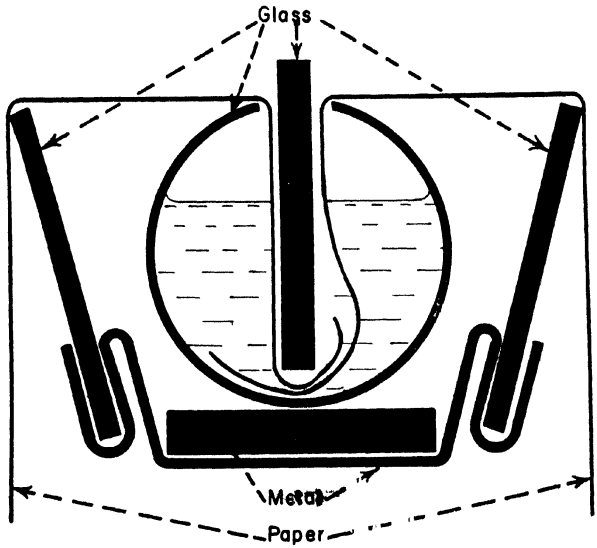


FIG. 8.—Trough for two-dimensional chromatography (Consden, Gordon and Martin).

of a glass-sided lead box, about 75 × 75 × 12·5 cm, made airtight with a lead cover. A completely airtight tin plate box which had a tray as a water seal was used when coal gas atmosphere was required.

The solution (6 to 12 μl.), corresponding to 200–400 μg. of protein hydrolysate, is placed near a corner of the paper, 6 cm. from either edge. The paper is held with one edge slightly overlapping the opening of the trough and pressed into it with a strip of sheet glass somewhat longer than the paper. Then both are transferred to the chamber which has been prepared as follows.

A removable lead tray, the bottom of which is covered with a two-phase layer of water and the solvent, is laid on the floor of the box, in order to secure a saturated atmosphere. The chromatogram is allowed to develop for 24 to 72 hours. The paper is then dried in a drying cupboard (through which hot air is sucked by a fan exhausting to the outside), turned through a right-angle and returned to the trough, in order to be developed by the second solvent. For overnight runs the chamber should be lagged. If phenol is used, the contaminant mentioned above may distort the fast-moving bands. To avoid this, the top 12·5 cm. of the strip or sheet is sprayed with phenol before the trough is filled. Finger marks should be avoided in all operations. After drying, the paper is sprayed with 0·1 per cent ninhydrin in *n*-butanol, and again dried and then heated at 80° for 5 min. The spots are outlined with pencil because of eventual fading. Protein hydrolysates or amino acid mixtures with a high content of inorganic salts do not yield satisfactory chromatograms. Excess HCl should be removed by repeated distillations in vacuo. Furthermore, it is essential to treat with ammonia vapour after the hydrolysate has been applied to the paper since some of the amino acids are insoluble in neutral media.

According to Phillips the fluorescence of amino acids and peptides can also be used for their location on the paper, in

which event no material is sacrificed for the ninhydrin reaction. However, the latter colour test appears to be somewhat more sensitive than the fluorescence test, which requires a minimum of 20 μg. substance per square inch.

Examples

"*Glycoleucin.*" The identity of Thudichum's glycoleucin with *dl*-leucine and not with norleucine can be proved by two-dimensional chromatography (Consden, Gordon, Martin, Rosenheim and Synge). A few mg. of each sample to be compared was stirred with a drop of water and suspensions of the copper salt were then treated with H_2S. Without filtering, 3–4 μl. of each solution was put on a strip of paper (Whatman No. 1) which was hung from a trough containing benzyl alcohol (saturated with water) in an atmosphere saturated with water, benzyl alcohol, and containing HCN. After 48 hours the paper was dried, sprayed with 0·1 per cent ninhydrin in *n*-butanol, dried and kept at 105° for 5 min.

Gramicidin S. The developer was phenol and collidine. Only *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine, and *d*-phenylalanine were observed. A one-dimensional paper chromatogram (butanol-benzyl alcohol) proved the absence of isoleucine in the leucine fraction (Synge 1, 2).

The Chromatographic Boundary Method (Tiselius–Claesson Method)

This new and important procedure which was described by Tiselius (1–13); Tiselius and Claesson; Tiselius and Hahn; Claesson (1–4, 6–8; cf. Claesson and Claesson) has so far been mainly employed for analytical purposes. For many details cf. the original papers.

According to Tiselius, if a dilute solution of a single solute is forced through a layer of suitable adsorbent, e.g., penetrating a column from below, then the following processes take place. Pure solvent will appear first above the column and its meniscus will rise continuously while the appearance of the solute will be retarded compared with this meniscus. The extent of the retardation will, of course, depend on the adsorption affinity.

The stronger the adsorbability, the later will the solute appear above the column, and the larger will be the distance between the pure solvent-solution boundary and the meniscus. An essential feature is that (provided secondary disturbances are avoided) there will be a sudden " break through " for each solute ; the boundaries formed will gradually rise above the surface of the adsorbent column (Fig. 9). If a suitable observation cell, whose volume is small compared to the total volume

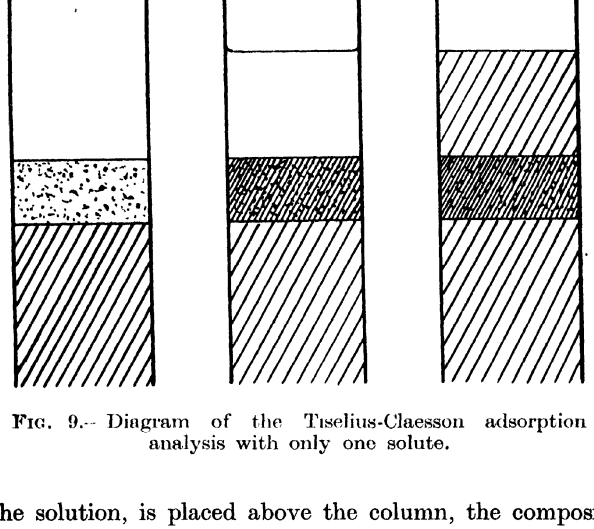


FIG. 9.- Diagram of the Tiselius-Claesson adsorption analysis with only one solute.

of the solution, is placed above the column, the composition of the liquid passing this cell can be observed. In principle, any physical method would be suitable for a continuous series of such readings, e.g., conductivity, spectrum, refractive index measurements, and especially the Toepler " schlieren " method, which has been used with success in electrophoretic experiments.

For the purpose of the following discussion it is assumed that the column has a length of a cm., a cross-sectional area of 1 cm.^{2}, a weight of s grams per cm^3., and a pore volume of

$\triangle$ cm^3. Furthermore, it is supposed that the top boundary of the solution of the substance A (concentration per ml., c_A) is in contact with the bottom of the column which had been completely moist with pure solvent. When the boundary of A has swept through the full length of the column, the meniscus in the observation tube on top will have to rise l cm. where $l \geqslant a$. Consequently, the quantity of A which penetrates the column from below, is lc_A. Suppose that of this the fraction $\triangle c_A$ is in solution and $(l - \triangle)\, c_A$ is adsorbed. The adsorbed amount per g. of the dry adsorbent will then be $m_A = (l - \triangle)\, c_A / as$. If a_A = adsorption coefficient for A (depending on the concentration), then $m_A = a_A\, c_A$ and $l = \triangle + a_A\, as$.

This gives an important meaning to the " retention volume " (" retardation volume "), $l - \triangle$, since the latter multiplied by c_A is the amount of the substance A adsorbed in the column, viz. $a_A c_A as$. If the Langmuir adsorption isotherm is valid, the adsorption coefficient a_A and the retardation volume will be independent of the concentration, if this is sufficiently low.

If there is more than one compound present, a mutual effect on the adsorption coefficients will take place. If a solution contains in addition to A the compound B which possesses a stronger adsorbability than A ; and if in the course of the experiment the boundary of B has just reached the top of the column and is located at a distance of l_B below the meniscus ; and if at the same time the boundary of A is higher up in the observation tube, viz. at a distance of l_A from the meniscus, then the solution between the lines l_A and l_B will contain only A. Its concentration c'_A will be different from c_A.

It follows from the foregoing that in this case

$$l_2.c_A = \triangle.\, c_A + \text{a.s.}\ a'.c_A + (l_2 - l_1)c'_A$$
$$c'_A/c_A = [l_2 - (\triangle + a.s.\ \alpha')]l_2 - l_1$$

In general $c'_A > c_A$, since in the presence of the more strongly adsorbed B the compound A will be less adsorbed than when it is the only solute. The solution following the B boundary in the observation tube will have the original concentration.

That the rate of flow must be sufficiently slow compared with the establishment of the adsorption equilibrium is a self-evident postulate that in some instances has been experimentally checked by Tiselius.

On the basis of the last equation this boundary method would permit quantitative analysis by estimation of the total concentration for each layer between two following boundaries and taking the successive differences, provided that $c = c'$. However, this will occur only if the adsorption displacement is negligible, i.e., if the concentration is low or/and the adsorbent is weak and possesses a large adsorption area. In the absence of these conditions the method may have a diagnostic value in a great number of instances.

For moving boundary systems formed by inorganic electrolytes, see Longsworth ; Dole.

Some characteristic features of the possibilities of quantitative estimations were worked out by Tiselius with, e.g., glucose-lactose mixtures. The glucose concentrations in the separated layers are considerably higher than in the initial solution predicted by the theory. The necessary corrections seem to be approximately proportional to the concentration and a linear extrapolation to $c = 0$ should be satisfactory. The quantitative method is limited by the fact that the difficulties just mentioned disappear only if the concentrations are below 0·2 per cent. On the other hand, if the concentration is less than 0·1 per cent, the boundaries become rather labile. At the present time an empirical calibration for a given adsorbent seems to be the most practical method for handling higher concentrations, such as 1–2 per cent.

We can differentiate between three types of such adsorption analysis, which were recently treated by Claesson (4, 8) in detail, viz., (*a*) *Frontal analysis*, (*b*) *Elution analysis*, and (*c*) *Displacement analysis*, the latter being especially effective.

(*a*) This is the simplest form (and has been discussed above) ; with it only a partial separation occurs, while with the other two methods complete separations may be reached. The original solution is forced through the column until all components appear, without further development operations. This procedure may well be compared with the initial phase

of an ordinary chromatographic experiment (introduction of the solution) (Fig. 10).

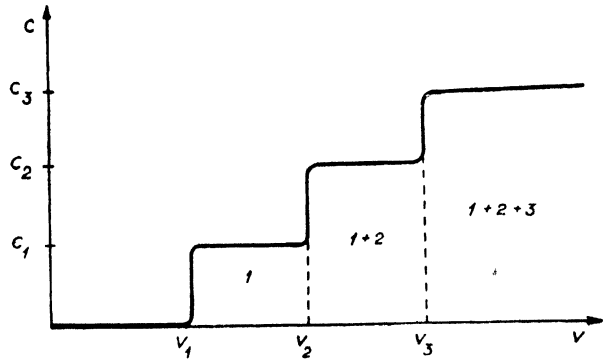


FIG. 10.—A frontal analysis diagram (Claesson 3).

(*b*) In this type a small volume of the solution is introduced and pure solvent is applied as a developer, as in one usual phase of the classic Tswett experiment. The individual compounds will pass as separate zones through the column (Fig. 11). The quantitative data are obtained from the area

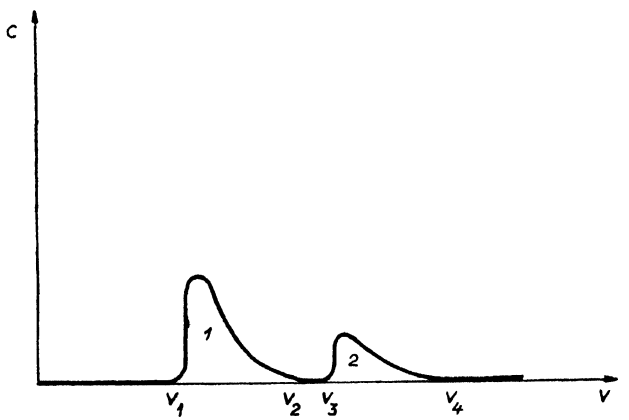


FIG. 11.—An elution analysis diagram (Claesson).

under the peaks; however, with strong adsorption the peaks will show considerable tailing and will not separate completely.

(*c*) Development is carried out by means of the solution of an introduced substance that possesses much stronger adsorption affinity than the solutes present. The latter are *displaced* from the adsorbent and then form sharply defined layers that are not disturbed by the displacer which finally follows. The individual compounds reach, after an adequate migration, stationary concentrations which remain constant, i.e., independent of the height of the liquid column. In Fig. 12 each

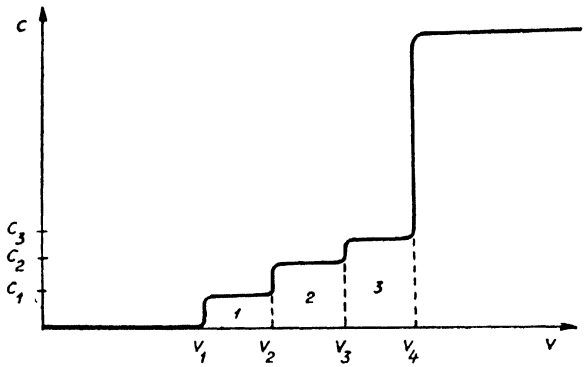


FIG. 12.—A diagram of displacement development (Claesson).

step corresponds to one compound and its area is a measure of the quantity. Since the heights are constant, the lengths of the steps are directly proportional to the amounts. (Great losses may occur, however, with very strong adsorption and incomplete displacement.)

Apparatus : For aqueous solutions the apparatus shown in Fig. 13 was used. The solution (25–35 ml.) is forced at a rate of

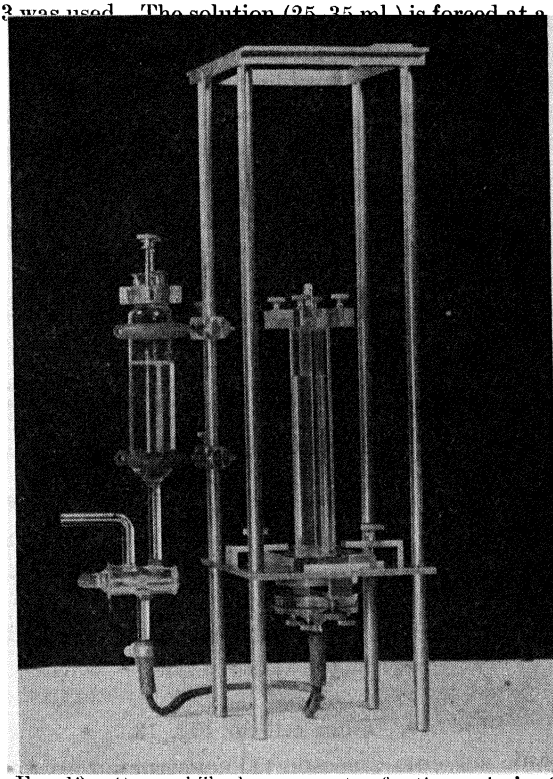


FIG. 13. Original Tiselius apparatus for the analysis of aqueous solutions.

about 50 ml. per hour through the filter cell ; in experiments with active carbon, pressures of 0·1–1 atm. were required. The usual diameters of the filter cells (made of transparent resin) were, e.g. 2, 1, and 0·5 cm., and their lengths were 4, 2, 1, and 0·5 cm. The top surface of the filter cell is pressed against the bottom of the observation cell (" cuvette "), which has a circular hole of the same diameter as the filter. The rectangular cell (cross section $5 \times 0{\cdot}5$ cm.) is made of transparent

resin (perspex, lucite or plexiglas). The final solution may be collected by an outlet at the top of the cell. In order to drive out all air, it is preferable to press, e.g., water through the column first. A correction is then made for the dead volume of water by introducing a little NaCl (adsorption negligible), which causes a " schlieren " band or gradient curve in the optical readings discussed below.

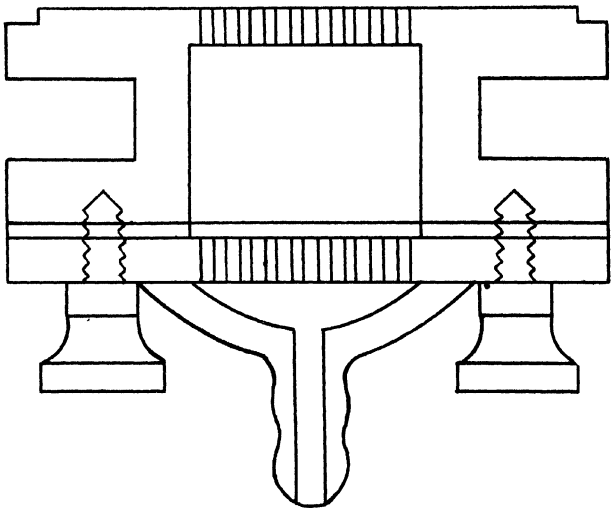


FIG. 14.—Filter cell for Fig. 13.

For organic solvents Claesson (1) constructed an apparatus with metal filter cells and made entirely of metal and glass. Its removable glass windows are sealed with thin cellophane or mipolam gasket rings. Since solutions may have lower densities than the pure solvent, it is also possible to use this device by introducing the solution at the top of the cuvette. All apparatus is kept thermostatically at 20°.

For observing and photographing the boundaries an optical system based upon the Toepler schlieren method for electrophoretic experiments was used, as described by Tiselius. Using the Philpot-Svensson modification of the method (cf. Claesson ; Svensson) one can obtain the concentration gradient

distribution in the cell as a photographed curve. Concentration estimations can be made without sampling.

Recently, an automatic registration device for the determination of the refractive indices was described by Claesson (2).

It seems that the schlieren method has not fulfilled expectations for some organic solutions. Because of some difficulties, e.g. the great dependence of the stability of the liquid column in the cuvette on differences in specific gravities, Tiselius and Claesson constructed a new apparatus in which the solution flows from the column into a micro-interferometer and thus permits continuous readings of the refractive index (Fig. 17). Cuvette and filter surfaces which come in contact with liquids have been covered with gold. Disturbances caused by convection are negligible, because the total volume is usually 20–50 ml. Furthermore, the different fractions can be conveniently collected from what has flown past the interferometer. The adsorbent is contained in a gilded brass cylinder packed between perforated plates and filter paper. The solution is forced from a burette into the adsorbent and continuous readings of refractive index and volumes are made. When organic solvents are used, small air bubbles may disturb the reading. This is avoided by introducing a pressure flask to protect the solution from air, as in an injection syringe. The volumes are then read in graduated collector tubes by means of a small telescope.

The whole equipment is shown in Figs. 15–16 and is manufactured by the firm " LKB-Produkter," Alvik, Stockholm.

For the handling of large amounts of substances on several hundred grams of adsorbent, "multiple filters" were introduced by Claesson (7). His system includes three or more filters whereby each smaller filter is below the larger one, their volume ratio being 5 : 1. Between each pair there is a small chamber with the same diameter as the larger filter of the pair.

Recently Dutton described an apparatus by means of which periodic refractometric readings of filtrates of ordinary chromatographic columns can be carried out. He thus extended the applicability of the Tiselius principle. A highly sensitive, differential, continuous flow refractometer is used by this

author who also is planning automatic recording. Solvents must have different refractive indices than that of the solute, and it is desirable that the developer had the same refractive index as the solvent in the outer cell of the differential refractometer. As a first example, the separation of stearic and oleic acids on Darco has been described.

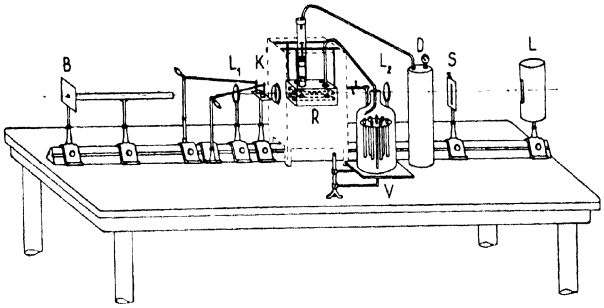


FIG. 15.—Schematic arrangement for the interferometric adsorption analysis (Tiselius-Claesson). L=light source, S=split, D=pressure flask with manometer, L_1=L_2=lens, V=collector with revolving

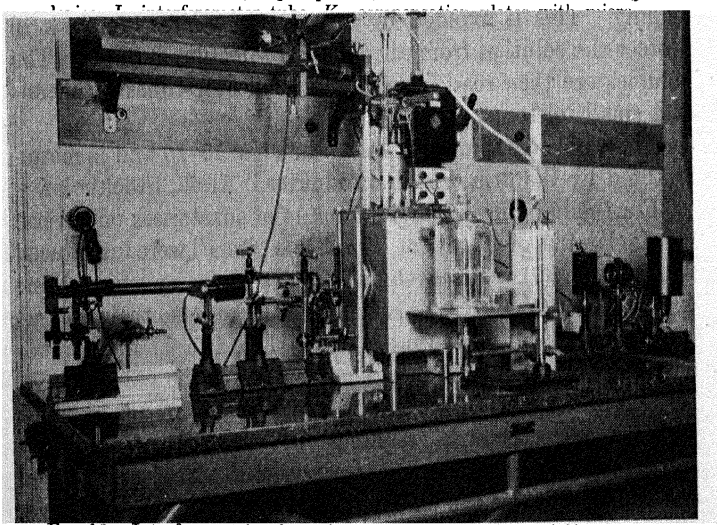


FIG. 16.—Interferometric adsorption apparatus (Tiselius and Claesson).

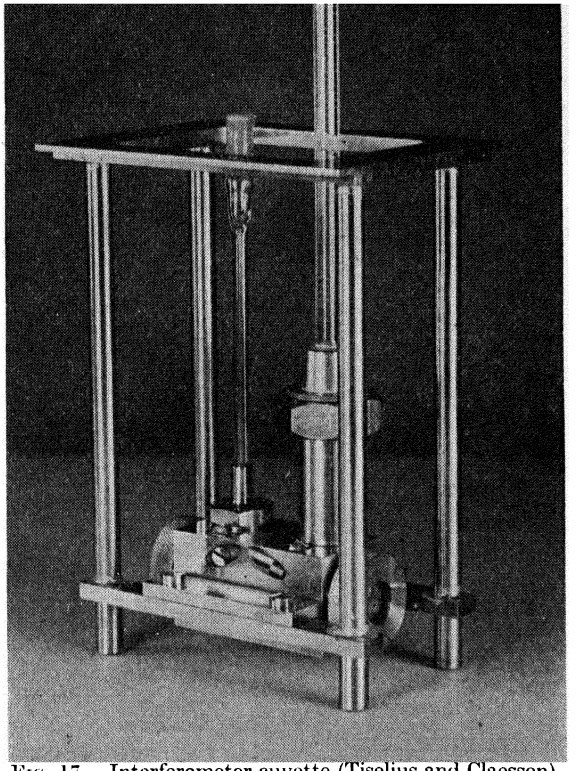


FIG. 17.—Interferometer cuvette (Tiselius and Claesson).

The cuvette consists of a rectangular brass block with four parallel channels drilled at 5 mm. distance from each other. The solution flows from the burette through the filter with the adsorbent directly into one of the upper channels. The outlet tube carries the solution to a sampling arrangement. The other three channels contain the solvent. The lower pair gives rise to the reference interference image. The inlet and the outlet of each channel are drilled at an angle through the block so that they end at the windows to avoid air bubbles. Gold tubing of 0·1 mm. wall thickness has been inserted into the channels. Inner channel diameter, 1·4 mm., channel length, 80 mm.; volume, 0·13 ml.

SPECIAL SECTION

CHAPTER III

CHLOROPHYLL

The non-existence of " chlorophyll *c* " was recently confirmed by Zscheile (1) who suggests that earlier observations were due to the decomposition of chlorophyll *a* to phæophytin and the inadequacy of the chromatographic method employed. Seybold and Egle (2) pointed out that after the adsorption of acid leaf extracts phæophytin may appear on the column, where it forms a grayish rim below the xanthophyll zone. These authors suggest that in some instances the total chlorophyll content should be converted into phæophytins, by the addition of oxalic acid before chromatographic estimation.

The term " chlorophyll c " is now being used for a pigment, also called chlorofucin or chlorophyll γ, which occurs in algæ (diatoms, dinoflagellates and brown algæ) and is not identical with the " *c* " of higher plants mentioned above (Strain, Manning and Hardin).

Adsorbents. Powdered sugar as used by earlier authors is still the most reliable adsorbent for chlorophyll (cf. Simonis ; J. Myers). Seybold and Egle (2) stress that each brand of powdered sugar should be tested because of great variations in adsorption behaviour. Furthermore, too slow filtration rate can be avoided by the admixture of 30–50 per cent sugar crystals with the powder. According to Zscheile and Comar it is advisable to dry the sugar at 90° and store it in an inert atmosphere. The same treatment was found to be satisfactory by Strain and Manning, who used confectioner's powdered sugar which contains 3 per cent corn starch. Different brands showed various filtration rates. Sugar can be also used for the microchromatography of chlorophylls. Strain and Manning found that 1·3 μg. of chlorophyll *b* (barley extracts) formed a definite zone on a 12 × 1·4 cm. column, even when 2,000 times more chlorophyll *a* was present.

Mackinney (5) prefers inulin to sucrose and, in order to save inulin, also uses magnesium citrate (+ 6 H_2O). With the latter adsorbent he removed the bulk of chlorophyll *a*; and *b* was then readsorbed on inulin (solvent, light petroleum containing 20 per

cent ether). According to Strott, the reported destruction of chlorophyll on starch does not take place if the pigment is eluted without delay. Starch was also used by Bukatsch. The use of urea (ground to 170 mesh, dried at 56°; mixed with 4 parts of talc, same mesh) was advocated by Masood, Siddiqi and Qureshi. L. A. Moore uses dicalcium phosphate.

The behaviour of crude chlorophyll on a number of adsorbents was studied by Eyster who found talc to be the best adsorbent as used by Willstätter and Stoll. The chlorophyll shows red fluorescence also in the adsorbed state. When retained on different adsorbents (moistened with water) it shows a similar spectral shift towards longer wave lengths an it also does when contained in living leaves.

Chlorophyll a and b. In order to obtain spectroscopically pure solutions, Zscheile and Comar proceeded as follows. After some previous operations the total chlorophyll from 1 kg. of fresh leaves, in about 200 ml. of light petroleum + ether (7: 3), was adsorbed on sucrose (35 × 5 cm.), without developing. Within 40 min. a green chlorophyll *b* zone (4–10 cm. broad) and below it a contiguous blue *a* zone (2–6 cm.) were differentiated. Only the centre half of the *a* zone was eluted with ether, filtered, dried over sodium sulphate and sucked through a fine sintered glass plate. If chlorophyll *b* has also to be obtained (best in a separate experiment), then it is advisable to wash the column with 1–15 l. of the light petroleum+ether mixture, until a 23–27 cm. green zone of *b* has been developed. Its centre portion is twice rechromatographed as indicated until only one homogeneous zone is present. Each chromatogram requires 2 hours.

Isolation of Chlorofucine (Chlorophyll *γ*) (Strain and Manning 2). The pigment, extracted by methanol from diatoms or brown algæ, was transferred to ether and, after concentration to a small volume and dilution with light petroleum, was developed on sugar with the latter solvent containing 2–4 per cent methanol. The sequence was: chlorofucine (top; pale green); fucoxanthins (orange); other carotenoids and chlorophyll *a* passed rapidly through. The

chlorofucine zone was eluted with ether. Its upper portions showed spectral variations because of secondary alterations. Maxima in ether, 627, 579·5 and 446 mμ. In similar experiments 20 × 3 cm. columns were used. The chromatograms can also be developed with light petroleum containing 0·5 per cent and then 2–4 per cent *n*-propanol (Strain, Manning and Hardin).

Separation of Chlorophyll d. Manning and Strain found that in various species of red algæ chlorophyll *b* is replaced by a new pigment termed chlorophyll *d* which is extracted by methanol more rapidly from *Gigartina agardhii* than is chlorophyll *a*. Such extracts can be worked up more easily on the Tswett column than can extracts of the entire pigment. One kg. of slightly moist, fresh algæ (pieces, 2–3 mm.) was agitated frequently with 2 l. of methanol, and after 30 min. this treatment was repeated with 1 l. for 5–10 min. The combined green extracts were mixed with 300 ml. of light petroleum (b.p. < 75°); 0·5 l. of 10 per cent NaCl was added and the bottom layer was re-extracted with 200 ml. of light petroleum. After washing and drying the combined solution was poured on to dry powdered sugar (20 × 6·7 cm.) where it was washed with a little light petroleum, then with the same solvent containing 0·5 per cent of *n*-propanol and 0·5 per cent of dimethylaniline. The diffuse green bottom zone (chlorophyll *d*) was contaminated by *a*. The *d*-zone was eluted with light petroleum + ethanol; the pigment was transferred with water into light petroleum which was dried and concentrated at 20° to 40 ml. (if a precipitate appears, a few ml. of ether should be added). The pigment was twice rechromatographed (8 × 4·7 cm., then 18 × 4·7 cm.). The first column was washed with light petroleum, the second (begun with 10 ml. solution) was developed with this solvent containing 0·5 per cent *n*-propanol + 0·5 per cent dimethylaniline. In the last chromatogram only traces of chlorophyll *a* preceded *d*, and all yellow pigments were adsorbed above *d*. When chlorophyll *d* reached 2/3 of the column (from top), its middle portion was removed and used (maxima in ether, 686 and 445 mμ). These operations require 7–8 hours.

Isomerization of Chlorophyll. Strain and Manning (2) made the remarkable observation that the chlorophylls *a* and *b* can be reversibly isomerized to yield chlorophylls *a'* and *b'*. For example, at 95–100° in *n*-propanol an equilibrium mixture is rapidly formed containing 1/5 of the pigment in the form of the new isomers. In order to demonstrate this chromatographically, 20 g. of green leaf tissue is scalded in boiling water for 1 min. and, after cooling, extracted with acetone or methanol. The pigments are transferred with a little water into light petroleum, which is then extracted twice with 80 per cent methanol and thrice with water, dried and filtered through powdered sugar (18 × 6·7 cm.). Upon developing the column with light petroleum containing 0·5 per cent *n*-propanol and 0·5 per cent dimethylaniline, it shows the following sequence: chlorophyll *b* (yellow-green; top); *b'* (yellow-green); *a* (green); and *a'* (green). It is observed that *a'* separates slowly from the *a*-zone.

Chlorophyll *d* which is contained in red algæ, undergoes a similar isomerization (Manning and Strain). After this chlorophyll had been kept for 9 days in methanol solution, the light petroleum extract was chromatographed as described above. At least three new zones were observed below that of unchanged chlorophyll *d*, viz., *d'* (yellow-green), isochlorophyll *d* (blue-green), and isochlorophyll *d'* (blue-green). The separation of the two middle zones was unsatisfactory, the other differentiations were excellent. Chlorophyll *d* seems to isomerize slowly even on the sugar column when washed with light petroleum + 0·5 per cent *n*-propanol.

Some data concerning ISOMERIZED PHAEOPHYTINS were given by Manning and Strain. Phæophytin *a* and isophæophytin *d* do not separate on the powdered sugar column when developed with light petroleum + 0·1 per cent propanol.

Precursor of Chlorophyll. Seybold and Egle (3) have further improved their technique. The green cotyledons of the gourd seeds were ground with sand and extracted with 95 per cent methanol. The pigments were transferred with water into benzene which was concentrated in a current of N_2. After

development, three well separated zones appeared on the sucrose column. The narrow, yellowish-brown middle zone contained protochlorophyll *b*, and the bluish-green bottom one protochlorophyll *a*. The top zone is claimed to be a mixture of *a* and *b* ; this requires further investigation. The middle and bottom zones were chromatographically homogeneous.

Bacterio-chlorophyll. A particular difficulty in this field is the easy formation of some artifacts during the isolation ; they cause the appearance of a complex chromatogram. In this connection mention should be made of the observation of Seybold and Egle (6) who observed three chlorophyll zones when light petroleum extracts of red *Thiocystis* bacteria were developed on sucrose with benzene. However, only the middle zone (bacterio-chlorophyll *a*) was genuine.

Bacterio-methylphæophorbide can be crystallized after a development on alumina with ether + acetone + methanol (20 : 10 : 1). A similar technique can be used for the isolation of its hydrogenation product as well as of some by-products of the preparation of bacterio-2-vinyl chlorin (Mittenzwei).

Animal Chlorophylls. Intact chlorophylls in the fæces of various animals were detected by chromatography on sugar (Seybold and Egle 4). Rats receiving chlorophyll by mouth excreted about half of it intact. Kohler, Elvehjem and Hart developed extracts of fæces pigment with benzene + light petroleum (1 : 9) on anhydrous sodium sulphate. The top zones were chlorophyll *a* and *b* while a third zone yielded, by rechromatography on powdered sugar and development, first with benzene + light petroleum 1 : 9 and then with a 1 : 4 mixture, two zones spectroscopically identical with the probophorbides *a* (or *c*) and *b*. The elutions were carried out with ether and water (which dissolved the column).

Breakdown Products of Chlorophyll

The dark green pigment which occurs in the tegument of *Bonellia viridis* was removed by Lederer (16) with cold alcohol ; this solution was extracted with light petroleum (removing impurities) and then with ether. From the latter the

pigment, *bonellin*, was extracted by 1 per cent ammonia and, upon neutralization, transferred into ether. It was taken up by 6 per cent HCl and, after neutralization, transferred into ether. This solution formed a broad green main zone on calcium carbonate from which the pigment could be eluted by HCl (which dissolves the column) and ether. A crystalline green compound was obtained and seems to be a dihydroxy-mesopyrrochlorine.

SOME CHLOROPHYLL DERIVATIVES (for Porphyrins see p. 66). The following compounds can be purified on alumina (ether) (Fischer and Gibian 1, 2): Mesodesoxo-pyrophæophorbide a (and methylester), desoxophyllerythrin, mesodesoxopyroisophæophorbide-α-methylester, mesopyrophæophorbide-α-methylester, mesophyllochlorine-methylester, mesorhodochlorine - dimethylester, mesopyrrochlorine - methylester, mesochlorine-p_6-dimethylester-6-carboxylic acid piperidide, chlorin-e_6-dimethylester-carboxylic acid-ethylamide. Acetone + ether (1 : 1) should be used for mesopurpurin-18-methylester-imide or mesopurpurin-18-methylester-benzoyloxime.

Meso-pyrophæophorbide β-methylester (talcum; ether + acetone 3 : 1). Crude 2,-α-hydroxy-meso-chlorine-e_6-trimethylester was developed with ether on talc, which retained some degradation products near the top and traces of porphyrins in the middle; the main product passed into the filtrate (Fischer, Mittenzwei and Hevér).

The chlorin e_6, obtained from methylphæophorbide with hydrazine, was chromatographed on talc using ether + chloroform (4 : 1) (Fischer and Conrad). Mesopurpurine-3-methylester (top) can be separated from 7 : 8-dihydroxy-mesophyllochlorinester on alumina, from ether (Fischer and Gerner). For further compounds cf. Fischer and Strell, Fischer and Pfeiffer (1, 2). Purification on the alumina column from ether: meso-isochlorine-e_4-bromodimethylester (Fischer and Gerner); 7-(or 8)-methoxy-meso-isochlorine-e_4 and similar compounds (Fischer, Kellermann and Baláz). For 2, α-hydroxy-meso-phyllochlorinester and tribromo-2-desvinyl-phyllochlorinester cf. Fischer and Baláz.

Appendix: Hæmoglobin

The possibility of the existence of two forms of hæmoglobin was revealed when Altschul, Sidwell and Hogness observed two red zones in an alumina chromatogram obtained with dialyzed solutions of horse blood serum. These zones separated upon washing with water. A similar observation was made later by Schwerdt on defibrinated and hemolyzed rabbit blood. It remains to be seen whether or not the adsorbent used, viz., Lloyd's reagent, may have influenced this result.

CHAPTER IV

PORPHYRINS

Some data on the adsorption behaviour of porphyrins and on the spectroscopy of adsorbates were given by Bandow (1–3). It seems that chromatography is about to become a diagnostic tool in some clinical cases which show an accumulation of urinary porphyrins.

Purification of Protoporphyrin IX (ex Hæmoglobin) (Grinstein and Watson ; Grinstein and Camponovo ; Grinstein 2). The crude methyl ester was adsorbed from chloroform + light petroleum (1 : 10) on calcium carbonate (e.g., Merck's heavy powder) and developed with a 1 : 3 mixture. Whilst most impurities remained in the upper section of the column, the ester formed a red-violet lower zone from which it was eluted with chloroform.

Porphobilinogen in Urine. This colourless precursor of porphyrins, which is insoluble in organic solvents, was obtained by Waldenström and Vahlquist by chromatographing porphyria urines on alumina. From the adsorbent porphobilinogen can be eluted by treatments with weak ammonia. Upon acidifying with HCl (congo red) and boiling, it forms porphyrins.

Uroporphyrins. Recently, Grinstein, Schwartz and Watson successfully used calcium carbonate columns from which the individual pigment zones were cut out ; earlier investigators mostly employed the liquid chromatogram technique. The authors report the purification of uroporphyrin I-octamethylester, m.p. 284°. Furthermore, they have shown that the porphyrin of m.p. 258–260° as obtained by Waldenström from porphyria urines can be resolved into uroporphyrin I-octamethylester, m.p. 284°, and an unidentified porphyrin, m.p. 208°.

Urine was brought to pH 3–4 with glacial acetic acid and sufficient talc was added. The porphyrin adsorbate was sucked off, dried on a Buchner funnel, pulverized and treated over night with HCl-saturated methanol. After filtration through sintered glass, the pigment was quantitatively eluted by

washings with methanolic HCl and then chloroform. Upon dilution with 1 vol. of chloroform the liquid was washed repeatedly with water, ammonia and 7 per cent NaCl. After filtration through $CHCl_3$-moistened paper and evaporation, the residue was dissolved in benzene + light petroleum (3 : 1) (solution of residues from fæces dissolved in a 1 : 2 mixture), and poured on to a calcium carbonate column. Urine extracts (obtained as described) are best developed with benzene ; for Waldenström porphyrins (extracted with ethyl acetate) benzene + chloroform (10 : 1 to 10 : 3) is satisfactory ; finally, for fecal pigments, benzene + light petroleum 1 : 2. A typical chromatogram of the crude mixture of urine porphyrin esters follows (all zones were separated by empty interzones) :

(*a*) red brown, not porphyrin
(*b*) rose, not porphyrin
(*c*) violet red, m.p. 284°, uroporphyrin-octamethylester-I
(*d*) violet red, m.p. 208°, unidentified porphyrin
(*e*) violet red, unidentified porphyrin
(*f*) violet red, unidentified porphyrin
(*g*) violet red, coproporphyrin III-methylester
(*h*) blue, not porphyrin.

The zones were eluted with chloroform but (a) required chloroform + acetic acid. Zones (c) and (d) were also obtained from Waldenström samples ; they crystallized from chloroform + methanol. Zone (c) showed the analytical data expected for $C_{48}H_{54}O_{16}N_4$. Upon rechromatography no further resolution occurred.

It should be stressed that Watson, Schwartz and Hawkinson also describe some failures in resolution of the Waldenström type porphyrin. Chromatography of artificial mixtures showed that such a porphyrin possesses a limited binding power of the 208° compound; larger added quantities were separable in the Tswett column.

Chromatography was also applied to some *decarboxylation* products of porphyrins.

Isolation of Uroporphyrin from the Fæces in Idiopathic Porphyria (Schwartz and Watson). This material was treated with methanolic HCl ; extracted porphyrins were transferred into chloroform on the next day and washed with water, ammonia and NaCl. After drying and dilution with 10 vol.

of light petroleum, the solution was filtered through Brockmann's alumina and developed with increasing proportions of chloroform in light petroleum. This proportion was 1 : 1 for a good differentiation of the red zone of uroporphyrin methylester (second from top).

Resolution of a Mixture of Uroporphyrin III and Coprophyrin I. Rimington found that acid treated kieselguhr easily adsorbs these and similar porphyrins from dilute HCl solution. An artificial mixture dissolved in 5 per cent HCl (w/w) was placed on a column and washed thoroughly with water. When acetate buffer pH 5·25 was applied, the uroporphyrin passed readily into the filtrate while the sharp zone of coproporphyrin remained at the top ; it was washed down with a phosphate buffer pH 7·15. The isomers I or II cannot be separated.

When applied to urine, this method has the advantage that a number of other urinary pigments are not adsorbed. To a nearly black urine (idiopathic porphyria) HCl was added until the concentration was 1 per cent. The chromatogram was washed with 1 per cent HCl, then with glacial acetic acid and water. Uroporphyrin remained adsorbed and was eventually eluted with acetate buffer pH 5·25. It precipitated upon an adjustment to pH 3·0.

Separation of Small Quantities of the Coproporphyrins I and III (Watson and Schwartz). Quantities as small as 10 mg. may be differentiated by esterification of the mixture with methanol + HCl, dilution with 1 vol. of water, neutralization with saturated sodium acetate (until the blue reaction of congo-red disappeared) and then with ammonia (pink colour with phenolred). The copro-III-ester can be removed from Brockmann's alumina (Merck) after washing with water, by repeated washings with 35 per cent acetone, and then the I-ester with pure acetone. This separation may be prevented by some impurities in urine extracts.

Chromatography was also used in a micro-estimation method of urinary coproporphyrins I and III by Schwartz, Hawkinson, Cohen and Watson. The crude product was

adsorbed from 1–2 drops of chloroform + 3–4 ml. of benzene on calcium carbonate (Cenco, U.S.P., precipitated powder; 6–8 cm. high) and developed with a 1 : 10 to 1 : 15 solvent mixture. The zone of the two isomers is cut out from the middle of the column. The limit of detection is 5μg. in daylight but much less in ultraviolet light (red fluorescence).

FURTHER REPRESENTATIVES. A 2-ethyl- and a 2-vinyl-porphyrin were separated on alumina by Fischer and Strell. Chloroform solutions of compounds like vinyl-phylloporphyrinester or oxo-phylloporphyrin-methylester can be purified by filtration through alumina (Fischer and MacDonald). For 2-desetyl-phylloporphyrine-methylester (alumina, chloroform) cf. Fischer and Baláz; Fischer, Kellermann and Baláz. Benzoxy-protoporphyrin-dimethylester or benzoxy-phylloporphyrin methylester can be developed on alumina with chloroform + ether 1 : 3 (Stier). Vinyl-rhodin-porphyrine-g_7-3-methanol-trimethylester or 2-α-hydroxy-chloroporphyrin $e_{(6)}$-trimethylester were developed on alumina with ether + glacial acetic acid (30 : 1) by Fischer and Oestreicher.

PORPHYRINS FROM BIRDS' FEATHERS (Volker 2–4). After extraction and esterification with HCl in methanol and developing on alumina with ether, the main pigment zone was eluted with methanol + ether and crystallized.

SPIROGRAPHIS PORPHYRIN (Fischer and Wecker). Crude 4-vinyl-deuteroporphyrin-dimethylester, obtained from this annelid, when developed on alumina with ether + chloroform 4 : 1 showed the following zones: destruction products (top); unesterified porphyrin; and the dimethylester.

PORPHYRIN-LIKE ARTIFACTS (from pyrrole and benzaldehyde) were resolved by Aronoff and Calvin into six components on talc with trichloroethylene as developer (elution with pyridine).

CHAPTER V

BILE PIGMENTS

For the elimination of disturbing red pigments in the estimation of urine *bilirubin*, no adequate chromatographic procedure has so far been found (With 2). From a mixture of monomethoxy- and dimethoxy-bilirubin-dimethylester on alumina the latter is washed into the filtrate with chloroform; the monomethoxy compound follows when methanol-containing chloroform is applied. Addition products of diazoacetic ester to *biliverdin*-methylester (or similar compounds) can be freed from unreacted diazoacetic ester by filtering the ether solution through alumina. The pigment is then developed with ether + chloroform (Fischer, Plieninger and Weissbarth).

Products of the Gmelin Reaction. In their investigations of this reaction (a series of colorations produced by nitric and nitrous acids with bile pigments) Siedel and Frövis, as well as Siedel and Grams, repeatedly used chromatography. A solution of 0·3 g. of mesobilirubin-XIII-α-dimethylester dihydrochloride in 200 ml. of chloroform was purified by washings with alkali and water and shaken with 40 ml. of 2 *N*-nitric acid + 50 mg. of sodium nitrite, until the solution turned green and then blue. When a violet shade began to show, the reaction was stopped by the addition of 40 ml. of 2 *N*-NaOH. The chloroform solution was then washed, dried, concentrated, diluted with 3 vol. of abs. ether and developed on talc with chloroform + ether (1 : 2). Three zones appeared: reddish violet (top), blue (glaucobilin) and violet. The top zone yielded, after elution with methanol, rechromatography, evaporation and ether extraction, red needles of mesobilipurpurin-XIII-α-dimethylester. The separation can also be carried out on Brockmann's alumina (ether + chloroform 6 : 1); a reddish-orange zone (second from top) then contains the ester mentioned.

Similar experiments with glaucobilin-XIII-α-dimethylester or its bromination product gave complicated chromatograms. In the presence of pyridine a red crystalline pigment was obtained. When the copper

salt of this glaucobilin in chloroform was treated with 2 *N*-HCl and the product developed on alumina with chloroform, two main blue zones appeared; the first contained a copper salt and the second glaucobilin ester.

Glaucobilin and Stercobilin. The following compounds have been purified on alumina by Stier: deutero-glaucobilin-dimethylester (developed with chloroform + ether until the contaminants passed into the filtrate, and then eluted with pure chloroform); diacetyl-hematoglaucobilin-dimethylester (adsorbed from acetone and developed with acetone + ether 1 : 3).

Stercobilin hydrochloride can be accumulated from alcohol-ether extracts of pathological fæces (hæmolytic icterus) on alumina and can be eluted with 2 per cent ammonia (Fischer and Stachel).

Crude **Mesobilifuscin**-methylester (0·5 g.) in 20 ml. of chloroform was developed on a 20 × 3 cm. Brockmann alumina column with chloroform + ether 1 : 3. Sequence: mesobilifuscin-methylester (top, brown); urobilin (yellow); mixture (violet); glaucobilin (blue); and mesobilipurpurin (red) (Siedel and Möller).

Mesobilifuscin from Mesobilirubinogen (Siedel and Möller). The crude reaction product, obtained with lead tetra-acetate was, after esterification, treated as above: mesobilifuscin-methylester (top, brown); mesobiliviolin- and mesobilirhodin-methylester (violet); glaucobilin-methylester (blue); mesobilipurpurin- and porphyrin-methylester (pink).

Myobilin (crude, from the feces of myopathics) was developed with chloroform + ether (1 : 2) on talc, whereupon urobilinoids separated from the myobilin and fats passed into the filtrate. The bottom zone, containing myobilin, was eluted with chloroform or alcohol. Further purification can be achieved by adsorbing myobilin from chloroform on Brockmann's alumina and developing with chloroform + ether (1 : 10) (a protein containing fraction filters through) and then

with a 1 : 2 mixture. The following sequence was observed: mesobilifuscin-methylester (top, brown), urobilin + stercobilin-methylester (yellowish); and much lower: myobilin (pale) (Meldolesi, Siedel and Möller).

Porphobilinogen. For estimations the fresh urine is adjusted to 1 per cent acetic acid and adsorbed on alumina. The zones containing porphobilinogen are cut out, washed with water and eluted with 1 per cent ammonia. If the presence of urobilinogen is suspected, the acidified eluate is extracted with ether and the main solution is rechromatographed as described (Vahlquist).

A *chromogen in pregnancy urine* yields a red pigment with iodine and passes unadsorbed through a column of aluminium hydroxide, but is quantitatively removed by 1 g. of decolorizing charcoal per 20 ml. of urine (Schales and Schales).

FURTHER BILE PIGMENTS. Some pigment products of glandular excretions of *Aplysia punctata* (sea hare or sea slug) were classified by Lederer and Huttrer as bile pigments. The chromoproteids can be obtained when the animals are kept in water. Upon filtration through alumina of moderate activity a violet and a red zone appear. It seems not to be possible to elute these native pigments. If acid is applied, the pigment mixture then becomes blue and chloroform soluble, and can be extracted and developed on calcium carbonate with butyl alcohol + chloroform (1 : 3). The violet and red zones are eluted with butanol containing 2 per cent HCl. Maxima in chloroform: violet pigment, 538, 496 mμ.; red pigment, 536, 498 mμ. They give a positive Gmelin reaction. Likewise, two green pigments contained in some *fishbones* can be purified by developing their esters on alumina with HCl-containing chloroform (Willstaedt 14).

CHAPTER VI

CAROTENOIDS

The use of chromatographic methods in this field has become so general that an exhaustive review would require a special monograph. Therefore, only a limited number of investigations can be considered in detail, and further material is condensed in Tables 4–8. General surveys : E. R. H. Jones ; Mackinney (6) ; Heilbron ; Strain (8, 14).

Estimation of Carotene. Out of a great number of papers only a few can be mentioned : Austin and Shipton ; Bickoff and Williams ; Booth ; Charkey and Wilgus ; Davies ; Fraps and Kemmerer ; Fraps, Kemmerer and Greenberg (1, 2) ; Griffith and Jeffrey ; Kemmerer and Fraps ; Kemmerer, Fraps and Meinke ; Kernohan ; Mann ; L. A. Moore ; O'Connor, Heinzelman and Jefferson ; Ramasarma, Hakim and Rao ; Sherman ; Silker, Schrenk and King ; Wall and Kelley ; Wilkes ; Zscheile, Beadle and Kraybill ; Lassen et al.

For the elimination of non-carotenes, with a view to the eventual estimation of carotene in various PLANT MATERIALS, the procedure as described by Kemmerer seems to have been officially adopted in the United States. As adsorbents $MgCO_3$ or $CaHPO_4$ are used. They should adsorb less than 5 per cent of the carotene when tested as follows : Pass through 2 g. of the adsorbent (15 × 1 cm. glass tube) 50 ml. of a solution containing 0·50–0·075 mg. of crystalline carotene in light petroleum, wash with the same solvent and estimate carotene in the filtrate. If the adsorbent is too retentive, it should be rejected ; if later in the analysis it turns out to be too weak, 2·5 per cent MgO is added. The chromatographic retention of the non-carotene pigments is carried out in the same manner using aliquots of concentrates of extracts in light petroleum.

For the retention of non-carotenes heat-treated silicious earth (filter aid 501) is used by Strain.

In the author's laboratory the carotene content of plant materials is estimated as follows : The well divided tissues are dehydrated by standing in methanol for a few hours and

are then submitted to a two-phase extraction by repeated mechanical shaking with methanol + light petroleum (b.p. 60–70°) 1 : 1 (later less methanol). All pigment is transferred from the combined extracts with water into the upper (petroleum) phase which is then washed methanol-free, dried with sodium sulphate, concentrated and developed on a calcium hydroxide column with light petroleum containing 0–3 per cent acetone, according to the strength of the adsorbent. The carotene zones are cut out, eluted with methanol, transferred into light petroleum or hexane and estimated photometrically. Isomerized zones can be estimated upon iodine catalysis, using published figures for the quasi-equilibrium mixture (survey : author 7). If a saponification is necessary, this is carried out before the adsorption, by keeping the light petroleum solution over 10–15 per cent methanolic KOH (in a broad conic flask) over night, at 20°, in N_2, washing the extract free of alkali and drying.

In FÆCES the carotene can be best determined, according to With (1, 2), by adsorbing (after saponification) the light petroleum extract on alumina. The column is washed with benzene until the rose-pink carotene zone has passed into the filtrate, while the other carotenoids and vitamin A remain adsorbed. This can be achieved by selecting a column of proper length (diameter, 1 cm.).

Some impurities which interfere with the Carr-Price reaction of carotenoids can be eliminated by chromatography (Johnson and Baumann).

Separation of Algal or Diatom Pigments (Strain, Manning and Hardin 2). Fresh acetone extracts were transferred into petroleum ether solution which, after concentration, was adsorbed on dry powdered sugar, where it was developed with petroleum ether containing 0·5 per cent propanol and 0·5 per cent dimethylaniline. The sequence of the diatom pigments was, e.g. : chlorophyll *c* (top), neofucoxanthins, fucoxanthin, diadinoxanthin, diatoxanthin, chlorophyll *a*, traces of mixture, carotenes (bottom). In order to accelerate the procedures and avoid isomerization as far as possible, the strongly adsorbed xanthophylls were prepared on separate columns. For the

resolution of the former type petroleum ether + 2 per cent propanol can be used advantageously. All pigments were purified by rechromatography and the carotenes were identified upon adsorption on magnesia + heat-treated diatomaceous earth (filter aid No. 501).

Astaxanthin-ester A from Hæmatococcus pluvialis (Kuhn, Stene and Sörensen). 20 g. of wet aplanospores were rubbed with sand and dry ice and extracted 4–5 times with a total of 750 ml. of acetone. The pigments were transferred into light petroleum ; the solution was washed, dried, concentrated to 80 ml. and chromatographed on calcium carbonate (40 × 5 cm.). The figures on the left designate height of the zones in mm. :

50 orange (Ester A).
25 gray-greenish (chlorophylls + Ester " II ").
25 deep violet (Ester A containing).
230 light salmon red (Ester A containing).
Filtrate, Ester C and β-carotene.

The combined first four zones were eluted with alcohol, transferred into light petroleum and rechromatographed on sucrose ; the esters of astaxanthin were retained under the two chlorophyll zones and many colourless contaminants were washed down the column. The whole ester A content appeared to be in a single zone which was eluted with benzene ; it crystallized upon evaporation of the eluate.

For bacterial carotenoids cf. Table 5 (p.80) as well as Sobin and Stahly.

TABLE 4

EXAMPLES OF THE CHROMATOGRAPHY OF CAROTENOIDS OCCURRING IN HIGHER PLANTS

Source	Carotenoid	Adsorbent ; developer	Literature
Badami Mango fruit . .	β-carotene	MgO ; light petr. ; ethanol	Ramasarma, Rao and Hakim
Grevillea robusta Cunn. (silk oak flower)	β-carotene	$Ca(OH)_2$; light petr.	Author and Polgár (2)
Daucus carota L. (carrot) .	α-, β-, γ-carotene, lycopene, etc.	$Ca(OH)_2$ + supercel ; hexane + 5 per cent acetone	Harper and Zscheile
Concentrates	β-carotene	Al_2O_3 ; light petr. (+ benzene)	Devine, Hunter and Williams
Cucumis citrullus L. (water melon)	β-carotene, lycopene	$Ca(OH)_2$; light petr., then benzene	Author and Polgár (1)
Rose hips, etc. . . .	carotene, lycopene	coll. Al(OH) ; light petr. + 1 per cent acetone	Jacoby and Wokes
Chara ceratophylla Wallr. and *Nitella syncarpa* Kützing	lycopene, α-, β-, γ-carotene	Al_2O_3 ; light petr.	Karrer, Fatzer, Favarger and Jucker
Lycopersicon sp.	α-, β-, γ-carotenes, lycopene, etc.	MgO or $Ca(OH)_2$; hexane + 1–10 per cent acetone ; alcohol	Porter and Zscheile (1)
Lycopersicum esculentum L. (tomato)	carotene	MgO + diatomaceous earth ; light petr.	Ellis and Hamner
Lycopersicum esculentum L. (tomato)	lycopene, β- and γ-carotene	$Ca(OH)_2$; light petr. + 0–10 per cent acetone	LeRosen, Went and author
Lycopersicum esculentum L. (tangerine tomato)	prolycopene	$Ca(OH)_2$; light petr. + 10 per cent acetone	Author, LeRosen, Went and Pauling ; LeRosen and author (2)
Butia capitata Becc. (palm fruit)	pro-γ-carotene	$Ca(OH)_2$; light petr.	Author and Schroeder (4)
Pyracantha angustifolia Schneid. (fruit)	prolycopene, pro-γ-carotene	$Ca(OH)_2$; light petr. + 2 per cent. acetone	Author and Schroeder (3)
Pyracantha augustifolia Schneid. (fruit)	poly-*cis*-lycopenes	$Ca(OH)_2$ + celite ; light petr. + 0–4 per cent acetone	Author and Pinckard
Evonymus fortunei Rehd. (seeds)	prolycopene, pro-γ-carotene	$Ca(OH)_2$; light petr. + 2·5 per cent acetone	Author and Escue (2)
Palm Oils	α-, β-, γ-carotenes, lycopene, xanthophylls	Al_2O_3 ; benzene—light petr. (1 : 4), etc. ; benzene + ether + alcohol	Hunter and Scott (1–2) ; Hunter, Scott and Edisbury ; Hunter, Scott and Williams ; Hunter and Krakenberger ; Blackie and Cowgill.
Mimulus longiflorus L. (monkey flowers)	β- and γ-carotene, lycopene, cryptoxanthin	Ca(OH) ; light petr. + 5 per cent acetone	Author and Schroeder (2)
Mimulus longiflorus L. (monkey flowers)	pro-γ-carotene, prolycopene	Ca(OH) ; light petr. + 2 per cent acetone	Schroeder
Prunus persica Sieb. (yellow peach)	carotene, lycopene, lutein	Al_2O_3 ; light petr.	Thaler and Schulte
Prunus persica Sieb. (peach)	lutein, zeaxanthin, cryptoxanthin, β-carotene	MgO ; light petr.	Mackinney (4)
Crude preparations . .	zeaxanthin, cryptoxanthin, lutein	MgO ; light petr. (+ ethanol)	Zscheile, White, Beadle and Roach
Celastrus scandens L. (false bitter-sweet, fruit)	celaxanthin, zeaxanthin ester	$Ca(OH)_2$; light petr. + acetone ; benzene + acetone	LeRosen and author (1)
Gazania rigens R. Br. (flowers) (Portugal)	gazaniaxanthin, rubixanthin, lutein, β- and γ-carotene	Al_2O_3 ; benzene + light petr. (1 : 3)	Schön (3)
Gazania rigens R. Br. (flowers) (California)	gazaniaxanthin, lutein, cryptoxanthin, lycopene, β- and γ-carotene	$Ca(OH)_2$; light petr. (in part) with acetone	Author and Schroeder (5)

TABLE 4 (*continued*)

EXAMPLES OF THE CHROMATOGRAPHY OF CAROTENOIDS OCCURRING IN HIGHER PLANTS

Source	Carotenoid	Adsorbent; developer	Literature
Zea mays L. (yellow corn).	α-, β-carotene, cryptoxanthin, etc.	MgO; light petr.	Kemmerer, Fraps and Mangelsdorf
Zea mays L. (yellow corn grain)	lutein, γ-carotene, etc.	MgO + supercel; hexane	White, Zscheile and Brunson
Leaves	xanthophylls (carotenes)	sugar; benzene	Seybold and Egle
Leaves	carotene, xanthophyll	starch; light petr.	Strott
Sarothamnus scoparius Kch.	chrysanthemaxanthin	Al_2O_3; benzene + ether (1 : 1)	Karrer and Jucker (2)
Chrysanthemum indicum L.	chrysanthemaxanthin	$ZnCO_3$; benzene	Karrer and Jucker (1)
Pyracantha coccinea L. .	lycopene, carotenes, xanthophyll-epoxide	$Ca(OH)_2$; benzene	Karrer and Rutschmann (7)
Tragopogon pratensis L. .	α-carotene-epoxide, xanthophyll-epoxide	$ZnCO_3$; benzene	Karrer and Jucker (8)
Ranunculus acer L.	α-carotene-epoxide; xanthophyll-epoxide	$ZnCO_3$; benzene	Karrer and Jucker (8)
Elodea canadensis Rich. .	eloxanthin (xanthophyll-epoxide)	$Ca(OH)_2$ then $ZnCO_3$; benzene	Karrer and Rutschmann (6)
Trollius europaeus . .	trollixanthin	$ZnCO_3$; benzene + ether; methanol + ether	Karrer and Jucker (9)
Caltha palustris	trollixanthin, xantophyll-epoxide	$ZnCO_3$; benzene + ether	Karrer and Jucker (12)
Tagetes patula L. . .	rubichrom, xanthophyll-epoxide	$ZnCO_3$; benzene + ether	Karrer, Jucker and Steinlin
Taraxacum officinalis L. (dandelion)	flavoxanthin	$ZnCO_3$; benzene	Karrer and Rutschmann (1)
Taraxacum officinalis L. (dandelion)	tareoxanthin	MgO + silicic earth; light petr. + 25 per cent acetone	Strain, Manning and Hardin (1)
Iris pseudacorus L. (yellow)	violaxanthin, lutein, β-carotene	$CaCO_3$; CS_2	Drumm and O'Connor
Viola tricolor L. (yellow pansy)	auroxanthin	$ZnCO_3$; benzene	Karrer and Rutschmann (2)
Viola tricolor L. (yellow pansy)	violeoxanthin	sugar; light petr. + 1 per cent propanol or MgO; light petr. + 25 per cent acetone	Strain, Manning and Hardin (1)
Capsicum annuum L. (red pepper)	capsorubin-, capsanthin- and zeaxanthin-ester, carotenes	$Ca(OH)_2$ + $CaCO_3$; light petr.	Cholnoky (4)
Bixa orellana L. . . .	bixin	$CaCO_3$; light petr. + methanol	Villela

TABLE 5

EXAMPLES OF THE CHROMATOGRAPHY OF CAROTENOIDS OCCURRING IN LOWER PLANTS

Source	Carotenoid	Adsorbent ; developer	Literature
Algæ	carotenes, xanthophylls	Al_2O_3 ; light petr.	Carter, Heilbron and Lythgoe
Oscillatoria rubescens	oscilloxanthin	$ZnCO_3$; chloroform	Karrer and Rutschmann (4)
Aphanizomenon flos-aquae	β-carotene, aphanin, aphanicin, flavacin, aphanizophyll	Al_2O_3 ; light petr.	Tischer (6)
Dinoflagellates	diadinoxanthin, dinoxanthin, peridinin, β-carotene	sugar ; light petr. + propanol	Strain, Manning and Hardin (2)
Diatoms	diatoxanthin, diadinoxanthin, fucoxanthin, β-carotene	sugar ; light petr (+ 0·5 to 2 per cent propanol)	Strain, Manning and Hardin (2)
Diatoms (in gross plankton)	carotenes, xanthophylls	Al_2O_3 ; light petr.	Gillam, El Ridi and Wimpenny
Navicula torquatum	ε-carotene	MgO + sil. earth ; light petr.	Strain and Manning (4)
Nitzschia closterium	carotenes, xanthophylls	sugar ; light petr.	Pace ; Dutton and Manning
Brown algæ	diatoxanthin, diadinoxanthin, violaxanthin, flavoxanthin-like pigment, fucoxanthin, β-carotene	sugar ; light petr. + propanol	Strain, Manning and Hardin (2)
Euglena (red)	carotenes	Al_2O_3 ; light petr.	Tischer (1)

Allomyces	γ- and β-carotene	$CaCO_3$ (top) + $Ca(OH)_2$ (bottom) or MgO ; light petr.	Emerson and Fox
Gymnosporangium	γ- and β-carotene	MgO + hyflo-supercel	Smits and Peterson
Rhodotorula sanniei	β- and γ-carotene, lycopene, torulene, acid pigment	Al_2O_3 ; light petr.	Fromageot and Tchang
Rhodotorula (torula) rubra	torularhodin	Al_2O_3 ; light petr.	Karrer and Rutschmann (3)
Rhodotorula rubra (several mutants)	torulene, γ- and β- carotene, etc.	Al_2O_3 + $Ca(OH)_2$; light petr. + acetone	Bonner, Sandoval, Tang and author.
Mycobacterium phlei	leprotene, α- and γ-carotene	Al_2O_3 ; benzene + light petr. (1 : 3)	Takeda and Ohta (1, 2)
Mycobacterium (from mineral oil)	astacin, carotenes	MgO + sil. earth ; light petr.	Haas, Bushnell and Peterson
Micrococcus tetragenus	γ-carotene, lycopene, rubixanthin, xanthophyll, rhodoxanthin	$CaCO_3$; light petr.	Reimann and Eklund
Rhodospirillum rubrum (purple bacterium)	spirilloxanthin	$Ca(OH)_2$ + $CaCO_3$ (1 : 2) ; benzene + 0·5 per cent to 2 per cent acetone or light petr. + 5 per cent to 10 per cent acetone	Polgár, van Niel and author

TABLE 6

Examples of the Chromatography of Animal Carotenoids

Source	Carotenoid	Absorbent ; developer	Literature
Man (serum)	carotene (etc.)	Al_2O_3 ; light petr.	Emmerie and Wolff; Hoch; Jensen and With ; Willstaedt and With (3, 4)
Man (liver)	carotene	Al_2O_3 ; benzene + light petr.	Jensen and With
Man (fæces)	carotene, xanthophyll	Al_2O_3 ; light petr.	van Eekelen and Pannevis
Cow (butter, colostrum)	β-carotene	Al_2O_3 ; light petr.	Gillam and Kon
Cow (fæces)	lycopene	Al_2O_3 ; light petr.	Gillam and Kon
Sheep (fæces)	carotene, xanthophyll	sugar ; light petr.	Seybold and Egle (4)
Rat (fæces)	β-carotene	Al_2O_3 ; light petr.	Ramasarma and Hakim
Regalecus glesné	astaxanthin and ester	$CaCO_3$; light petr.	Kuhn and Sörensen (2)
Flamingo (fat)	fenicotterin	CaO ; light petr.	Manunta (1)
Bird's feathers	lutein, red pigment	$CaCO_3$; light petr.	Völker (3) ; Test
Bird's liver	carotene	Al_2O_3 ; benzene + light petr.	Jensen and With
Egg yolks	carotene, xanthophyll	MgO + supercel ; ether	Hauge, Zscheile, Carrick and Bohren
Egg yolks	β-carotene	bone meal ; light petr.	Mann (1)
Reptiles (liver)	carotene	Al_2O_3 ; benzene + light petr.	Jensen and With
Snakes (blood plasma)	carotenoids	$CaCO_3$ and Al_2O_3	Villela and Prado
Pila glauca (eggs)	chromoproteid		Comfort
Astacus gammarus (lobster shell)	ovoverdin	$Al(OH)_3$; water	Kuhn and Sörensen (1)
Salmo trutta (brown trout)	astacene (ex astaxanthin)	MgO or Al_2O_3 ; light petr.	Steven
Trombidium (mite)	red pigment	CaO ; benzene	Manunta (2)

Bee's wax	β-carotene, lutein-ester, etc.	Al_2O_3 ; light petr.	Tischer (3)
Silk worm	carotene, xanthophyll	sugar ; light petr.	Seybold and Egle (4)
Tetigonia viridissima (grasshopper)	carotenes, xanthophylls (with glaucobilin)	Al_2O_3 ; light petr.	Junge
Paroctopus bimaculatus (liver-pancreas)	xanthophylls and esters	$Ca(OH)_2$, $CaCO_3$	Fox and Crane
Paroctopus bimaculatus (ink)	xanthophyll-esters	$Ca(OH)_2$, $CaCO_3$	Fox and Crane
Mytilus californianus (sea mussel)	mytiloxanthin, etc.	$CaCO_3$; benzene	Scheer
Echinonderms	carotenes, xanthophylls echinenone	$Ca(OH)_2$, $CaCO_3$	Fox and Scheer
Anemones	astacene-esters, mitridene-esters, carotenes, xanthophylls	$Ca(OH)_2$ or MgO, $CaCO_3$; light petr.	Fox and Moe ; Fox and Pantin
Sponges	carotenes, echinenone	Al_2O_3 ; light petr. + benzene	Drumm, O'Connor and Renouf

Marine Muds. The chromatographic study of the carotenoids in oceanic muds was continued by Fox *et al.* Fox and Anderson eliminated greenish pigments from their epiphasic light petroleum extracts by filtration through calcium carbonate and adsorbed the carotenoid hydrocarbons, mainly β-carotene (and some red pigments), on calcium hydroxide forming the lower part of the column. The hypophasic lipochromes were resolved on calcium carbonate from light petroleum or benzene solution (fucoxanthin, lutein, zeaxanthin). Similar methods (using in part MgO instead of $Ca(OH)_2$) were applied in recent extensive studies by Fox, Updegraff and Novelli.

Cis-trans Isomeric Carotenoids

In stereochemical work in this field chromatographic methods occupy a unique position. No other existing procedures seem to make it possible to separate such isomers and to test the homogeneity of the individual compounds. A review of the literature extending to 1944 was given by the author (7).

Separation of α-Carotene Stereoisomers. To 6 mg. of pigment in 50 ml. of light petroleum a solution of 0·1 mg. of iodine was added and the liquid kept in intense daylight for 30 mins. The chromatogram was then developed with light petroleum + 0·5 per cent acetone on calcium hydroxide (24 × 4·8 cm.) (author and Polgár 4). (Height of zones in mm.)

10 colourless.
40 pale orange : neo-α-carotene U.
7 colourless.
10 light yellow : neo V.
7 colourless.
40 yellow : neo W.
2 colourless.
36 light orange : unchanged α-carotene (all-*trans*).
5 colourless.
25 yellow : neo B.
2 colourless.
2 pale yellow : neo C.
2 colourless.
5 pale yellow : neo D.
2 colourless.
5 pale yellow : neo E.

Separation of β-Carotene Stereoisomers. (Polgár and author 1). A solution of 25 mg. pigment in 150 ml. of light petroleum, after refluxing for 30 min. in a slow CO_2 stream, was developed on a 28 × 7 cm. calcium hydroxide column with light petroleum + 2 per cent acetone (on the left, height of the zones in mm.) :

2 brownish, irreversible.
15 colourless.
35 reddish orange : neo-*β*-carotene U.
2 almost colourless.
0·5 yellow, irreversible.
2 almost colourless.
85 dark orange : unchanged all-*trans*-*β*-carotene.
40 dark yellow : neo-*β*-carotene B.
2 almost colourless.
20 pale reddish : neo *β*-carotene E.
2 almost colourless.
12 yellow : a labile isomer.

A similar chromatogram can be obtained upon the addition of iodine (1 to 2 per cent of the pigment) to the solution at room temperature and illumination by a Mazda lamp from 60 cm. distance, for 15 min.

The relative efficiency of a great number of developers in the separation of all-*trans*-β-carotene, neo-β-carotene U and neo-β-carotene B was tested by Bickoff who made use (on lime) of LeRosen's technique. A small addition of anethole or *p*-cresyl-methylether to light petroleum was found to be most efficient, and it made the separation of stereoisomeric carotenes possible even in the liquid chromatogram.

Isolation of the Polycis compounds, Prolycopene and Pro-γ-carotene from Pyracantha angustifolia. The air-dried berries (1 kg.) were extracted with ether and, after saponification, transferred into light petroleum and chromatographed on lime, in a percolator (45 × 20 × 8 cm.). After developing with 5 l. of light petroleum and then with the same solvent + 1 per cent acetone, three main coloured sections appeared ; the middle one (7 cm. high) contained, among others, the desired poly*cis* carotenoids. The percolator was inverted and the cone removed by tapping on the glass. The main section was eluted with alcohol, transferred to light

petroleum, and developed with the same solvent containing 2 per cent acetone on a 28 × 7 cm. column.

80 minor zones near top.
35 bright orange : prolycopene.
5 traces of pigment.
12 yellow (unidentified).
10 minor zones.
50 orange : pro-γ-carotene.
10 yellow (unidentified).
20 minor zones.

Each of the pro zones was eluted with alcohol, and re-chromatographed as above. Finally, the solvent was evaporated, the residue dissolved in the minimum amount of benzene and crystallized by cautious addition of abs. methanol. Yield, 28 mg. of each pigment (author and Schroeder 3).

Stereoisomerization of Prolycopene by Iodine. To 5 mg. of pigment in 50 ml. of light petroleum 0·03 mg. of iodine (in the same solvent) was added and 2 min. later the solution was sucked rapidly into a 24·5 × 4 cm. calcium hydroxide column. Upon developing with petroleum ether + acetone (9 : 1) fourteen coloured zones appeared, each of which contained stereoisomers of lycopene. The top zone (all-*trans* form) was followed by a neolycopene A zone. The third zone from bottom contained unchanged prolycopene which corresponded to half of the total pigment. In contrast, if the catalyst was allowed to act for half an hour, a subsequent chromatogram was free of prolycopene, and it included three zones only, viz., all-*trans*-lycopene, neo A and neo B (LeRosen and author 2).

Separation of Poly-cis Lycopenes. From the ripe fruit of *Pyracantha angustifolia* seven poly-*cis* lycopenes (including prolycopene) were separated which all contain 4 to 7 *cis* double bonds. Their adsorption affinities are much weaker than those of all-*trans*-lycopene and neolycopene A. The chromatographic resolution of such stereoisomeric mixtures has been described in detail by the author and Pinckard. A suitable column material is calcium hydroxide + celite. As developer light petroleum containing 0 to 4 per cent acetone

was used. The isolation of the individual stereoisomers required repeated chromatographic separations.

Separation of cis-trans Isomeric Zeaxanthins (author and Tuzson 19; author, Cholnoky and Polgár; author and Lemmon). The solution of 114 mg. of homogeneous zeaxanthin in 200 ml. of benzene was kept with 0·4 mg. of iodine in daylight for several hours and developed with 80 ml. of benzene on calcium carbonate of medium strength (30 × 7·5 cm.):

12 orange yellow: neozeaxanthin A.
4 interzone.
30 light yellow: neozeaxanthin B.
50 interzone (with traces of neo C).
150 orange yellow (darker than A): unchanged all-*trans*-zeaxanthin.

The zones of neo A and B were eluted with alcohol and rechromatographed (26 × 4 cm.). After displacement of the benzene from the column with light petroleum, elution of the two neo zones with ether and evaporation, each residue was dissolved in cold methanol and made to crystallize by adding 50 per cent methanol until cloudiness appeared. In the ice box 13 mg. of neozeaxanthin A and 10·5 mg. of neo B crystallized out.

Separation of cis-trans Isomeric Fucoxanthins (Strain and Manning 3). Fucoxanthin when treated with iodine in light petroleum containing dimethylaniline is partially converted into the *cis* compounds *b* and *c*. The sequence on powdered sugar is: fucoxanthin *c* (top), fucoxanthin *b*, and ordinary (all-*trans*) fucoxanthin *a*.

Separation of cis-trans Isomeric Capsanthins. A solution containing 5 mg. of pigment in 50 ml. of benzene and 0·05 mg. of iodine was kept in daylight for an hour and developed with benzene on calcium carbonate (about 23 × 4 cm.) (author and Cholnoky 8).

10 red: neocapsanthin A.
6 red (different shade): neo B.
2 red: neo C.
20 colourless.
80 dull pink: unchanged capsanthin (all-*trans*).

Stereoisomerization Products of Natural Methylbixin (a mono*cis* form). The pigment (8 mg.) was fused, kept in a sealed capillary tube (filled with CO_2) at 165° for 1 min., dissolved in 5 ml. of benzene, diluted with 15 ml. of light petroleum and developed with benzene + light petroleum 1 : 5 (later 1 : 3) on calcium carbonate (Merck's heavy powder, 18 × 2·5 cm.) :

15 yellow : irreversible product.
3 colourless.
5 pink (traces).
50 red-orange : unchanged pigment.
10 pink : all-*trans*-methylbixin.
10 orange : neomethylbixin A.
9 yellow : mainly neo B.
2 darker orange : irreversible.
32 orange : neo C.
Filtrate : yellow (irreversible).

Exposure to *sunshine* of a solution of natural methylbixin in benzene + light petroleum, in a transparent glass tube, for 15 mins. yielded 8 per cent neo C and some minor isomers (author and Escue 3).

TABLE 7

EXAMPLES OF THE SEPARATION OF *cis-trans*-ISOMERIC CAROTENOIDS

Stereoisomeric set	Adsorbent ; developer	Literature
α-Carotene . .	$Ca(OH)_2$; light petr. + 0·5 per cent acetone	Author and Polgár (4) ; Nash and Zscheile
	Al_2O_3 ; hexane + 2 per cent ether	
β-Carotene .	$Ca(OH)_2$; light petr.	Carter and Gillam ; author and Tuzson (19) ; Polgár and author (1) ; Beadle and Zscheile
	Al_2O_3 ; hexane	
Carotene .	$Ca(OH)_2$; light petr. + 2 per cent acetone	Author and Polgár (6)

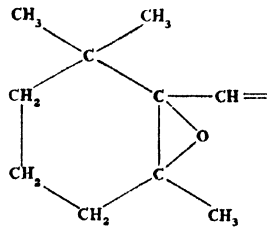
TABLE 7—(*continued*)

EXAMPLES OF THE SEPARATION OF *cis-trans*-ISOMERIC CAROTENOIDS

Stereoisomeric set	Adsorbent; developer	Literature
Lycopene	$Ca(OH)_2$; benzene + light petr. 3 : 1	Author and Tuzson (19); LeRosen and author (2); author, LeRosen, Schroeder, Polgár and Pauling
	$Ca(OH)_2$; light petr. + 10 per cent acetone	
	$Ca(OH)_2$; light petr. + 0–4 per cent acetone	Author and Pinckard
Lutein	$CaCO_3$; benzene + light petr. (1 : 1)	Author and Tuzson (19)
	MgO; dichloroethane	Strain (11)
Cryptoxanthin	$Ca(OH)_2$; benzene + light petr. (1 : 1)	Author and Tuzson (19); author and Lemmon
	$Ca(OH)_2$; light petr. + 10 per cent acetone	
Gazaniaxanthin	$Ca(OH)_2$; light petr. + 5-25 per cent acetone	Author and Schroeder (5)
Celaxanthin	$Ca(OH)_2$; benzene + 20 per cent acetone	LeRosen and author (1)
Zeaxanthin	$CaCO_3$; benzene	Author and Tuzson (19); author, Cholnoky and Polgár; author and Lemmon
	$CaCO_3$; benzene + light petr.	
Physaliene	$CaCO_3$; light petr.	Author, Cholnoky and Polgár
Taraxanthin	$CaCO_3$; benzene + light petr. (1 : 1)	Author and Tuzson (19)
Fucoxanthin	Sugar; light petr.	Strain and Manning (3)
Capsanthin	$CaCO_3$; benzene (+ 1 to 2 per cent acetone)	Author and Cholnoky; Polgár and author (3)
Capsanthin-dipalmitate	$CaCO_3$; light petr.	Author and Cholnoky (8)
Capsorubin	$CaCO_3$; benzene	Author and Cholnoky (8)
Capsorubin-dipalmitate	$CaCO_3$; light petr.	Author and Cholnoky (8)
Spirilloxanthin	$Ca(OH)_2 + CaCO_3$ (1 : 2); benzene + 0·5 per cent to 2 per cent acetone or light petr. + 5 per cent to 10 per cent acetone	Polgár, van Niel and author
Torulene	$Ca(OH)_2$; benzene	LeRosen and author (1)
Methylbixin	$CaCO_3$; benzene + light petr. (1 : 3)	Author and Escue (3)

Epoxides of Carotenoids

In a series of remarkable investigations Karrer *et al.* described epoxides of natural polyenes which undergo several interesting reactions and also lead to the interconversion of some carotenoids.



Characteristic end group of β-carotene mono- and di-epoxide.

Preparation of β-Carotene-mono- and di-epoxide; Mutatochrome, Aurochrome, and Luteochrome (Karrer and Jucker 5). To an abs. ether solution of 2·1 g. of β-carotene (containing a little α) perphthalic acid in ether was added (1·5 active O-atoms per mole). After 20 hours, the solution was repeatedly washed with bicarbonate solution and water and dried over sodium sulphate. The evaporation residue was adsorbed from light petroleum on calcium hydroxide (2 tubes, 70 × 4·5 cm.) and developed with the same solvent. The longest wave-length maximum of each zone in CS_2 is given below (thickness of zones in mm.):

Zone A (top), 20, orange yellow, 454mμ.
B 40, reddish orange, 498mμ.
C 30, yellow, 457mμ.
D 80, orange, 481mμ.
E 90, orange, 505mμ.
F 20, yellow, 507mμ.

After elution with methanol + ether, the zones A, B and C could not be made to yield crystalline material. Zone D gave, after recrystallization from benzene + methanol, 80 mg. of

crude luteochrome crystals. From zone E a crystalline mixture was obtained (700 mg.), in which β-carotene-di-epoxide was preponderant; and a similar mixture (100 mg.) from zone F with β-carotene-mono-epoxide as the main component. LUTEOCHROME (zone D) was rechromatographed from light petroleum on calcium hydroxide and the upper (main) zone (150 mm.) was divided into three equal parts, the maxima of which lay at 482, 482 and 502mμ respectively. The zones with maxima at 482mμ were combined (46 mg.) and rechromatographed in the same manner :

80 mm., yellow-orange; cut into five parts: 482, 482, 482, 482 and 485mμ.

15 mm., yellow : 482mμ.

5 mm., orange : 509mμ.

The four first sections yielded, upon crystallization from benzene + methanol, 20 mg. of pure luteochrome, $C_{40}H_{56}O_3$, m.p. 176° (uncorr., vac.).

β-Carotene-di-Epoxide (zone E). Rechromatography of the 700-mg. sample mentioned from light petroleum on calcium hydroxide gave two zones which were divided into seven and three parts respectively :

200 mm., yellow-orange, 481, 504, 504, 504, 504, 504 and 507mμ.

30 mm., yellow, 509, 508 and 508mμ (contained 220 mg. of crude mono-epoxide).

From the combined third to sixth sections of the upper zone, 200 mg. of well crystallized but not yet homogeneous di-epoxide (from benzene + methanol) was obtained; this was rechromatographed :

300 mm., yellow-orange, five sections : 480, 480, 502, 501 and 501mμ.

80 mm., orange, three sections : 508, 504 and 505mμ.

The third to fifth sections of the top zone (combined with another corresponding sample) were twice rechromatographed in an analogous manner, with very prolonged development

(light petroleum). The uppermost zone then contained pure di-epoxide, below which some mono-epoxide was adsorbed ; at the bottom another pigment appeared. Yield, 40 mg. of di-epoxide ; m.p. 184°.

β-Carotene-mono-epoxide (zone F). The development of 320 mg. of the crude product with light petroleum on calcium hydroxide gave :

250 m., orange, six sections: 505 (blurred), 506 (blurred), 506 (blurred), 503 (sharp) and 503 (sharp) mμ.

80 mm., orange, two sections : 502 and 507mμ.

The combined sections with maxima at 503mμ were used for the isolation of the di-epoxide, while the section with a maximum at 507mμ (combined with 60 mg. of similar material from other experiments) gave :

10 mm., yellow, 504mμ.
150 mm., orange, three sections : all 511mμ.
50 mm., yellow, 504mμ.

The centre zone yielded pure, crystalline β-carotene-mono-epoxide, $C_{40}H_{56}O$, m.p. 160° (after repeated crystallizations from benzene + methanol).

Conversion Products of the β-Carotene Epoxides with Acids. A fresh solution of 50 mg. of the mono-epoxide in 75 ml. of chloroform (old solvent, containing HCl) was allowed to stand for 4 minutes and was then de-acidified with sodium bicarbonate. The evaporation residue gave the following zones from light petroleum on calcium carbonate :

70 mm., yellow, 489mμ.
40 mm., reddish-orange, 521mμ. (yielded 10 mg. of *β*-carotene).

From the top zone, upon elution and two recrystallizations from benzene + methanol, 21 mg. of mutatochrome, $C_{40}H_{56}O$, were obtained, m.p. 164°. In an analogous experiment with 50 mg. of the di-epoxide the top zone yielded aurochrome, $C_{40}H_{56}O_2$ (10 mg.), the second zone 10 mg. of mutatochrome ; the third and fourth zones did not yield crystals, while from the bottom zone 3 mg of β-carotene was isolated.

Oxidation Products of α-Carotene (Karrer and Jucker 6). Under conditions analogous to those described for β-carotene, the oxidation mixture of 320 mg. of α-carotene gave the following zones (light petroleum, two columns, 70 × 4·5 cm. ; it is advisable not to increase the pigment content per column) :

30 mm., orange-brown.
80 mm., yellow, 482mμ. ; yield, 80 mg. of flavochrome.
20 mm., orange-yellow, 502mμ.; yield, 10 mg. of α-carotene-epoxide.
50 mm., yellow orange, 510mμ.

For further artificial conversion products of carotenoids, cf. Table 8, pp. 94–95.

TABLE 8

EXAMPLES OF THE CHROMATOGRAPHY OF ARTIFICIAL CONVERSION PRODUCTS OF CAROTENOIDS

Starting Material	Product	Adsorbent ; developer	Literature
α-Carotene	β-carotene	$Ca(OH)_2$; light petr.	Karrer and Jucker (10)
α-Carotene	5 : 6-dihydro-α-carotene	$Ca(OH)_2$; light petr.	Polgár and author (2)
α-Carotene	α-carotene-epoxide, flavochrom	$Ca(OH)_2$; light petr.	Karrer and Jucker (6)
β-Carotene	dihydro-β-carotene	$Ca(OH)_2$; light petr.	Karrer and Rüegger (2)
β-Carotene	5 :6-dihydro-β-carotene	$Ca(OH)_2$; light petr.	Polgár and author (2)
β-Carotene	β-carotene-epoxides, mutatochrom, aurochrom, luteochrom	$Ca(OH)_2$; light petr.	Karrer and Jucker (5)
β-Carotene	some vitamin A and other H_2O_2-oxidation products	Al_2O_3 ; light petr.	Hunter and Williams
Lycopene	bixin-dialdehyde, apo-1-bixin-dialdehyde, apo-2-lycopinal, apo-3-lycopinal	Ca(OH) ; benzene + light petr. (1 : 1)	Karrer and Jaffé
Lutein	acid conversion products	MgO ; light petr.	Quackenbush, Steenbock and Peterson
Lutein	desoxyluteins	$Ca(OH)_2 + CaCO_3$ (1 : 1) ; light petr. + 5 per cent acetone	Author and Sease
Xanthophyll-diacetate	α-citraurin	$(CaOH)_2$; benzene + ether (1:4)	Karrer, Koenig and Solmssen
Xanthophyll	zeaxanthin	$ZnCO_3$; benzene	Karrer and Jucker (10)
Xanthophyll	flavoxanthin, chrysantheraxanthin	$ZnCO_3$; benzene	Karrer and Jucker (4)
Zeaxanthin	antheraxanthin ; violaxanthin, auroxanthin, mutatoxanthin	$ZnCO_3$; benzene	Karrer and Jucker (4)
Capsanthin	capsanthin-epoxide, capsochrome	$ZnCO_3$; benzene + ether (1 : 1)	Karrer and Jucker (8)
β-Apo-2-carotinal	ketone with capsanthin chromophore	Al_2O_3 ; benzene	Karrer and Jucker (3)
Rhodoviolascin	bixin-dialdehyde	$Ca(OH)_2$; benzene + light petr. (1 : 1)	Karrer and Koenig
Crocin	tetradecaacetyl-crocin	Al_2O_3 ; methanol	Kuhn and Wang
Crocetin	*trans*-crocetin-bis-(2 : 3 : 4 : 6-tetraacetyl-β-*d*-glucose)-ester	Al_2O_3 ; methanol	Kuhn and Wang
Crocetin	*trans*-crocetin-(2 : 3 : 4 : 2'' : 3'' : 4'' : 6''-heptaacetyl-β-gentiobiose)-ester	Al_2O_3 ; methanol	Kuhn and Wang

Naturally Occurring Colourless Polyenes

Phytofluene ($C_{40}H_{64}+H_2$?). This colourless polyene-hydrocarbon is widespread in plants and is recognized on chromatographic columns by its intense greenish-gray fluorescence in ultraviolet light. It shows an unusually strong adsorbability considering that only five conjugated and two isolated double bonds are present. On calcium hydroxide its position coincides with those members of the stereoisomeric α-carotene set located directly below α-carotene. It is not easy to separate phytofluene from α-carotene itself by developing with light petroleum on calcium hydroxide, alumina or magnesia. On silicic acid (Merck, with 33 per cent celite) the sequence is inverted and phytofluene appears directly above α-carotene. When light petroleum + 0·25 per cent acetone is used as a developer on activated alumina (150–200 mesh), phytofluene forms a narrow zone which overlaps with α-carotene. The difference in the adsorbabilities increases on alumina + calcium hydroxide mixtures. Phytofluene spreads out on pure calcium hydroxide and at the same time a differentiation of some stereoisomers may take place. Using calcium hydroxide and light petroleum, the following sequence was observed: vitamin A (top); phytofluene; anhydrovitamin A; isoanhydrovitamin A. For the isolation of phytofluene from canned tomato paste, repeated adsorptions on alumina + calcium hydroxide mixtures and on pure alumina are recommended (author and Sandoval 1–3; author and Polgár 5; cf. Strain 6, 9, 15). The adsorption affinity of phytofluene is much weaker than that of some other fluorescing compounds that occur in green plants.

Further data on the chromatography of phytofluene: Mackinney and Fratzke; Porter and Zscheile (2); Sandoval, Meserve, Deuel and author; author and Haxo; Bonner, Sandoval, Tang and author.

Appendix: Some Synthetic Polyenes

Approximately the same methods as in the field of the carotenoids are being applied in that of synthetic polyenes; in particular, chromatographic filtration through alumina is used. For example, 1 : 6-diphenyl-1 : 6-dimethyl-hexadiene-(1 : 5), in toluene (Schmitt), and undecatetraene-(3 : 5 : 7 : 9)-one-(2), in benzene, were treated in this manner. For the latter treatment the adsorbent had to be freed from air by heating to 150-200° in high vacuum (F. G. Fischer and Schulze).

Diphenyloctatetræne: p. 196; diphenylbutadiene: p. 15.

CHAPTER VII

MISCELLANEOUS NATURAL PIGMENTS

Naphthaquinone, Anthraquinone and Related Pigments

Crystalline **Echinochrome,** $C_{12}H_{10}O_7$, was obtained by Lederer and Glaser from the ovaries of *Arbacia aequituberculata*. The violet ovaries were extracted with 80 per cent aqueous acetone containing 1 per cent acetic acid. After evaporation of the acetone, the aqueous residue was purified by a light petroleum extraction and the pigment was then extracted with ether. From this the pigment was transferred into aqueous sodium bicarbonate and, after acidification, again into ether which was washed, dried and chromatographed on calcium carbonate. The main brown zone was dissolved in HCl and the pigment was taken up with ether. After evaporation of the solvent, the residue can best be purified by vacuum sublimation. Dark red crystals ; yield, 1–1·5 mg. per ovary. The violet coloured quills of *Strongylocentrotus lividus* can be handled in the same manner ; however, above echinochrome there appears also a blue-violet zone of spinochrome, $C_{12}H_{10}O_8$, in this case.

Ether solutions of crude synthetic echinochrome A (2-ethyl-3 : 5 : 6 : 7 : 8-pentahydroxy-naphthaquinone-1 : 4) were chromatographed by Wallenfels and Gauhe on calcium carbonate.

Alkannan. The synthesis of this pigment was achieved by Brockmann and Müller who condensed isohexylaldehyde with naphthazarin. The crude product was dissolved in benzene + ligroin (high b.p.), 1 : 50, and developed on an " acid " silicic acid column. Three of the four zones were washed down with light petroleum, with frequent spectroscopic control of the flow. The second coloured zone contained the alkannan which was isolated by extracting with alkali, acidifying the blue solution and extracting it with benzene.

PTERINS

Uropterin (=Xanthopterin). The "xanthin-base" fraction obtained from urine can be used more conveniently than described earlier for the isolation of uropterin (according to Koschara 7), by means of a pressed layer of only 120 g. of frankonite KL on a 30 cm.-Buchner. The adsorbent is washed with 1 *l.* of phosphate buffer pH 7·6, whereupon 2 *l.* of the solution (adjusted with trisodium phosphate to pH 7·6) is introduced. The column should be washed with 2 *l.* of the pH 7·6 buffer and the pterin eluted with borate buffer pH 9·2. The methods were, with some modifications, used by Schöpf and Kottler for the isolation of xanthopterin from *Catopsilia rurina* (males).

A CLEAVAGE PRODUCT of pterins, ALLANTOIN, was isolated by Schöpf, Kottler and Reichert. From the wings of 16000 *Catopsilia rurina* (males) a crude xanthopterin sample was prepared which was dissolved in 2·8 *l.* of 2 *N*-ammonia, poured into 17 *l.* of boiling *N*/5 baryta and heated again to the boiling point. The hot solution was rapidly filtered (with exclusion of CO_2) into a bottle filled with N_2. Upon cooling *β*-xanthopterin barium crystallized out. Its filtrate was neutralized with 2*N*-sulphuric acid, the barium sulphate was eliminated in the centrifuge and the solution was concentrated to 1 *l.* in vacuo. The precipitated crude GUANOPTERIN was then sucked off, the filtrate acidified with acetic acid and filtered, first through frankonite KL and then through a 1-cm. layer of carboraffin. The allantoin (together with a blue fluorescing impurity) was eluted with 115 ml. of 2 *N*-ammonia + 115 ml. of alcohol. Evaporation in vacuo and trituration of the crystals with water resulted in the isolation of 0·25 g. of crude product and from this 0·14 g. of pure allantoin.

PTERIN-LIKE PIGMENTS were observed on small scale when Crowe and Walker chromatographed on alumina some ethyl acetate extracts of ultra-filtrates which had been obtained from diphtheria bacillus cultures.

UROTHIONE, $C_{11}H_{13}O_3N_5S_2$, (Koschara 8), occurs in crude pterin samples prepared from urine. A concentrate was obtained from 1,000 *l.* of urine by adsorption on 2 kg. of carboraffin C (nearly free of iron), elimination of uric acid and pterins by washing with strong alkali, and elution of the urothione with acetone + 0·4 *N*-ammonia (1 : 1). The concentrate was neutralized with H_2SO_4 until a small precipitate appeared. It was then adsorbed on 400 g. of Bleicherde XXF (diameter, 30 cm.). Upon washing with 2 *l.* of water the urothione was eluted with 50 per cent acetone. After concentration of the pigment solution to 0·3 *l.*, addition of 18 g. of sec. phosphate and dilution to 1 *l.* with 0·15 *M*-phosphate buffer pH 7·6, it was rechromatographed. This second column was washed with the same buffer and developed with 0·15 *M*-borate buffer pH 9·2. The solution was then ready for precipitation with mercuric sulphate.

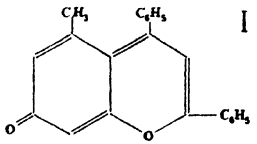
Anthocyanins and Related Pigments

Nudicaulin, the pigment of *Papaver nudicaule* (yellow Iceland poppy), an N-containing diglucoside with an anthocyanin-like structure was extracted by Price, Robinson and Scott-Moncrieff with methanolic HCl and, after neutralization with NH_3 developed with warm water on alumina.

For some *flavonol* glucosides cf. Bielig.

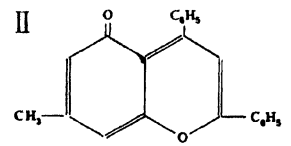
Dracorhodin, a 2-phenylbenzopyrilium derivative, which was isolated by Brockmann and Junge from " dragon's blood ", was purified by adsorbing 0·5 g. in 600 ml. of benzene on 80 g. of gypsum (dried at 120°). When developed with benzene + 0·5 per cent methanol, the red dracorhodin zone migrated into the filtrate and was ready for crystallization.

Synthetic Compounds, related to Anthocyanidins, were prepared by Brockmann and Junge (2, 3). The condensation product of orcine and benzal-acetophenone when chromatographed from benzene on gypsum showed below the red zone of the main *p*-quinoid compound (I) also the blue zone of the *o*-quinoid base (II). Similar observations were made on the condensation product of dibenzoyl-methane and phloroglucinol-monomethylether.

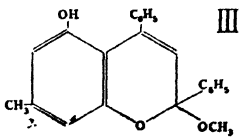


I

II

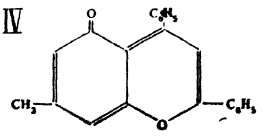


It is remarkable that the pyranol ether (III), which loses methanol and yields the anhydro base (IV) when heated in some solutions, undergoes the same change at 20° when adsorbed on alumina. The colourless benzene solution gives rise to a blue zone which can be eluted without colour change.



III

IV



Anhydro-7-hydroxy-2 : 4-diphenyl-benzopyranol could not be separated from chloranil-hydroquinone. However, by adsorption of the pigment on some aluminas, washing with benzene containing 2 per cent methanol and concentration of this filtrate, the red crystals of the anhydro base appeared. On alumina were also chromatographed : anhydro-7-hydroxy-6-methoxy-2 : 4-diphenylbenzopyranol and 7-hydroxy-8-methoxy-2 : 4-diphenylbenzopyrilium picrate.

PENITRINIC ACID. $C_{15}H_{17}O_5N$, a yellow pigment from *Penicillium notatum* was purified by filtering its ether solution through Brockmann's alumina (acid-treated, pH 4·5). The percolates yielded crystals (Stodola, Wachtel, Moyer and Coghill).

The eye pigment of *Drosophila melanogaster* (fruit fly) was resolved by developing with aqueous Na_2CO_3 on talc + celite (Wald & Allen).

AZULENES

Crude synthetic vetivazulene or 1 : 4-dimethylazulene can be purified by filtering cyclohexane solutions through alumina (Pfau and Plattner 2 ; Plattner and Wyss) ; 4 : 8-dimethyl-6-(hydroxy-isopropyl)-azulene and 4 : 8-dimethyl-6-isopropenyl-azulene were adsorbed from light petroleum on alumina and eluted with ether + acetone (Plattner and Roniger). Alumina was likewise used for the isolation of verdazulene from *Lactarius deliciosus* (Willstaedt 12, 13). Coats and Cook adsorbed vetivazulene from hexane and, after elution with alcohol and concentration, precipitated the reddish-brown needles of the 1 : 3 : 5-trinitrobenzolate. The latter type of compounds can be resolved on alumina by filtering a cyclohexane solution through the column as shown, e.g., for 1-(or 2- or 5-) methylazulene, 4 : 8-dimethyl-azulene and 4 : 8-dimethyl-azulene-6-carboxylic ester (Plattner and Fürst ; Plattner and Roniger 1, 2 ; Plattner and Wyss).

PHOTODYNAMIC PIGMENTS

Hypericin which shows brilliant red fluorescence in ultraviolet light occurs in St. John's wort (*Hypericum perforatum*). According to Betty and Trikojus it is there accompanied by a non-fluorescing precursor which can be converted rapidly, by exposure to light, into hypericin itself. The two compounds separate on calcium carbonate.

Pace and Mackinney as well as Brockmann, Pohl, Maier and Haschad believe hypericin to be a helianthrone or naphthodianthrone derivative. While Brockmann's sample was

chromatographically homogeneous, the substance obtained by Pace and Mackinney consisted of at least six components which could be separated by prolonged washing of, e.g., 150 mg. of crude hypericin, dissolved in 200 ml. of 80 per cent aqueous acetone, on a magnesium carbonate (Merck)-hyflo supercel (3 : 2) column (5 × 4 cm.). Several hours were required until this liquid chromatogram was completed. Quercetin, when present, was located between the two main pigment zones, termed hypericins X and Y.

Brockmann adsorbed his hypericin from nitrobenzene solution on calcium oxalate ; upon further developing with the same solvent, the pigment migrated slowly through the column. Chromatography was also used for the purification of some derivatives and cleavage products ; e.g., the crude hexabenzoate was dissolved in benzene + light petroleum (1 : 1) and filtered through calcium sulphate. The corresponding product of the benzoylation in a reducing medium formed a blue main zone on calcium carbonate.

The zinc dust distillation product of hypericin was adsorbed from benzene on alumina II. The main red zone, obtained by developing with benzene + light petroleum (1 : 1) showed golden-yellow fluorescence ; it was eluted with boiling benzene. Similar was the chromatographic technique for the investigation of analogous products prepared from 2 : 2′-dimethyl-helianthrone or 2 : 2′-dimethyl-naphthodianthrone. From a mixture, obtained by the HI-reduction and subsequent dehydrogenation of hypericin, anthrodianthrene was isolated by adsorbing the benzene filtrate from naphthodianthrene on alumina III, then on II. A red zone was eluted from the latter with benzene + methanol and yielded anthrodianthrene upon sublimation in high vacuum.

Three crystalline PHOTOSENSITIZING COMPOUNDS of unknown structure were obtained by Wender, Gortner and Inman from buckwheat (*Fagopyrum esculentum*). An 80 per cent acetone extract was transferred into ether which was washed and evaporated. Upon adsorption on a talc column, with anhydrous sodium sulphate packed on the top, one blackish-purple substance was retained by the sulphate ; two other pigments on the talc were poorly separated. From the sulphate the compound was isolated after solution of the adsorbent in warm water. It was then dissolved in glacial acetic acid, and precipitated with ether. The talc was eluted with alcohol and the pigments were transferred into ether from which solution 15 per cent HCl removed some impurities, and 23 per cent acid the second photosensitizer ; the third was extracted from the ether with bicarbonate.

SYNTHETIC DYESTUFFS

The chromatographic information available in this important field is still very scattered. In a short survey Wykypiel stressed the use of chromatography for testing common dyes for uniformity in aqueous or pyridine solution. For the fluorescence of certain dyestuffs on different fibres, cf. Grant.

Toluylene brown samples prepared by different methods were chromatographed on alumina by Ruggli and Fischer and shown to have great heterogeneity, depending on the method of preparation.

Congo red in water separates on calcium carbonate into a blue adsorbate and a red filtrate. **Diamine Blue 3R** gives a red and a less stable blue component, which perhaps differ in dispersion (Haller).

The azo compounds obtained from opsopyrrole and benzene diazonium salt were adsorbed from chloroform on alumina; only the monoazo dye passed into the filtrate (Fischer, Reinecke and Lichtenwald).

Rieman recommends as a demonstration experiment the separation of **Victoria Blue B** (top), crystal violet and **Auramine** from aqueous solution on alumina-hyflo supercel (developer, phosphate buffer pH7).

For the resolution of azo dye mixtures cf. also Ruiz.

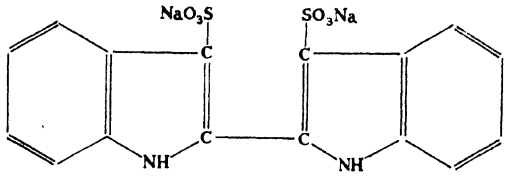
Di-chaulmoogroyl-fuchsin (Wagner-Jauregg and Reinemund). 3·3 g. of fuchsine (dried at 100°, in vac.) was refluxed in 150 ml. of abs. pyridine with 5·7 g. of chaulmoogroyl chloride for 1–2 days. The decanted solution was evaporated and the residue was dissolved in a little alcohol + HCl. After dilution with ether, the solution was washed with dilute NaOH, HCl, and water, then filtered. The evaporation residue of the dried ether solution was dissolved in benzene and adsorbed on alumina. Brown impurities were removed by washing with benzene containing 2 per cent methanol; the

main product could be eluted with benzene + 32 per cent methanol. Yield, after reprecipitation from ether + petroleum ether, 0·3 g.

Estimation of Evans blue in Plasma and Serum (Morris). A 2-cm. column of magnesium oxide (Anala R) + aluminium hydroxide 1 : 20 retains this dye quantitatively from a mixture of 1 ml. of dyed plasma or serum + 0·3 ml. of 2 *N*-NaOH. After washing with water (removes proteins, etc.) and with glacial acetic acid + 70 per cent alcohol (1 : 9) (removes haem pigments) the blue zone can be eluted with abs. ethanol + conc. HCl + water (4 : 1·25 : 1) and estimated colorimetrically.

Oxazine Pigments obtained by condensation of β-naphthol-3 : 6-disulphonic acid (or the corresponding 6 : 8-acid) with *p*-nitrosodimethylaniline can be chromatographed in 80 per cent alcohol solution on Brockmann alumina. Suitable eluents are : water, or (for the 6 : 8 compound) *M*/15-KH_2PO_4 (Eggers and Dieckmann).

Indigo Sols (Ruggli and Stäuble).



Indigo sol O

(" Indigo sol O 4B " is a tetrabromo derivative of sol O ; " Indigo sol rose IR extra " is a dimethyl-dichloro derivative.)

The " indigo sols " are alkali salts of acid sulphates of leuco vat dyes. Some representatives of this group were chromatographed on calcium carbonate (activated according to Ruggli and Jensen) or on Merck's alumina. For 2 mg. in 5 ml. of water a 17 × 1·3 cm. column was needed ; as developer water was added in 5-ml. portions. The three compounds mentioned passed fairly rapidly through the column. In mixtures the highly dispersed indigo sol rosa appeared first in the filtrate

(it also has the highest diffusion coefficient in gelatin), then indigo sol O, and very close to it O 4B.

The filtrate (collected in 5 ml. portions) was filtered into a thrice diluted ferric-ion reagent (100 g. of cryst. ferric-ammonium sulphate, 500 ml. of 10 per cent sulphuric acid, and 1500 ml. of water) which oxidizes each indigo sol to the corresponding insoluble dye. This reaction takes place with indigo sol rosa or O 4B only on heating but with O at room temperature. For the detection of the " rosa " the ferric solution of 60–70° is applied, while to differentiate between O and O 4B each 5 ml.-portion of the chromatographic filtrate is first treated with the cold reagent and then the dye formed is filtered off and the filtrate heated to test for O 4B. Compound O appeared in the fourth 5 ml. portion ; the fifth to seventh fractions contained both O and O 4B, but the eighth only O 4B (in the last fraction a precipitate appeared upon heating only).

A more complicated polycyclic compound, viz. " indigo sol-green IB " is adsorbed near the top of the column, forming a red zone which, upon washing with the ferric reagent, turns green.

CHAPTER IX

MISCELLANEOUS ALIPHATIC SUBSTANCES

The results in this field are rather limited because the low adsorption affinity of many simple compounds excludes the use of chromatography proper. Frontal analysis : Claesson (8).

The presence of NaCl seems to increase the adsorbability of lower fatty acids on animal carbon (Quartaroli).

Hydrocarbons. Some data on the adsorption of saturated light hydrocarbons on activated charcoal were reported by Crawley. Tetracosane (or octadecyl-cyclohexane) can well be separated from di-*sec.*-butyl-decahydro-naphthalene using floridin and pentane ; the paraffins are adsorbed more strongly (Nederbragt and de Jong). A paraffin, $C_{18}H_{38}$ (pristane?), obtained from gray amber was purified by a liquid chromatogram (alumina, light petroleum ; Lederer 19).

***d*-α-Hexadecyl-glycerylether** (=Chimylalcohol = "Testriol"), which was obtained from a fraction of pigs or bulls testes and contained the lipoids with a negative Girard T reaction, was isolated by a liquid chromatogram on alumina. Chimylalcohol was present partly in the last fractions, and the remainder could be obtained by boiling the adsorbent with 5 per cent acetic acid (Prelog, Ruzicka and Steinmann). The di-*p*-nitrobenzoate can also be purified on alumina. Prelog and Beyermann isolated chimylalcohol from pig's spleen.

Some dehydration products of a hexenyl-acetylenic glycol were separated in the form of their dinitrophenylhydrazones by Marvel and Walton (alumina, benzene).

Some volatile **Carbonyl compounds** (acetaldehyde, propionaldehyde, etc.) occurring in the urine were partially separated by Matthiessen, Lipp, Lipp and Vorwerk. After an acid distillation of 3600 *l.* of urine and isolation of crystalline 2 : 4-dinitrophenylhydrazones, some of these fractions could be resolved on frankonite from light petroleum or chloroform solution. Roberts and Green recommend for the separation of the 2 : 4-dinitrophenylhydrazones of simple aliphatic compounds the adsorption from benzene + light petroleum (1 : 2)

and developing with light petroleum + 4 per cent ether on silicic acid-super cel. The top to bottom sequence of the dinitrophenylhydrazones was: acetaldehyde; acetone and propionaldehyde (not separable); and methylethylketone. The zones can be eluted with ether, and the products crystallize from alcohol. Laurylaldehyd-2 : 4-dinitrophenylhydrazone can be purified by developing with benzene on alumina "Alorco" and eluting with alcohol + ether (Haagen-Smit, Redemann and Mirov). For the chromatography of some condensation products of citral, cf. Heilbron, Johnson, Jones and Raphael. The following aldehyde derivatives can be conveniently purified on alumina from benzene solution: *p*-nitrophenylhydrazone of propionaldehyde, and the *p*-phenyl-phenacylester of α- or β-hydroxybutyric or isobutyric or isovalerianic acids (Ruzicka, Dalma, Engel and Scott).

Lower molecular Saturated Aliphatic Acids (formic, acetic, propionic, butyric and valerianic) show selective adsorbabilities when their 1 per cent aqueous solutions are chromatographed on activated carbon (Claesson 1); however, no quantitative and sharp separations can be obtained. Some exploratory work has been done by Tiselius (9) on the separation of aliphatic acids which were usually dissolved in 0·05 to 0·1 *N* sulphuric acid, in order to avoid dissociation. Alkali salts are much less adsorbed, e.g., sodium acetate gives practically no retardation.

Caliri adsorbed a mixture of acetic and oxalic acids (each 0·06 *N*) on charcoal (16 × 2 cm.). Upon washing with water 50-ml. portions were taken. At first only acetic acid appeared in the filtrate; its maximum quantity was reached in the 8. portion when oxalic acid just became detectable. The latter became predominant from the 18th portion on. In an experiment with a ternary mixture, boric acid appeared in the 3., acetic acid in the 6., and oxalic acid in the 8. fraction.

E. L. Smith briefly pointed out that it is possible by using partition chromatography to achieve quantitative separations of formic, acetic, propionic, butyric and valerianic acids. For example, 1 per cent of acetic in propionic acid or the same

amount of formic in acetic can be detected, because the first acid of each pair possesses the stronger adsorption affinity. If the quantities were reversed, the lower zone would be engulfed by the main one.

The estimation of volatile fatty acids by partition chromatography in the silica gel, water, chloroform system, with bromcresol green as an indicator, was recently described by Elsden, especially for propionic, *n*-butyric and *n*-valeric acid. Formic acid could not be determined and acetic acid only indirectly.

In order to overcome some difficulties in the one-dimensional partition chromatography of acids (possessing low volatility) on a moist paper sheet, Lugg and Overell recommend as the mobile phase the use of *n*-butanol which has been saturated with acetic acid. The latter is eventually removed from the paper by drying.

A more detailed study of the separation on the micro scale of FORMIC, ACETIC, PROPIONIC AND BUTYRIC ACIDS by partition chromatography was recently published by Ramsay and Patterson (*n*-butyric and isobutyric acids could not be separated from each other). The adsorption system consists of silica gel, saturated with water and containing an indicator.

For amounts of acid up to 2 ml. $N/10$ a tube, 30 × 1·1 to 1·3 cm., is used; but for 2–6 ml., a 15–20 × 2·2 to 2·5 cm. tube. The procedure for the smaller column is as follows. To 5 g. of commercial silicic acid (or 3 g. of a gel prepared in the laboratory) 1 ml. bromcresol-green (100 mg. in 25 ml. of water + 1·5 ml. of $N/10$ ammonia) and sufficient NH_3 for producing the alkaline colour are added, then water, the amount of which must be determined for each batch (e.g., 1·5 ml.). A slurry is made with a few ml. of 1 per cent butanol + chloroform in a mortar and then 25–30 ml. of solvent are added. A wet column is prepared and the excess liquid is pressed or sucked off without letting the material dry out. The acid solution is introduced. The latter can be obtained from distillates by evaporation of the neutralized solution to dryness, acidifying with a small excess of sulphuric acid, adding sodium sulphate to form a semi-solid mass, and extracting the material thrice with

1 per cent butanol in chloroform. The individual zones can be titrated in the filtrate but the final identification of the acids must be achieved by methods other than chromatography.

The separation of the zones can be easily observed if the ammonium salt of 3 : 6-disulpho-β-naphthalene-azo-N-phenyl-naphthylamine (" R-NH_4 indicator ") is applied. The initial blue band is resolved into four distinct zones, the higher molecular acids moving faster.

Formic acid cannot be desadsorbed in the usual manner ; consequently, it is eluted (after the other acids have passed through) by extruding the column and extracting it with excess alkali. After acidification and distillation the formic acid can be titrated.

Small amounts of Acetic Acid, e.g., in distillates, can be detected, according to Shoppee and Reichstein (3) as follows. The solution containing about 75 mg. of the acid was neutralized with *N*/1-NaOH, evaporated and dried at 50°. After elimination of phenolphthalein by repeated ether extractions the residue was refluxed with 330 mg. of *p*-phenyl-phenacyl-bromide (*p*-phenyl-ω-bromoacetophenone) in 7·5 ml. of abs. alcohol for an hour. Upon evaporation the substance was transferred with water into benzene which was washed, dried with sodium sulphate and evaporated. Its pentane solution was chromatographed on 9 g. of alumina (Merck, Brockmann) which was prepared with pentane. Thirty ml. of benzene + pentane (3 : 10) washed down some *p*-phenyl-phenacylbromide ; the next 30 ml. of the same mixture gave only little material, whereupon 150 ml. of benzene + pentane (4 : 10) eluted the *p*-phenyl-phenacylacetate. Yield, after recrystallization from ether + pentane, 130 mg. ; m.p. 110–1°.

Separation of the *p*-Phenylphenacyl esters of Fatty acids (Kirchner, Prater and Haagen-Smit). These esters of acetic to caproic acid can all be separated from the following or preceding homolog. In this case, the higher molecular weight esters are less strongly adsorbed. The corresponding C_4, C_5, and C_6 iso-acids form an analogous series. Since the separation is based on the blue fluorescence of adsorbates, a

previous, thorough removal of fluorescent contaminants is necessary. For this purpose shaking the alcohol solution with decolorizing charcoal is recommended, followed by chromatographic purification.

Example for the separation: 16·9 mg. of butyric derivative and 17·1 mg. of acetic derivative were developed with a 1 : 1 mixture of benzene + light petroleum (b.p. 80–90°) on Merck silicic acid (reagent grade: 18 × 1 cm.). The upper fluorescing zone (25 mm.) was separated by a 6-mm. interzone from the lower fluorescing zone (25 mm.). The first zone yielded 16·3 mg. of acetyl derivative, and the second one 16·8 mg. of the butyric derivative. Capric and undecylic or lauric and myristic phenylphenacyl esters did not separate.

For higher fatty acids see p. 110.

Some $\triangle^1$- and $\triangle^2$- **Unsaturated Dicarboxylic Acids** (analogues of traumatic acid) were synthesized by English. The ether solution of the crude products was passed through decolorizing carbon + supercel (1 : 1). Only 4–5 parts of carbon were needed for 1 part of substance. Upon washing with dry ether and then with ether + acetone (3 : 1) some by-products and $\triangle^2$-acids first appeared in the filtrate and were followed by the fairly pure $\triangle^1$-acid, e.g., $\triangle^1$-octene-1 : 8-dicarboxylic acid.

Oxinite. Öhman carried out a Tiselius frontal analysis on "ethylene-oxinite" which was obtained by electrochemical nitration of ethylene at an anode giving a high over-potential. By integration of the curve and comparing with a control experiment (mixture of pure nitroglycol, butyl nitrate and diglycol dinitrate) the ratio of these three components in the sample could be calculated.

CHAPTER X

LIPOIDS

Higher Fatty Acids

For the purification of crude samples a chromatographic filtration may be of considerable use and could well precede further analytical operations. Kurz found that the filtration of a 5 per cent. light petroleum solution through silica gel eliminated various contaminants such as pigments, oxidation or polymerization products. Furthermore, chromatographic homogeneity will certainly become a standard test for highly purified fatty acids. According to Kaufmann (1) even the purest samples now available could be divided into fractions with different iodine numbers by the use of benzene or light petroleum solutions and silica gel.

The important problem of a rapid and complete resolution of fatty acid mixtures is unsolved. For example, Manunta's (1) results with palmitic and stearic acids on magnesium sulphate or frankonite were evidently far from being quantitative. As matters stand, adequate methods will have to be found empirically, since individual adsorption isotherms do not constitute a reliable basis for prediction. According to Cassidy (2, 3) the reciprocal influence of the individual fatty acids present in the same solution is quite considerable. It seems that the dependence of adsorption affinity on chain length is also a complicated one, as illustrated by the following facts (Cassidy). Lauric acid was adsorbed more strongly from light petroleum on certain brands of silica gel or carbon than stearic acid while other samples showed the opposite behaviour Evidently, some adsorbents prefer the lower molecular fatty acids while others adsorb the higher molecular ones more strongly. In numerous instances no differentiation occurs. Some alumina samples adsorbed about equal molecules/g. of lauric, myristic, palmitic and stearic acids.

Some separations required as much as 100 days. In a nine-hours' experiment with a mixture of lauric, myristic, palmitic and stearic acids in light petroleum pure lauric and nearly pure stearic acid were obtained with an overall recovery of 91 per cent.

The solution in 100 ml. of light petroleum of lauric and stearic acids (about 0·15 g. of each) was adsorbed on Carbon " 1 " (activated, Merck, 3 × 1·3 cm, ; air pressure, 20 cm. Hg) and washed with the same solvent. The first 300 ml. eluted almost pure lauric acid (0·24 g.), the next 100 ml. some impure acid ; the next 300 ml. a mixture of about equal parts of the two acids ; finally 200 ml. of light petroleum containing 2 per cent methanol brought down 0·12 g. of pure stearic acid.

Graff and Skau used heavy magnesia impregnated with an alcohol solution of phenolsulphonphthaleine (phenol red) and dried at 100° ; the individual acids formed yellow zones.

On Brockmann's alumina Kaufmann (2) carried out some separations ; the composition of the fractions was established by melting point diagrams, refraction data and iodine numbers. A mixture of equal parts of stearic and myristic acid, in 10 parts of benzene, was sucked slowly through 6 parts of the adsorbent. The upper third of the column contained pure stearic acid, the filtrate nearly pure myristic acid, while mixtures occurred in the rest, as shown after (imperfect) elutions with acetone. In contrast, the system silica gel-trichloroethylene allowed an accumulation of the higher molecular acids in the filtrate. It is rather surprising that, according to Kaufmann's iodine numbers, some saturated acids were more strongly adsorbed than unsaturated ones with comparable chain lengths.

The frontal analysis of fatty acids has been studied by Claesson (4).

Two monobasic LACTONIC ACIDS, viz. nephrosterinic acid, $C_{17}H_{28}O_4$, and its dihydro derivative, nephrosteranic acid, which occur in the lichen, *Nephromopsis endocrocea* were separated by Asahina and Yanagita.

One gram of the mixture in 50 ml. of benzene was developed with 100 ml. of benzene on Brockmann alumina (50 × 1 cm.). After the column had been cut into five parts, each was eluted with warm glacial acetic acid and precipitated with water. The top fraction contained 0·1 g. of the unsaturated and the bottom part 0·1 g. of the saturated acid mentioned (m.p. 96° and 95°).

Frontal analysis of fatty acid esters : Claesson (4).

Some *cis-trans* isomeric unsaturated acids, e.g., oleic and elaidic, can be separated by fractional elution from silica gel (benzene, petr. ether ; Kaufmann and Wolf).

Autoxidation of Methyl Oleate. Atherton and Hilditch stirred 55·4 g. of this ester with oxygen during daylight for

about 800 hours. The solution of the product in 250 ml. of light petroleum (b.p. 40–60°) was developed on a 36-cm. column of activated and dried silica gel with 450 ml. of the same solvent. The filtrate contained chiefly unoxidized methyl oleate. On the basis of peroxide and iodine values the column was divided into an upper (19·5 cm.) and a lower part. After elution with acetone and permanganate oxidation in the same solvent, it was found that the product from both sections consisted of a mixture of suberic and azelaic acids (isolated from the upper part: 0·7 g. and 1·0 g.; lower: 0·8 g. and 1·6 g.).

Various Constituents of Lipoids

A general survey has been published by Heinz.

It is desirable to work out efficient methods for a quantitative separation of the different types of compounds that occur in fats.

Recently, this aim was partially achieved by Trappe (2). He found that the sequence of decreasing adsorption affinities on alumina or silicic acid is: phosphatides, fatty acids, cholesterol, triglycerides, cholesterol-esters, aliphatic hydrocarbons. On acid earths the sequence of cholesterol and triglycerides was inverted. The technique consisted in preparing a liquid chromatogram by using a series of specific developers to separate each type of compound.

(*a*) On Brockmann's alumina (Merck) the difficulty arose that unsaturated fatty acids underwent autoxidation and, furthermore, that triglycerides (e.g., in CCl_4) suffered a partial saponification. Because of the latter process some triglycerides could not be eluted by benzene, although they are but poorly adsorbed from this solvent. Alumina, however, can be used for a separation of the hydrocarbons and cholesterol-esters from other fat components. For example, 350 mg. of an artificial mixture of oleic acid, palmitic acid, cholesterol, cholesterol-ester, paraffin and cetene was adsorbed from 25 ml. of light petroleum on a 5·2 × 2·5 cm. column. Development with 50 ml. of light petroleum carried the hydrocarbons, and a subsequent washing with 50–75 ml. of CCl_4 the cholesterol esters quantitatively into the filtrate.

A complete elimination of free acids is possible by using chloroform, which washes down all the other types. The free fatty acids also remained on the column, when the lipoid was first saponified and the chromatogram was washed with light petroleum, CCl_4 and benzene ; the free cholesterol could then be eluted with chloroform + 10 per cent. alcohol.

In all these experiments the breaking of the alumina column during washings was prevented by a thin layer of kieselguhr + $BaSO_4$ on the top.

(*b*) On silicic acid (Schering ; heated at 125° for several hours) 265 mg. of an artificial mixture was chromatographed (2·1 × 2 cm.). Twenty-five ml. of light petroleum removed the hydrocarbons quantitatively, whereupon the elimination of cholesterol esters was achieved with 50 ml. of trichloroethylene. Finally, 50 ml. of ether eluted the triglycerides + cholesterol + fatty acids.

(*c*) From adsorbates on commercial acid earths, e.g., frankonite KL (treated with warm dilute HCl, water, acetone, ammonia, water, and dried at 120°), the hydrocarbons were washed down with CCl_4, then the cholesterol esters with benzene and, finally, the triglycerides + free cholesterol + fatty acids with ether + 10 per cent alcohol ; phosphatides remained on the column. (On earths containing mineral acid cholesterol undergoes a catalytic alteration.)

These methods should be further improved. It seems that for many purposes a preliminary saponification would be a clear advantage. It must also be noted that the reported method does not allow individual separations within each class. It may be of some use in the future that, according to Trappe, the adsorbability of oleic acid, triolein, and free or esterified cholesterol on aluminium silicate, silicic acid, kaolin and Brockmann's alumina increases in the following series of solvents : methanol, ethanol, propanol, acetone, ethyl acetate, ether, chloroform, methylene chloride, benzene, toluene, trichloroethylene, carbon tetrachloride, cyclohexane, and light petroleum (b.p. 30–50°).

Glycerides. Claesson (1) investigated the behaviour of (mostly binary) mixtures of trilaurin, trimyristin, tripalmitin and triolein in ether solution on activated carbons.

A separation of glycerides from fatty acids was carried out by Kaufmann and Schmidt with a view to a possible freeing of commercial fats (cf. Tischer and Illner).

A mixture (acid number, 44) of 8 g. of olive oil with 2 g. of stearic (or oleic) acid in 75 ml. of benzene was sucked through 60 g. alumina (Merck; diam., 1 cm.). The filtrate possessed an acid number of 0·4, and the four fourths of the column (eluted with acetone), from top to bottom, 129, 1·8, 1·6, and 0·8.

From *linseed oil* Walker and Mills (1,2) isolated some unsaturated triglycerides on the basis of the iodine numbers of eluates obtained from different parts of the alumina column.

For the isolation of some constituents from rye germ oil cf. Friese, Benze, Pommer and Wiebeck.

Squalene, $C_{30}H_{50}$, as obtained from liver oils, can be freed from oxygenated impurities by adsorption on alumina from light petroleum (Farmer and Sutton). It has been separated from ergosterol by Täufel, Thaler and Widmann. When a pure hexane solution (5 ml.) containing 1 g. of each compound was washed down an 8-cm. alumina column with 15 ml. of hexane, the evaporation residue of the filtrate (0·98 g.) was free of ergosterol. However, because of the presence of contaminants, chromatography alone seems to be unsatisfactory for an estimation of the squalene content in the unsaponifiable residue of fats. A preliminary chromatographic accumulation may be useful before the squalene, e.g., in yeast fat, is estimated as its hexa-chlorohydrate.

UNSAPONIFIABLE SUBSTANCES IN LIVERS. Some preliminary experiments with human livers were reported by Bürger and Plötner. Stanger, Steiner and Bolyard fractionated cholesterol-free residues (75 g. in 1 *l.* of light petroleum, Brockmann's alumina, 24 × 4·6 cm., developed with the same solvent). No chemically pure substances were obtained.

Some higher alcohols and hydrocarbons that occur in the unsaponifiable residues of some liver oils were separated on alumina by Nakamiya. The sparse data available refer to batyl-, chimyl-, selachyl- and oleyl-alcohols, also to squalene, pristane, gadusane, etc.

Wax. In an alumina chromatogram, prepared by Tischer and Illner from beeswax in benzene, the sequence was: free acids (top), wax esters, high molecular wax alcohols, hydrocarbons.

P- and N-containing Lipoids. The elimination of phosphatides (plus free fatty acids) was carried out by Trappe (3) who filtered the chloroform solution of a lipoid mixture through alumina. The other constituents were scarcely adsorbed and the filtrate was found N- and P-free (ratio, fat : adsorbent = 1 : 15).

When crude soybean oil is treated with a synthetic aluminium silicate, complete removal of the phosphatides takes place (Thornton, Kraybill and Mitchell).

A quantitative separation of the choline-containing phospholipoids (lecithin, sphingomyelin) from the corresponding choline-free compounds (cephalin) of liver extracts was reported by Taurog, Entenman, Fries and Chaikoff. They found that only the latter are adsorbed on Merck's U.S.P. light magnesia from methanol solution. First, both types were adsorbed from light petroleum, whereupon the choline-containing fraction was eluted with methanol. The adsorptive capacity of the MgO used was about 2 mg./g. No columns were employed and the operations were carried out in centrifuge tubes. (A cloudiness of methyl alcohol eluates can be cleared up by small quantities of NaCl.)

The cerebroside content of sphyngomyelin samples was eliminated by Thannhauser, Setz and Benotti by filtering the methanol and light petroleum (1 : 10) solution through alumina ; the cerebroside remained adsorbed. On the other hand, cerebrosides as well as gangliosides (a class of sugar-containing brain lipoids) can be freed from a phosphatide content by filtering the pyridine solution through Brockmann's alumina, which retains the phophatides only. The filtrate is then P-free. Some other solvents do not produce this effect (Klenk ; Klenk and Rennkamp).

CHAPTER XI

CARBOHYDRATES

Adsorption isotherms of sugars have been repeatedly studied (e.g., on charcoal by Gyani) but chromatographic work proper has been carried out on free carbohydrates only recently. A significant development for which Wolfrom and his group must be credited includes the chromatography of free sugars and sugar derivatives, the zones of which are located by brushing the column with alkaline permanganate. Aqueous solutions of some sugar phosphates have been submitted to chromatographic treatment on ion exchangers. In non-aqueous media acetylated or methylated sugar derivatives, etc., are being investigated in different laboratories. Since these compounds occur in cleavage products of high molecular carbohydrates, such experiments will contribute to the structural clarification of polysaccharides. A different aspect of sugar chromatography has been opened up by the study of coloured esters as well as by some applications of the Tiselius-Claesson method. For paper chromatography, cf. Partridge ; Flood, Hirst and Jones. Bibliography : Binkley and Wolfrom (4).

Free Sugars

Tiselius Analysis. According to Tiselius (6), it is not possible to resolve, e.g., glucose and fructose or sucrose and lactose mixtures on active carbon. Arabinose and glucose gave a small separation. Evidently the glucose and galactose molecules behave in such a similar manner that they may substitute for each other in the adsorption isotherm. In one experiment the retention volumes were : 1 per cent solution of glucose alone, 4·37 ml. ; 1 per cent galactose alone, 4·15 ml. ; however, a mixture of the two solutions showed a single schlieren band corresponding to 3·10 ml.

Using glucose and lactox on Carbo active (Kahlbaum), the result as represented in Fig. 18 was obtained within 1 to 2 hours.

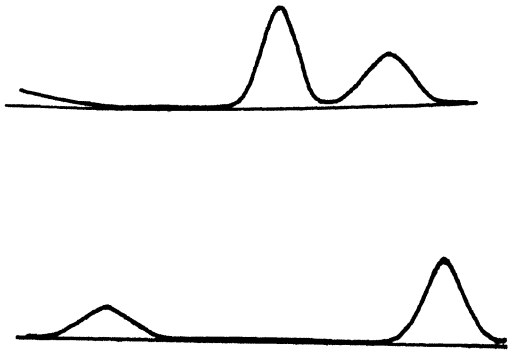


FIG. 18.—Diagrams obtained by the Philpot-Svensson method: 1% NaCl, 1% glucose, 1% lactose, small apparatus; filter cell, 10 mm. Flow from left to right. Upper diagram: glucose and NaCl, lower diagram (taken later), left to right, lactose and glucose. (Ordinate, difference in the refractive index between the solution and pure solvent.) (Tiselius).

The separation of di- to hexasaccharides can be carried out according to Tiselius (6) and Tiselius and Hahn who used ephedrine as a displacer. Fig. 19 shows that glucose (which is

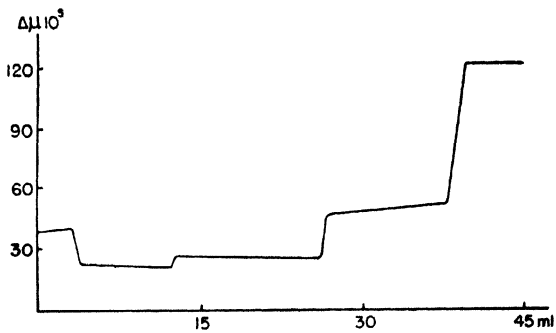


FIG. 19.—Artificial mixture of 15 mg. of glucose, 7·5 mg. of sucrose, and 30 mg. of raffinose, displaced by 0·5% ephedrine, on carboraffin (Tiselius and Hahn).

very weakly adsorbed when leaving the column) forms a " free " zone, while the higher saccharides appear in stationary concentrations and form horizontal sections of the curve whose height is characteristic for each saccharide type. In contrast, the individual variations within the respective classes of saccharides are irrelevant.

Irreversible adsorption on the carbon may bring about considerable losses. Tiselius and Hahn found that a previous treatment of the adsorbent with a dilute solution of the displacer is helpful. Furthermore, such treatment diminishes the individual stationary concentrations to various extents and thus increases the chances of separation.

A mixture of different saccharides, obtained by the enzymatic cleavage of corn starch and subsequent fractionation with alcohol, was adsorbed on active carbon (treated with 0·25 per cent ephedrine), whereupon the displacement was carried out with 0·5 per cent ephedrine (Fig. 20). On the basis

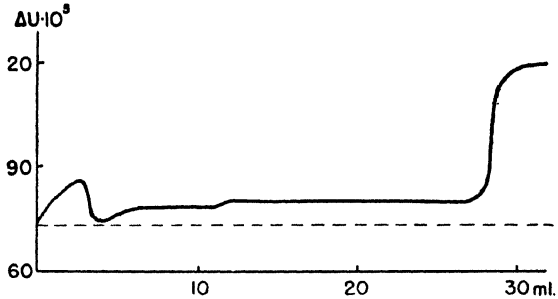


FIG. 20.—Separation of the components of a saccharide mixture obtained by enzymatic hydrolysis of corn starch, on active carbon. Starting material, 10 mg. Found, 6·8 mg. of hexasaccharide, 1·9 mg. of pentasaccharide, and 1·3 mg. of tri- and tetrasaccharide (Tiselius and Hahn).

of the stationary concentrations two components of the mixture could be identified as tri- and tetrasaccharides, while the molecular weights of two others were found by testing the reducing powers before and after hydrolysis.

Chromatographic Separation of Free Sugars by the Brush Method. The use of free sugars for chromatographic

purposes on a preparative scale has the great advantage that no derivatives have to be synthesized, thus avoiding losses and complications. The main task was to find an adsorbent suitable for aqueous media. Lew, Wolfrom and Goepp first employed native clays, such as Florex XXX, on which the sugar zones can be located by using a fresh 1 per cent permanganate solution in 2·5 *N*-NaOH. Since the indicator streak was not easily detectable and, furthermore, the extrusion properties of the clay were found to be unsatisfactory, Georges, Bower and Wolfrom now use with success a commercial synthetic, hydrated calcium acid silicate, named "Silene EF" (Columbia Chemical Division, Pittsburgh Plate Glass Co., Barberton, Ohio) in admixture with Celite 535 (5 : 1).

The following standard conditions are suitable :

11 × 0·9 cm. column ; 0·5 ml. of 90 per cent dioxane (purified according to Fieser, Experiments in Organic Chemistry), followed immediately by a solution of 2 mg. of the substance in 0·2 ml. of 90 per cent dioxane. The chromatogram is developed with 10 ml. of 90 per cent dioxane (for *l*-fucose and the compounds following it, with only 5 ml.).

Under these conditions the compounds enumerated below show decreasing adsorption affinities :

α-*d*-Galacturonic acid ; lactose monohydrate ; lactitol ; dulcitol ; melezitose, raffinose pentahydrate, gentiobiose, *d*-gluco-*d*-guloheptose ; sucrose, maltose monohydrate, cellobiose, *d*-glucitol (sorbitol) ; *d*-galactose, *d*-mannitol ; *d*-glucose, *d*-fructose, *d*-mannose, *l*-sorbose ; *l*-fucitol ; *l*-arabinose ; *l*-fucose ; *d*-xylose ; *l*-rhamnose monohydrate ; methyl-α-*d*-glucopyranoside. Some separations may be improved by changing the developer ; for example, in the separation of sucrose from raffinose 95 per cent alcohol, or, still better, 95 per cent isopropyl alcohol is far superior to 90 per cent dioxane.

When it is necessary to obtain the sugars in crystalline form, additional operations after chromatography may become necessary. For example, 500 mg. of *d*-galactose+500 mg. of *l*-rhamnose were adsorbed from 20 ml. of 90 per cent dioxane on a 19·5 × 4·4 cm. column and developed with 218 ml. of 92 per cent dioxane. Each zone was eluted with 200 ml. of water, filtered through a layer of celite 535, and 20 ml. of 10 per cent neutral lead acetate was added. The precipitate was filtered off, the excess lead precipitated with H_2S, and the charcoal-decolorized solution concentrated in vacuo to 50 ml. This solution was filte ed slowly through 50 g. of Amberlite IR-100 and then through

50 g. of Amberlite IR-4. After concentration *in vacuo* the crystalline residue was freed from traces of silica by treatment with a little water and filtration. Recovery, 78 to 80 per cent pure sugar.

Sugar Phosphates

Purification on Ion Exchange Adsorbents. McCready and Hassid found that compounds such as glucose-1-phosphate (Cori ester), fructose-6-phosphate (Neuberg ester) or fructose 1 : 6-diphosphate (Harden-Young ester) can be conveniently purified by passing the crude solution through a column which exchanges the cations for H-ions. When the filtrate is then brought into contact with an acid adsorbing column, the strongly acid sugar phosphate is adsorbed while proteins, dextrins, weak acids, etc., pass through. Finally the ester is eluted with alkali and crystallized as K-salt.

Columns. The synthetic resins amberlite IR-100 and IR-4 were used in a simple apparatus (Fig. 21). In the latter (tube, 100 × 10 cm.) *c* is connected with dist. water and *a* is an overflow. Fine particles are eliminated by washing the column in reverse direction with water at such a rate as to produce a 50 per cent "bed volume" expansion. ("Bed volume" = washed and drained volume, is measured by washing to a 50 per cent bed volume expansion followed by draining the liquid to 2·5 cm. of the top).

This resin (1200 ml.) was then submitted to 3–4 exchange cycles such as the following one. Four per cent HCl was passed through (7 bed volumes/hour) followed by washing until the resin became acid free. A subsequent treatment with 3 l. of 4 per cent NaCl (10 bed volumes/hour) substituted Na for H in the resin. The column was then washed with water and regenerated with 3 l. of the acid, etc. The titratable acid content of influent water and the filtrate were equal.

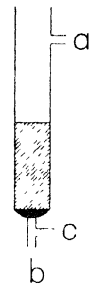


Fig. 21.—Apparatus for the exchange adsorption of sugar phosphates (McCready and Hassid).

The acid adsorbing IR-4 (a 200 ml. bed in a 100 × 3·5 cm. tube) was submitted to 4 exchange cycles as above, but ammonium hydroxide was used in the regeneration, which should be considered complete when pH 11 prevails in the filtrate. When subsequently the resin was treated with HCl, this value was used as an index of saturation with the acid.

Procedure. A crude solution (8 l.) obtained by the cleavage of 100 g. of potato starch with phosphorylase, passed through a 200 ml. bed volume of an IR-4 column (7 bed vol./hr.) which was then washed with 3 l. of water. A 4 per cent solution of NH_4OH was then employed (5 bed vol./hr.) and the pH was measured in each 50 ml. portion. After 1/2 l. has passed, pH 11 was attained, whereupon a further

200 ml. of the ammonia was applied to insure complete regeneration of the column; this filtrate was discarded. Fifty grams of potassium acetate was added to the first filtrate and the pH was adjusted to 12 with 10 per cent KOH. After the addition of 1·5 vol. of methanol, pure dipotassium glucose-1-phosphate dihydrate crystallized at 4° within a day. After washing with methanol and ether, and drying at 50° in vacuo, 40·5 g. was obtained (recovery, 94 per cent).

Sugar Acetates

Acetobromo Sugars. A chloroform solution of crude acetobromo-mannose can be decolourised and purified by filtering it through successive layers of anhydrous sodium sulphate, decolourising charcoal (Darco) and filter-Cel (Talley, Reynolds and Evans).

Purification of 6-β-*d*-Mannosido-β-*d*-Glucose Octa-Acetate (NORMAL FORM) (Talley, Reynolds and Evans). In order to separate the octaacetate from impurities which contain free hydroxyl groups, ignited aluminium oxide (—80 mesh) was satisfactory, after the adsorbent had been freed of alkali by washing with 1 per cent acetic acid, then with water, and had finally been heated to 240°. The column (30 × 3 cm.) was moistened with anhydrous ether, and a solution of 4 g. of material in 50 ml. of ether was introduced. The solvents used for the preparation of the liquid chromatogram were mixtures of ether, acetone and ethanol (finally, 95 per cent ethanol + 5 per cent water). The glucose tetraacetate migrated through the column and carried some triphenylmethane derivative, which fluoresced in ultraviolet light and was located directly below the main product, thus making it possible to follow the experiment. The receiver was changed after each 50 ml. portion, and the optical rotations of the fractions were determined.

α-Penta-acetyl-*d*-idose- < 1 : 5 > can be adsorbed on alumina from benzene + light petroleum and eluted with benzene + ether (Sorkin and Reichstein 2).

Separation of Sugar Acetates and Sugar Alcohol Acetates by means of the Brush Method (McNeely, Binkley and Wolfrom). This procedure, which can also be employed for complicated mixtures, has the advantage that the

inherent adsorption affinities of the sugars are less masked by the small acetyls than by heavier groups. The brush reagent, freshly prepared 1 per cent potassium permanganate in 2·5 *N*-NaOH, produces a yellow to brown colour where it crosses an acetate zone ; interzones appear greenish, because of much slower reaction of the column material. A convenient adsorbent is synthetic " magnesol ", a hydrated acid silicate, 2 MgO, 5 SiO_2 (Westvaco Chlorine Products Co., South Charleston, West Virginia) which was mixed with 1/5 of its weight of " celite No. 535 " (Johns-Manville Co., New York). It is desirable to extract this mixture with large amounts of acetone at room temperature and to use it in an air dry state. The column (23 × 3·5 cm.) contained about 50 g. of the adsorbent, the top of which was moistened with 10 ml. of benzene. About 250 mg. of an approximately 1 : 1 mixture of two peracetates was then introduced and the development was carried out with thiophene-free benzene containing a little absolute ethanol (Table 9).

TABLE 9

Separation of Some Sugar (and Sugar Alcohol) Acetate Pairs by Using the Permanganate Brush Method (McNeely, Binkley and Wolfrom).

Relative location of the zones	Peracetate of	Volume of developer and ratio, benzene : ethanol in it
upper lower	β-Maltose β-*d*-Glucose	450 ml., 200 : 1
upper lower	Sucrose β-*d*-Glucose	350 ml., 200 : 1
upper lower	Raffinose β-*d*-Glucose	400 ml., 200 : 1
upper lower	keto-*d*-Fructose β-*d*-Glucose	500 ml., 500 : 1
upper lower	Sucrose β-Maltose	300 ml., 100 : 0·75
upper lower	Raffinose β-Maltose	300 ml., 100 : 1
upper lower	Raffinose Sucrose	375 ml., 100 : 1
upper lower	(levo)-Sorbitol *l*-Rhamnitol	350 ml., 500 : 1

It must be stressed that some time is necessary for the full reaction with permanganate. While the peracetates of fructose, glucose and maltose attain this stage within a minute, 2 to 5 minutes are required for the other compounds. Since the acetates are separated by large interzones, they can be recovered nearly quantitatively, and in a high state of purity, without recrystallization. For example, a β-maltose-octaacetate zone (35 mm. broad) was separated by a 60-mm. interzone from glucose-pentaacetate (22 mm.). Both were eluted with 125 ml. of acetone which was then evaporated at room temperature.

It is probable on the basis of preliminary experiments that the peracetates of the following pairs could also be handled in the described manner: α- and β-*d*-glucose; α-*d*-arabinose and α-*d*-glucose; α-*d*-arabinose and fructose; *d*-mannitol and (levo)-sorbitol; finally β-*d*-glucose and the sorbitol.

Recently, Georges, Bower and Wolfrom worked out a suitable method for the separation of the sugar acetates using *Silene EF* (see above), which is in several respects superior to the clays employed earlier, e.g., Florex XXX (Lew, Wolfrom and Goepp). Using a 5 : mixture of silene-celite, and column dimensions of 11 × 0·9 cm., the separation of sugar acetates or sugar alcohol acetates are conveniently carried out by first wetting the column surface with 0·5 ml. of benzene followed, without delay, by a solution of 2 mg. of the substance in 0·5 ml. of benzene. The following developers were found to be satisfactory (the acetates are listed in the sequence of decreasing adsorbabilities; those in the same line cannot be separated):

(*a*) 15 ml. of benzene + alcohol (250 : 1):
Raffinose-hendecaacetate.
β-Melibiose-octaacetate; sucrose-octaacetate.
β-Maltopyranose-octaacetate.

(*b*) 15 ml. of benzene + alcohol (500 : 1):
keto-d-Fructose-pentaacetate.
d-glucitol (sorbitol)-hexaacetate; *d*-mannitol-hexaacetate.
β-*d*-Glucopyranose-pentaacetate.
α-*d*-Arabopyranose-tetraacetate.

(*c*) 15 ml. of benzene + alcohol (1000 : 1):
α-*l*-Fucose-tetraacetate.

The separation of d-glucitol-hexaacetate and d-mannitol-hexaacetate, was carried out under somewhat different conditions. The mixture containing 250 mg. of each compound

was dissolved in 33 ml. of benzene and developed with 1800 ml. of benzene + alcohol (1000 : 1) on a 26 × 5·1 cm. column. The zones were located with alkaline permanganate solution. The upper zone contained the glucitol derivative. Each zone was eluted with 100 ml. of acetone, followed by filtration and washing with 300 ml. of the same solvent. Evaporation yielded the two crystalline acetates. However, the glucitol derivative had to be rechromatographed on a 22·5 × 4·4 cm. column (1250 ml. of benzene + ethanol 1000 : 1). Recovery, 71 to 75 per cent.

Further literature on sugar acetates: Binkley and Wolfrom (2–4); Wolfrom and Thompson; Georges, Miller and Wolfrom.

Methyl Sugars including Cleavage Products of Methylated Polysaccharides

Considering the great adsorbability differences between di-, tri- and tetramethylated sugars or their methyl glucosides, the analysis of such mixtures constitutes a valuable addition to the classic distillation methods. Not only can the end groups in methylated polysaccharides be thus determined but a study of the partially methylated components which occur in the hydrolysate is also possible.

This principle was used by J. K. N. Jones for the differentiation of tri- and tetramethyl-methylglucosides on alumina and by Bell who carried out a separation of 2 : 3 : 4 : 6-tetramethylglucose, 2 : 3 : 6-trimethylglucose, and of dimethylglucoses, on a silica-water-chloroform column by partition chromatography. The results can be summarized as follows:

Glycogen from horse muscle: 12 repeating units (Bell).
Glycogen from *Ascaris lumbricoides*: 26-28 units (Bell).
Whole rice starch: 26-28 units (Bell).
Rice starch: 33 units (Jones).
Banana starch: 26 units (Jones).

Quantitative Separation of fully Methylated and partially Methylated Sugars on Alumina (J. K. N. Jones; Brown and Jones). The principle is to remove the tetramethyl derivative from the adsorbent with chloroform

and then the trimethyl compound with methanol. The method appears to be so sensitive that a partial separation even of α- and β- methylglucosides of tetramethyl-glucopyranose can be obtained. In the following resolutions the recovery of fully methylated sugar was 93 per cent.

A solution containing 530 mg. of 2 : 3 : 4 : 6-tetramethyl-α-methyl-glucoside, 515 mg. of the corresponding β-glucoside and 1020 mg. of 2 : 3 : 6-trimethyl-methyl-*d*-glucoside in light petroleum (b.p. 60-80°, pretreated with H_2SO_4 and NaOH) was adsorbed on alumina. (350 g.; 46 × 3·2 cm.; British Aluminium Co., Burntisland; activated at 360° for 4 hours.) The column was then washed with (dried and distilled) chloroform. The filtrate was collected in 100-ml. fractions and the evaporation residue of 5 ml. of each fraction was submitted to the Molisch test. After the passage of 1100 ml. of chloroform, seven fractions contained a total of 940 mg. of the tetramethyl compound (spec. rotations in chloroform: + 75°, + 110°, + 130°, + 83°, + 68°, + 20° and − 25°). The next fractions left no weighable residues. The trimethyl-methyl-*d*-glucoside was then washed down with methanol: yield, 1000 mg.

A mixture of 245 mg. of tetramethyl-methylglycoside ($n_D^{16} = 1{\cdot}4440$) and 283 mg. of the trimethyl compound ($n_D^{16} = 1{\cdot}4572$) (which can be expected from 5 g. of partly methylated starch) were separated in a similar manner. With chloroform, six fractions showing ^nD values between 1·4415 and 1·4462 indicated a 93 per cent recovery of the tetramethyl compound.

As seen above, the method works reliably for approximately equal quantities of the trimethyl and tetramethyl derivatives. However, in the investigation of polysaccharide repeating units, the following preliminary enrichment of the tetra fraction is recommended by the authors. After neutralization of the hydrolysate with a slight excess of *N*-NaOH, and filtration, the solvent is removed in vacuo, the syrup dissolved in 50 ml. of water and extracted with S-free light petroleum in an all-glass apparatus for 4 hours, which removes the fully methylated sugar as well as a similar quantity of the trimethyl derivative. The extraction is repeated with light

petroleum, b.p. 38–40°. After evaporation and weighing, the combined material is submitted to chromatography as described.

K. H. Meyer and Gürtler found that if a mixture of tetra- and tri-methyl-methylglucoside (ex lichenin) is developed with light petroleum on alumina, a mixed fraction first passes through, followed by fractions free from the tetra-derivative.

Separation of Methylated Sugars on Silene. According to Georges, Bower and Wolfrom, methylated sugars can be conveniently handled on Silene EF columns (see above). The top of the 11 × 0·9 cm. column is wetted with 0·5 ml. of benzene which is followed immediately with a solution of 2 mg. of the substance in 0·5 ml. of abs. chloroform. The developers are : for 2 : 3-dimethyl-*d*-glucose (top) or 2 : 3 : 6-trimethyl-*d*-glucose, 15 ml. of benzene + alcohol (100 : 1) ; and for 2 : 3 : 4 : 6-tetramethyl-*d*-glucose, 12·5 ml. of benzene + alcohol (250 : 1). The zones are detected by brushing with alkaline permanganate as mentioned above.

Separation of Methylated Sugars by Partition Chromatography (Bell). These experiments were based on the fact that the partition coefficients of 2 : 3 : 4 : 6-tetramethyl- and 2 : 3 : 6-trimethyl-glucose are very different in water-chloroform. Furthermore, the separation of the trimethyl compound from dimethyl-glucoses was made possible by the circumstance that a 9 : 1 mixture of chloroform + *n*-butanol extracts nearly 12 times as much of the trimethyl glucose from water as does pure chloroform.

The SILICA is ground thoroughly in a mortar and ½ part of water is stirred into the powder, after which the moist adsorbent is made into a slurry with chloroform and poured into the chromatographic tube. The supernatant chloroform is then allowed to drain through the gel and the column is washed by two " column-lengths " of chloroform (degreasing).

Each silica batch should be tested as follows. Three columns (diam., 1 cm.), each of 2·5 g. of silica, are prepared ; degreasing is unnecessary. One ml. of each chloroform solution of the tri- and tetramethyl glucose is pipetted on to the surface of each column. After the solution has been drawn in, one column length of chloroform is passed through. The second tube is treated with four times as much solvent, and the third held in reserve. The first and second columns are extruded and dried at 110°. After cooling, zones are located by fine drops of 2 per cent alcoholic α-naphthol, followed by conc. sulphuric acid (mauve coloration = Molisch reaction). If the silica is " good ", i.e., not too strong,

the first column should show a trimethyl zone at the top and a tetramethyl zone near the bottom while the second column should have let the latter zone pass into the filtrate. If this does not happen, the third column should be washed with eight column-lengths of chloroform; if some tetramethyl compound is still in the column, the silica batch should be rejected as too strong. While this standardization is certainly of use, it seems probable that some more convenient method could be found.

Hydrolysates of methylated gluco-polysaccharides, containing 100–200 mg. of tetramethyl-glucose in 10–15 parts of water were filtered through charcoal, diluted with water until the concentration was 5 per cent and extracted nine times with 1 vol. of chloroform; the latter was evaporated without previous drying (all such evaporations must be carried out in the presence of a little barium carbonate). The residue, which should not include more than 300 mg. of material, contains all the tetramethyl- and $\frac{1}{10}$ of the trimethyl-glucose present. Its chloroform solution is chromatographed on 25 g. of silica (diam., 4 cm.) by using the requisite amount of chloroform for the batch of adsorbent. The tetramethyl-glucose in the filtrate is dissolved in ether + light petroleum (1 : 3) and filtered through a 2-mm. layer of barium carbonate, covered by a similar layer of charcoal, into a special apparatus where the solvent is evaporated.

The aqueous phase from the first extraction of the hydrolysate is repeatedly shaken with 1 vol. of chloroform + butanol (9 : 1), until no more than 500 mg. of the trimethyl sugar remains in the water. The aqueous solution is evaporated in vacuo, the residue is dissolved in chloroform + butanol and chromatographed on the same column as before; the trimethyl compound is washed into the filtrate with chloroform + butanol, with an amount of solvent which has been previously established. The combined chloroform-butanol solutions are evaporated to dryness. The residue is dissolved in ether + acetone (2 : 1), evaporated and dried in high vacuum to yield trimethyl-glucose. Finally, the column is extruded and the dimethyl sugar is eluted with 5 × 100 ml. of acetone. It is transferred into dry ethyl acetate and evaporated to yield dimethyl-glucose.

Recovery experiments: An artificial mixture of 183 mg. of tetramethyl- and 114 mg. of trimethyl-glucose yielded 171 mg. and 107 mg. respectively; 160 mg. of hydrolyzed heptamethyl-β-methylcellobioside gave 71 mg. of tetramethyl and 66 mg. of trimethyl-glucose in pure form.

Separation of Methylated Sugars by Fluorescence-Chromatography. According to Norberg, Auerbach and Hixon, 2 : 3-dimethyl-, 2 : 3 : 6-trimethyl, and 2 : 3 : 4 : 6-tetramethyl-*d*-glucoses fluoresce on fibrous alumina in ultraviolet light and can be conveniently located. When a mixture of the tri- and tetra-methyl compounds was adsorbed from benzene and developed with the same solvent, a single fluorescent layer was first observed and later differentiated into two zones, the upper one containing trimethyl-glucose. Elution with methanol and evaporation gave quantitative recovery. Some qualitative observations were also made with a mixture of the three methyl compounds mentioned. Since dimethyl-glucose is not soluble in benzene, a mixture of benzene+acetone(1 : 1) can be used; it carries the tri- and tetra-methyl compounds into the filtrate while 2 : 3-dimethyl-*d*-glucose forms a fluorescing zone.

4 : 6-Benzal Derivatives of Sugars

Recently a number of such compounds were purified by adsorption on alkali-free alumina, and separations were carried out by means of a liquid chromatogram. An effective resolution sometimes required very large amounts of solvents.

Gulose and Altrose Derivatives. 2 : 3-Anhydro-4 : 6-benzal-α-methyl-*d*-guloside-<1 : 5> was separated from 4 : 6-benzal-α-methyl-*d*-idoside- < 1 : 5 > -2 (?)-methylether. The former compound was removed from alumina by benzene + petroleum, the latter only by benzene and benzene + ether (20 : 1) (Sorkin and Reichstein 2). The product obtained by hydrogenation under pressure of 4 : 6-benzal-α-methyl-*d*-altroside-<1 : 5>-monomethylether-(3) was adsorbed on alumina and the hexahydro derivative eluted with benzene + ether (2 : 1) and (1 : 1) (Grob and Prins).

Galactose Derivatives. A mixture of 4 : 6-benzal-α-methyl-*d*-galactoside-<1 : 5> can be separated from the corresponding β-form on alumina. Ether+methanol (99 : 1)

elutes first the α- and then a part of the β-compound which is quantitatively obtained with a 98 : 2 mixture (Sorkin and Reichstein 2).

Benzoylation Products of 4 : 6-Benzal-α-methyl-*d*-galactoside-<1 : 5> (Gyr and Reichstein). The crude product prepared from 1·35 g. of starting material was adsorbed on 56 g. of activated alumina which was washed with the solvents listed below; 200-ml. portions were evaporated for investigation of the residue :

200 ml. light petroleum + benzene (1 : 1) ; oil.
800 ml. light petroleum + benzene (1 : 9) } 70 mg. dibenzoate.
200 ml. abs. benzene }
200 ml. abs. benzene ; mixture.
600 ml. abs. benzene }
400 ml. benzene + ether (199 : 1) } 165 mg. 2-monobenzoate.
200 ml. benzene + ether (98 : 2) ; mixture.
400 ml. benzene + ether (98 : 2) ; 130 mg. 3-monobenzoate.
600 ml. benzene + ether (1 : 1) }
200 ml. abs. ether } 730 mg. 3-monobenzoate.
400 ml. chloroform ; mixture.
400 ml. chloroform + methanol (3 : 1) ; 240 mg. unchanged starting material.

Tosylation of 4 : 6-Benzal-α-methyl-*d*-galactoside -<1 : 5> (Reber and Reichstein, cf. Sorkin and Reichstein). The crude product obtained from 10 g. of starting material yielded 3·5 g. of the ditosylate by crystallization. The mother liquors were then chromatographed on 300 g. of alkali-free alumina and the column was washed with 52 × 1 litres of solvent. The first 17 fractions (benzene, and benzene + ether 99 : 1) gave, upon crystallization from acetone + methanol, 1·38 g. of the 2 : 3-ditosylate. The fractions no. 18-28 (benzene + ether 98 : 2, then 96 : 4) (1·84 g.) contained chiefly the 2-monotosylate, but no. 29-30 (benzene + ether 96 : 4), a mixture. The no. 31-50 (benzene + ether, then ether) yielded the 3-monotosylate (6·17 g.). Some starting material could be recovered by washing the column with ether + methanol, and methanol.

3-Monotosyl-4 : 6-benzal-β-methyl-*d*-galactoside-< 1 : 5 > was purified by adsorption on alumina and elution with benzene + ether (1 : 1), then with ether.

Of the two triacetates of unknown structure obtained by acetylation of *d*-altro-methylose-monomethylether-(3) one can be eluted from the neutral alumina with benzene + light petroleum (1 : 4 to 1 : 1) and then the other with benzene + ether (9 : 1 to 1 : 1) (Grob and Prins).

Glucose Derivatives. Methylation of 4 : 6-benzal-α-methyl-*d*-glucoside-<1 : 5> (Bolliger and Prins). The elution of the alumina with benzene yielded some dimethylether, that with benzene + ether the 3-monomethyl compound. 2-Tosyl-4 : 6-benzal-α-methyl-*d*-glucoside-<1 : 5> was obtained by tosylation and the crude product was adsorbed on alumina. From the latter benzene + light petroleum removed some ditosylate, whereupon the desired monotosylate was eluted with benzene and benzene + ether.

Some further chromatographic data given by Maehly and Reichstein refer to the following compounds: 3-carbethoxy-4 : 6-benzal-α-methyl-*d*-galactoside-<1 : 5>; 3-tosyl-4 : 6-benzal-α-methyl-*d*-galactoside-<1 : 5>; 2-methylthio-4 : 6-benzal-α-methyl-*d*-idoside-<1:5>-3-methylether; and 2-desoxy-4 : 6-benzal-α-methyl-*d*-guloside-<1:5>.

Coloured Sugar Derivatives

W. S. Reich (1, 2) must be credited with the chromatographic separation of some sugar esters which he obtained by reacting all hydroxyl groups with *p*-phenylazo-benzoyl chloride, $C_6H_5 \cdot N = N \cdot C_6H_4 \cdot COCl$. These products are briefly termed "azoyl esters" or "azoates" (penta-azoates, tetra-azoates, etc.). The Reich method has been employed recently by several authors for the separation of monosaccharides or of disaccharides from monosaccharides, etc. Azoates of some methyl and acetyl derivatives of sugars were also investigated. Silicic acid or magnesol seem to be suitable adsorbents, the behaviour of which is illustrated by Table 10.

Azobenzoyl esters can be freed from excess acid by filtering the crude chloroform solution through alumina (Freudenberg and Plankenhorn).

Separation of Glucose and Fructose Azoyl Esters (Reich). For the adsorption of such mixtures Brockmann alumina (after a treatment with ethanol for three days) is

TABLE 10

SEPARATION OF SUGARS AND SOME SUGAR DERIVATIVES BY MEANS OF THEIR AZOYL ESTERS

Separated Azoates of	Adsorbent	Solvent	Developer	Eluent	Literature
Glucose, fructose	silica	chloroform + benzene + light petroleum 1 : 2 : 2	benzene + light petroleum 1 : 3	chloroform + methanol 4 : 1	Reich
α-lactose and α-*d*-galactose; trehalose and β-*d*-glucose; α-lactose and sucrose; α-*d*-glucose and β-*d*-fructose; β-maltose and α-*d*-glucose; sucrose and α-*d*-glucose	magnesol (with dicalite)			chloroform + 5–10 per cent methanol	Coleman, Farnham and Miller; Coleman and McCloskey
β-*d*-glucose and β-*d*-cellobiose; α-*d*-glucose and β-*d*-fructose; α-*d*-galactose and β-*d*-fructose; sucrose and β-*d*-fructose; α-*d*-glucose and melezitose; β-*l*-arabinose, β-*d*-glucose, α.α-trehalose, and β-cellobiose. α-*d*-galactose and β-*d*-galactose; β-*d*-glucose and α-*d*-xylose; β-*d*-glucose and β-*l*-arabinose	silicic acid (Merck's reagent)	chloroform + 0·2 per cent alcohol; chloroform + benzene + light petroleum 1 : 1 : 1	same as solvent		
Methyl-β-*d*-cellobioside and methyl-α-*d*-glucoside; tetraacetyl-β-*d*-glucose and heptaacetyl-β-*d*-cellobiose					
2 : 3-dimethyl-*d*-glucose, 2 : 3 : 6-trimethyl - *d* - glucose, and 2 : 3 : 4 : 6 - tetramethyl-*d*-glucose	silica	chloroform + benzene + light petroleum 1 : 1 : 1	same as solvent	chloroform + alcohol 4 : 1	Mertzweiller; Carney and Farney

convenient ; however, elution is incomplete. Satisfactory is a silica column prepared as follows. Seventy grams are mixed with 300 ml. of benzene + light petroleum (b.p. 60–80°). A portion is poured on a 40 × 3 cm. tube and forced downwards by nitrogen pressure, until only little solvent remained over the surface ; then a new portion is introduced until a height of 30 cm. is reached. A perforated disc is placed at the top and 100 ml. of the solvent mixture is pressed through (6–10 ml. per min. ; during chromatography, 3–4 ml.). A solution of glucose and fructose esters (200 mg. each) in 40 ml. of chloroform + 80 ml. of benzene + 80 ml. of light petroleum was introduced and developed with 800–900 ml. of benzene + light petroleum (1 : 3). (The figures on the left denote the height of the zones, in mm.)

40 dark orange (172 mg.), fructose ester, $[\alpha]_{644}^{20} = -323°$.
65 almost colourless (17 mg.).
2 orange (16 mg.).
6 nearly colourless.
30 dark orange (166 mg.), glucose ester, $[\alpha]_{644}^{20} = +171°$.
105 nearly colourless (9 mg.).
2 orange (1 mg.).

The elution was carried out with chloroform + methanol (4 : 1), with 12 hours standing. The residue, after vacuum evaporation, was dissolved in a little CCl_4 and, after filtration, precipitated with light petroleum (b.p. 40–60°). Upon re-chromatography, complete purity was attained. On a micro scale, using a 15 × 0·7 cm. tube, 10 mg. of ester can be handled, corresponding to 2 mg. of sugar.

Separation of α-*d*-Glucose and Sucrose Azoates (Coleman, Farnham and Miller ; Coleman and McCloskey). From a mixture of 100 + 100 mg. an upper zone was obtained containing 106·7 mg. of the sucrose ester ($[\alpha]_{644}^{25} = +47°$ instead of + 35°) and a lower zone containing 88·1 mg. (+ 212° instead of + 225°).

Separation of α-Lactose and Sucrose Azoates. 120 + 120 mg. substance. Upper zone, 144·5 mg. of lactose azoate, $[\alpha]_{644}^{25} = +206°$ instead of + 274° ; lower zone, 89·2 mg. sucrose azoate (+ 36° instead of + 35°). Evidently, this separation was incomplete.

Separation of 1-Azoyl-tetraacetyl-β-*d*-glucose and 1-Azoyl-heptaacetyl-β-cellobiose. A mixture of 215 mg. from each ester was developed on silicic acid (diam., 2·7 cm.) with chloroform + 0·2 per cent alcohol. Recovered, from the top zone (2·5 cm. wide) 114·4 mg. of the cellobiose ester with a nearly correct rotatory power, viz. $[\alpha]_{644}^{25} = 54{\cdot}5°$. The zone of the glucose derivative was 2 cm. wide and was separated from the former by a 1·5-cm. interzone. Recovered, 115·9 mg., $[\alpha]_{644}^{25} = +\ 62{\cdot}6°$ (instead of + 63°).

Separation of β-*l*-Arabinose, β-*d*-Glucose, α,α-Trehalose, and β-Cellobiose Azoates. A mixture of 150 mg. of each ester was developed for 12 hours on silicic acid (diam., 4 cm.) with chloroform containing 0·1 per cent alcohol :

110 cellobiose ester, 152·7 mg. $[\alpha]_{644}^{25} = +\ 109°$ instead of + 105°.
10 interzone.
20 trehalose ester, 136 mg. ; + 199°, instead of + 210°.
150 interzone.
100 glucose ester, 138 mg. ; + 13° instead of — 50°.
70 interzone.
100 arabinose ester, 130·4 mg. ; + 721° instead of + 750°.

In similar chromatograms a lower zone usually leaves some material behind in the next higher one. It seems that at least two adsorptions will be needed in such instances.

TABLE 11

EFFECT OF ADSORBENT AND DEVELOPER ON THE SEPARATION OF β-*d*-GLUCOSE-PENTAAZOATE AND β-CELLOBIOSE-OCTAAZOATE (COLEMAN AND MCCLOSKEY)

Adsorbent	Developer	Chromatogram
Magnesol (with decalite)	chloroform + benzene + light petroleum (1 : 1 : 1)	no differentiation
,, ,,	same with 0·4 per cent alcohol	two good zones
,, ,,	same with 1–10 per cent alcohol	poor or no separation
Silicic acid . . .	chloroform + benzene + light petroleum (1 : 1 : 1)	two good zones, no migration of cellobiose zone
,, ,, . . .	same with alcohol	see above
,, ,, . . .	chloroform with 0·1 or 0·2 per cent alcohol	clear separation ; cellobiose zone migrates only with 0·2 per cent alcohol

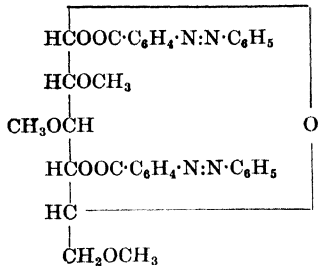
Azoyl-methyl-sorbitols. Boissonnas (1–3) investigated the separation of mixtures of tetra- and trimethyl-methylglucosides, which were first converted, by catalytic hydrogenation and azoylation, into the respective azoyl-methyl-sorbitols (" glucitols "). For example, from benzene, on alumina Grade III (Brockmann) 1 : 5-diazoyl-2 : 3 : 4 : 6-tetramethyl-glucitol forms a sharp zone near the top, while the more diffuse zone of a trimethyl derivative is located in a lower section. The six binary mixtures of the four triazoyl-trimethyl-glucitols were separated with an error up to 15 per cent, while an error of only 5 per cent is claimed in the separation of trimethyl derivatives from the di- or tetra-methyl-compounds.

Separation of 2 : 3-Dimethyl-, 2 : 3 : 6-Trimethyl- and 2 : 3 : 4 : 6-Tetramethylglucose Azoates (Mertzweiller, Carney and Farley). The silica was prepared by acidifying a 40 per cent solution of sodium silicate with HCl, igniting the gel over a Meker burner, washing with water and drying at 110°. About 100 mg. of azoates could be developed in a 35 × 2·2 cm. column by means of chloroform + benzene + light petroleum (1 : 1 : 1). (First, the column was washed with this mixture.) Four coloured zones appeared : tetramethyl (top), trimethyl and two forms of dimethyl compound. In order to elute, each zone was soaked with chloroform + ethanol (4 : 1) for an hour. A colorimetric estimation showed nearly quantitative recovery.

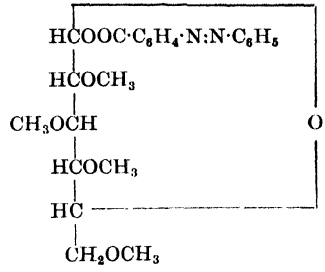
Some earlier experiments, using alumina, were reported by Myrbäck and Tamm.

Separation of the Methanolysis Products of Fully Methylated Disaccharides by means of the Azoates (Coleman, Rees, Sundberg and McCloskey). When, for example, methylheptamethyl-β-cellobiose is hydrolyzed with HCl, 2 : 3 : 6-trimethyl-*d*-glucose and 2 : 3 : 4 : 6-tetramethyl-*d*-glucose can be obtained and, upon azoylation, a mixture of

the following compounds which are separated chromatographically :



4-Azoyl-2 : 3 : 6-trimethyl-*d*-glucosyl azoate.



2 : 3 : 4 : 6-Tetramethyl-*d*-glycosyl azoate.

The method was applied to permethylated gentiobiose, melibiose, cellobiose, lactose and maltose. It is peculiar that the monoazoyl compound was generally more strongly adsorbed than the diazoyl derivative. The upper zone was contaminated by some amounts of the lower one and had to be submitted to a second adsorption. With the lactoside and maltoside a third coloured zone of unclarified nature appeared near the bottom. All zones were tested polarimetrically and evaluated on the basis of the rotatory power of corresponding synthetic azoates which were equilibrium mixtures of the α- and β- forms. These individual forms can also be obtained chromatographically. There does not seem to exist a general rule for the chromatographic sequence of the corresponding α- and β- forms.

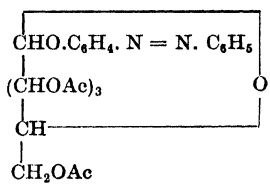
A slurry of 200 g. of silicic acid (Merck, reagent) of 1/2 *l.* of benzene was introduced and gas pressure was applied to pack the column (60 × 3·3 cm.) on to which a chloroform solution (10 ml.) of 0·5–3 g. of an azoate mixture was poured. A suitable developer is benzene containing 0·1 per cent abs. alcohol. During the development a pressure of 20–30 cm. mercury was maintained. The elution was made with chloroform + 10–20 per cent ethanol. Each filtered eluate was evaporated in vacuo, the chloroform solution of the residue was sucked through asbestos and fritted glass, evaporated again,

dried at 50° under 3 mm. pressure and weighed. The yields were nearly theoretical in several instances; they amounted, however, to 74–75 per cent in case of methyl-4-azoyl-2 : 3 : 6-trimethyl-*d*-glucoside or methyl-*d*-azoyl-2 : 3 : 4-trimethyl-*d*-glucoside, and to 53 per cent for methyl-2-azoyl-3 : 4 : 6-trimethyl-*d*-glucoside.

The limitation of the method, if only a single separation is carried out, can be illustrated by an example. After chromatography, from an artificial mixture of 0·16 g. of 2 : 3 : 4 : 6-tetramethyl-*d*-glucosyl azoate and 0·23 g. of 4-azoyl-2 : 3 : 6-trimethyl-*d*-glucosyl azoate, the following amounts were recovered: 0·14 g., $[\alpha]_{i\cdot 44}^{25} = +23°$ instead of $+36°$, and 0·21 g. (—8° instead of — 1°).

Coleman *et al.* also reported some data concerning hydrolysis, azoylation and chromatography of fully methylated disaccharides.

Separation of p-Phenylazophenyl Polyacetylglycosides (Hurd and Zelinski). The structure of these coloured sugar derivatives differs from that of the azoyl compounds already mentioned, since they carry only one azo group in the molecule. The compounds can be considered as *glycosides* of *p*-hydroxy-azobenzene, HO. C_6H_4. N=N.C_6H_5 and otherwise fully acetylated sugars:



Structure of *p*-phenylazophenyl polyacetyl-glycosides.

Several pairs of such sugar derivatives can be separated on silica gel columns by development with benzene or chloroform or benzene + chloroform + light petroleum 1 : 1 : 1.

Examples for a 52 × 2 cm. glass tube and 0.1 to 0.2 g. of each sugar derivative (only the sugars are mentioned). The first member of each pair occupies the upper position: glucose and xylose; galactose and glucose; lactose and glucose; maltose and glucose; lactose and maltose. Elution: with acetone (Soxhlet).

Separation of Sugars from Amino Sugars and Amino Acids

According to Freudenberg, Walch and Molter, glucosamine hydrochloride and glucose can be separated quantitatively by means of the exchange resin Wofatit K or (preferably) KS, which retains only the basic compound. The glucosamine is then eluted with excess HCl. In order to separate glucosamine from basic, neutral and acid amino acids, the adsorbent just mentioned was used from which the acid and neutral amino acids are displaced by dilute pyridine ; glucosamine as well as the basic amino acids remain adsorbed and can be removed with ammonia or HCl + much pyridine hydrochloride.

Th. Wieland (2) found that 10 mg. of alanine and 20 mg. of glucose can be separated in 80 per cent alcohol solution. This is first neutralized with 80 per cent alcoholic alkali (phenolphthalein) and then poured on to 4 g. of activated alumina (moist with 80 per cent alcohol). If this column is washed with 30 ml. of the same solvent, 96–98 per cent of the sugar is found in the filtrate. The amino acid can be easily eluted with water.

Derivatives of N-methyl-*l*-glucosaminic and -mannosaminic acids: Wolfrom and Thompson (2).

Polysaccharides

Some rather qualitative observations were reported by Levi who found that starch, Zulkovsky starch, glycogen, inulin and dextrins are adsorbed, e.g., on charcoal or magnesia, and that some zones show fluorescence. His starch samples could be eluted by boiling water from magnesia but only by glycerol or diluted glycerol from charcoal.

Separation of the two Constituents of Corn Starch. It was reported briefly by Samec that cotton adsorbs rapidly and in substantial amounts (1·7 per cent) β-amylose (" amylose ") while α-amylose (" amylopectin ") remains in solution. Independently, Pacsu and Mullen made the following observations which could well be the basis of a chromatographic technique. The β-component is preferentially adsorbed

by activated carbon, fuller's earth, alumina (Brockmann) and especially by cotton. If a cold 1 per cent starch paste is brought into contact with the latter, the adsorbent can be washed free of α-amylose with cold water. The β-compound is then eluted with warm water. Only α-amylose can be isolated in unchanged form. However, β-amylose solutions rapidly precipitate granules which are then insoluble in cold water. This change is prevented by the addition of pyridine. If β-amylose is precipitated by alcohol, it becomes insoluble when dried.

For the elimination of the CARBOHYDRATE content of a non-toxic, ALLERGENIC fraction ("CB–1A") obtained from castor beans, among various other procedures, chromatography of the picrate was used by Spies, Coulson, Chambers, Bernton and Stevens. The solution of about 12 g. of the picrate in 1·5 ml. of 50 per cent ethanol was passed slowly through Brockmann's alumina (13·5 × 4·4 cm.) and washed with 100 ml. of the solvent. The main (top) zone was then subjected to an alkaline elution procedure. Similar experiments with the picrate obtained from cottonseed were earlier reported by Spies, Coulson, Bernton and Stevens.

CHAPTER XII

AMINO ACIDS AND PEPTIDES

The biochemically important problem of the resolution of amino acid mixtures is being attacked from different sides. Although most of the present methods still require improvement, it is to be hoped that they will lead to a general and quantitative procedure for the resolution of partial and total protein hydrolysates. So far the technique has had to be developed empirically, since the knowledge of the adsorption isotherms of individual amino acids (cf. Cheldelin and Williams) does not permit general predictions to be made about the behaviour of mixtures (Lottermoser and Edelmann ; Schaaf and Reinhard).

While great variations in the adsorbability of the individual amino acids on indifferent (neutral) column materials, such as charcoal, have been revealed, adsorbents possessing definitely basic or acid character are especially useful for the division of mixtures into groups, based on typical differences between monoamino-monocarboxylic, diamino-monocarboxylic and monoamino-dicarboxylic acids. Several applications have been made of " basic " (untreated) and " acid " alumina columns ; the latter can be prepared by a treatment with acid or an acid buffer. A further application of the exchange adsorption principle is the successful use of synthetic resins such as wofatit and amberlite. Some data are available about chromatography of coloured derivatives and also about the Tiselius-Claesson method. Applications of partition chromatography will be outlined at the end of this chapter. The field under discussion was briefly reviewed by Th. Wieland and, recently, in more detail, by Cannan (2) as well as by Martin and Synge in their article " Analytical Chemistry of the Proteins " ; cf. Martin (4).

Separations by partition Chromatography on Silica Gel. This method has been outlined on p. 38. A thorough investigation of the factors influencing separations on silica gel, in the chloroform-17 per cent butanol, chloroform-2 per cent-butanol, ethyl acetate, cyclohexane-5 per cent propanol and cyclohexane-30 per cent propanol systems was reported by

Tristram, who also made use of the " hydrolysis " of artificial amino acid mixtures. Tristram tested the influence of the quality of the silica gel on the accuracy of the estimations and states that single controls are inadequate for checking individual results obtained in resolving protein hydrolysates.

Two main factors influencing the effectiveness of the gel are, its iron and water contents. While the preparation of silica according to Gordon *et al.* yields a product with 53 per cent water (w/w), samples up to 85 per cent water were tested by Tristram. He concludes that, especially in an iron-free gel, the water content must be increased to an optimum at which the silica still shows powder properties but only very little true adsorption.

Separation of Amino Acids on a Starch Column. An excellent method of partition chromatography on this adsorbent was recently announced by S. Moore and Stein. They use a starch column (15–30 × 2 cm.) and the liquid system, *n*-butanol-benzyl alcohol, nearly saturated with water. The effectiveness of their separations is much improved by the application of a fully automatic fraction collector, which includes a circular rack holding 80 test tubes. (A funnel with a capillary tip prevents evaporation.) Each drop of the filtrate intercepts a light beam focused on a photocell and the drops are counted by an automatic-reset impulse counter, which (after a preset number of drops) resets itself to zero and closes the switch on a motor. The next tube then moves below the column. An experiment may run from two to three days and may involve any fraction size between 1 drop and 20 ml. A recovery of about 98 per cent was obtained in the separation of *inter alia* phenylalanine (first fractions), leucine and isoleucine.

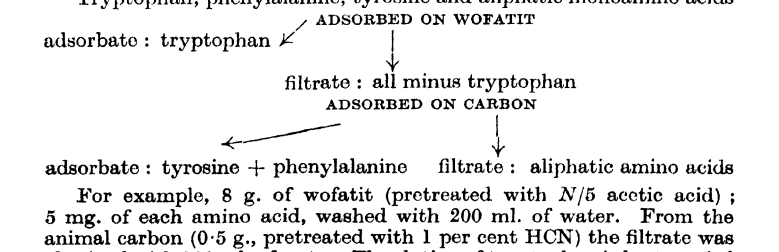
Traces of metals in the starch may disturb the regular separation of the amino acid emerging first; this is prevented if an aliphatic amine or 8-hydroxyquinoline is run through before the first amino acid fraction.

It should be stressed that the starch column operates both as a support in partition chromatography and as an ordinary adsorbing column, since the amino acids do not always emerge in the order of their partition coefficients. In fact, the column is also very effective when only water is used; in this instance a quantitative separation of alanine and glycine takes place.

Separation of some Aliphatic and Aromatic Amino Acids. A partial separation of glycine, *d,l*-leucine, *d,l*-phenylalanine, and *l*-tyrosine on Darco G–60 + filter paper pulp was described by Wachtel and Cassidy (1, 2). The aliphatic amino acids were washed into the filtrate within 4 hours. A treatment with 5 per cent aqueous acetone removed a part of the phenylalanine adsorbed. It seems that such lengthy operations on untreated carbons may involve a considerable decomposition of some amino acids.

Schramm and Primosigh treated their active carbon (Schering) first with boiling acetic acid and then, in order to prevent oxidative destruction, with KCN. After washing of the column (4 cm. high, 2 g.) with 5 per cent acetic acid, the amino acids were introduced (1–2 mg. in 2–5 ml. of 5 per cent acetic acid). By washing with 50 ml. of the same acid (without suction) the aliphatic amino acids reached the filtrate within 2–3 hours and were free of phenylalanine, tyrosine or tryptophan. These were eluted with 300 ml. of a 5 per cent phenol solution in 20 per cent acetic acid ; however, for phenylalanine 100 ml. of 20 per cent acetic was needed and a water jacket of 70°. (These were considerable volumes for the amount of material employed.)

Some experiments were also reported by Turba, Richter and Kuchar (cf. Freudenberg, Walch and Molter). They found the exchange resin " Wofatit M " to be a specific adsorbent for tryptophan and they propose the following scheme :



For example, 8 g. of wofatit (pretreated with *N*/5 acetic acid) ; 5 mg. of each amino acid, washed with 200 ml. of water. From the animal carbon (0·5 g., pretreated with 1 per cent HCN) the filtrate was obtained with 100 ml. of water. The elution of tryptophan is best carried out with aqueous alcohol or pyridine ; this eluate (after evaporation of the alcohol or pyridine) should be filtered through neutrol filtrol in order to eliminate resin impurities. Tyrosine and phenylalanine are eluted by means of a pyridine-acid mixture.

A new system for the separation of the amino acids into *four groups* (aromatic, basic, neutral, and acid) has been recently outlined by Tiselius, Drake and Hagdahl. It is based on the following facts: under the conditions applied only aromatic amino acids are adsorbed on carbo activatus Merck; from the filtrate only the basic ones are adsorbed on Wofatit C (and from this fraction only the acid amino acids on Amberlite IR–4); all the other amino acids are retained by Wofatit KS. For details cf. the original paper.

A considerable simplification of the technique has been achieved by coupling together several independent (glass or metal) columns that can be detached and separately eluted. For example, charcoal (top), Wofatit C and Wofatit KS are used. The solution is washed down with 5 per cent acetic acid, and the charcoal filter is removed; 20 per cent acetic acid is then sent through the remaining two filters in order to wash the Wofatit C free from neutral and acid amino acids. The following eluents are used: from charcoal, 5 per cent phenol (in 20 per cent acetic acid) and from Wofatit C or KS, *N*-HCl.

Separation of Monoamino-Monocarboxylic, Monoamino-Dicarboxylic, and Diamino-Monocarboxylic Acids on Exchange Resins. The adsorbents used for such purposes must have a definitely acid or basic character; they also include artificial ion exchange resins. It was pointed out by Freudenberg, Walch and Molter that all amino acids can be retained by acid exchangers like Wofatit K, KS or A. In contrast, on exchange resins of basic character (Wofatit M) only the acid amino acids are retained. A quantitative separation of alanine and aspartic acid or of similar pairs can be based on these facts.

Cannan (1) achieved a quantitative estimation of MONOAMINO-DICARBOXYLIC ACIDS in HCl-hydrolysates of egg albumin, β-lactoglobulin or edestin simply by shaking with Amberlite IR–4 until the pH had risen to 6–7. The aspartic and glutamic acids were then recovered by treating the washed adsorbate with a limited excess of HCl. De-acidite was used for similar purposes by Englis and Fiess.

In order to **separate the basic amino acids from other constituents of protein hydrolysates** (Block), the acid hydrolysate of 11·7 g. of commerical blood fibrin in 800 ml. of water was freed from acid by Amberlite IR–4 until pH 6 was reached. The solution including wash water (1·5 *l.*) was then filtered through the cation adsorbing resin, Amberlite IR–100 (25 × 2 cm. ; 40–50 ml./min.), and the column was washed. The basic amino acids were eluted from it by exchange with 7 per cent HCl until the phosphotungstic and the Sakaguchi reactions became negative in the filtrate. The latter was concentrated and then diluted to 100 ml. ; it was free from cystine, tyrosine, proline and hydroxyproline. It yielded the basic amino acids by the usual methods.

Exchange resins have been employed for the same purpose by Schramm and Primosigh (2, 3).

Recently, Th. Wieland (3, 5) used Wofatit C for the separation of basic amino acids contained in protein hydrolysates. In this exchanger, which contains carboxyl groups, the acid character is so weak that the material binds only the basic amino acids and displays in this respect a high degree of specificity. Its capacity is at least 0·1 g. of histidine per gram exchanger. Elution occurs easily with dilute mineral acids.

Of interest are the following observations. A separation of histidine from arginine + lysine is only successful if the exchanger is first converted, by washing with dilute KOH, into a potassium permutit. Such a column will adsorb only arginine and lysine from neutral solution, while histidine, whose isoelectric point is less on the basic side (pH 7·5), is carried into the filtrate by water. (An exchange with Na^+ or Li^+ was not successful.) The capacity of the neutralized K-exchanger is much less than that of the acid Wofatit C, viz., 2·4 mg. arginine per gram.

Commercial Wofatit C(I.G. Farben) should be dried at 100°, milled and sieved (diameter, 0·1 to 0·2 mm.). It is then purified by prolonged washings with 2*N*-KOH, 2*N*-HCl, and water to neutral reaction of the colourless filtrate. In order to prepare neutral material from this acid wofatit, this is kept for some time in contact with 2*N*-KOH and washed neutral with water.

The regeneration of a used acid column requires only a brief de-acidification with water.

Some basic amino acids can be separated from the non-basic ones and from each other by a single adsorption as follows (Bergdoll and Doty) :

To 10 ml. of solution (1–6 mg. of lysine, 1–4 mg. of histidine and 1–8 mg. of arginine ; 0·3 *N*-HCl), 30–50 mg. of zinc dust are added (in order to reduce cystine to cysteine) and the liquid is heated to 80° and then cooled. It is adsorbed on 20 g. of a Lloyd reagent-Hyflo Supercel mixture (1 : 2) in a 40 × 1·9 cm. tube ; a 0·6 cm. Hyflo Supercel layer is placed on top. The column is washed with 50 ml. of 1·7 *N*-HCl, then 10 ml. of the amino acid solution is introduced, followed by 180 ml. of 0·5 *N*-HCl ; 200 ml. of 1 *N*-HCl ; 150 ml. of 0·125 *M*-sodium bicarbonate ; 100 ml. of 10 per cent pyridine in 0·7 *N*-HCl ; and, finally, by 40 ml. of 0·5 *N*-HCl. The following filtrate fractions are taken : 80 ml. (discarded), 480 ml. (lysine), 625 ml. (histidine), and 730 ml. (arginine).

Separation of Aspartic and Glutamic Acids from Neutral and Basic Amino Acids on pH 3.3 buffered Alumina. Turba (cf. Turba and Richter) found their commercial aluminium oxide to be too weak for this purpose even after a treatment with *N*-HCl or acetic acid. However, washing Brockmann's alumina or " Aluminum oxydatum anhydricum " with an acetic acid-acetate buffer (pH 3·3) was satisfactory. Mixtures of aspartic and glutamic acid with glycine, alanine, leucine, serine, arginine, histidine, tryptophan, proline, cystine or methionine gave quantitative separations. Alumina (5 g.) was shaken with 10 ml. of the buffer for 10 min. After draining the liquid and washing the adsorbent with 2 × 10 ml. of water, the solid was introduced into the adsorption tube by means of 20 ml. of water. The solution containing glutamic and a monoamino-monocarboxylic acid (10–15 mg. in 2 ml. water each) was then cautiously added and the column washed with 3–5 × 10 ml. of water. When the filtrate had become negative to ninhydrine, it was concentrated in vacuo. The column was extruded into a fritted glass filter and the tube was rinsed out with 10 ml. of *N*/20-NaOH. The material was triturated with 40 ml. and then with 6 × 10 ml. of this alkali. The last filtrate was alkaline and free of amino acids. The eluate was acidified with strong HCl and evaporated in vacuo (40°) ; the residue was taken up with water and again evaporated. The

solution then showed pH5 and was ready for analysis. With cystine or other difficultly soluble amino acids a few drops of the pH 3·3 buffer were added to the initial solution and the elution was carried out with this buffer instead of water.

Separation of Glutamic from Aspartic Acid was made possible by the fact that a pH 3·3 buffer carries only the glutamic acid into the filtrate (Turba and Richter). Thirty grams of alumina were treated with a *N* acetic acid-acetate buffer (pH 3·3) in a bath (50°) for 10 min., then filtered, washed with 2 × 60 ml. of water and transferred with 120 ml. of water into the adsorption tube. A solution of the two acids (2 ml., 5 mg. each) + several drops of the buffer was introduced ; the adsorbent was washed with 160 ml. of the buffer and then with 30 ml. of water. The aspartic acid could be eluted by stirring with 30 ml. of *N*/2-NaOH and then with 5 × 30 ml. of *N*/10-NaOH.

These results were confirmed by Th. Wieland and Wirth, who also found that if an alumina column (10 g.) which had been pretreated with 0·5 *N*-HCl and washed with water is applied, 10 mg. of glutamic acid can be eluted with 0·5 *N*-acetic acid at room temperature ; aspartic acid (10 mg.) remains in the column and is eluted with 0·5 *N*-NaOH.

Aspartic acid, which would interfere with micro-estimation of glutamic acid, can be removed by using acid alumina (Prescott and Waelsch).

Isolation of Glutamic Hydrochloride from some Protein Hydrolysates (Th. Wieland 4, cf. Wieland and Paul). The solution (pH7) was adsorbed on acid alumina (50 g. per g. protein) and washed with 20 ml. of water, 50 ml. of saturated H_2S solution and again with 50 ml. of water (per g. protein). After the dark top zone had been discarded, the lower border line of the amino acids was located by boiling small samples of the column with neutral phosphate buffer and ninhydrin. The column was then appropriately cut and the section containing the amino acids was filled into a new tube. This

column, which should remain wet all the time, is washed with cold saturated baryta. As soon as the flow gives a positive barium reaction, the receiver is changed and the solution is collected until there is a strong reddening of phenolphtalein. After concentration in vacuo, correct elimination of Ba with sulphuric acid and centrifuging, the $BaSO_4$ is boiled out twice with 2*N*-HCl, and the combined liquids are evaporated to 2 ml./g. protein. Upon saturation with HCl at 0° and seeding with *dl*-glutamic acid hydrochloride, crystallization occurs.

Separation of Acid, Neutral, and Basic Amino Acids by Exchange Adsorption on Basic and Acid Alumina. Th. Wieland (1, 2) studied the behaviour on " basic alumina columns " (alumina Merck, washed with CO_2-free water) and " acid alumina columns " (treated with 3–4 parts of *N*-HCl and washed until slightly acid toward litmus). On the " basic " column lysine and arginine, for example, are adsorbed as cations while monoamino-dicarboxylic acids are adsorbed as anions on the " acid " column which lets the neutral or basic amino acids through. The diamino-monocarboxylic acids can be eluted from basic columns with dilute HCl until congo red paper attached to the tube turns blue ; the monoamino-dicarboxylic acids are released by alkali from an " acid " column.

The packing of the alumina suspensions required but little suction (a 5 cm.-layer = 5 g.). A neutralized (litmus) mixture of 25 mg. of aspartic acid, 10 mg. of lysine and 10 mg. of alanine was chromatographed on 4 g. of " acid " alumina. Of the filtrate (25 ml.) 4 ml. were employed for the titration of the free mineral acid and 10 ml. for that of lysine + alanine (evaporation in vacuo, titration in 90 per cent alcoholic solution with *N*/100 90 per cent alcoholic NaOH against phenolphthalein). Another 10 ml. of the filtrate were passed through 4 g. of the " basic " alumina which, upon washing with water, retained lysine only. A disadvantage of such titration methods is that HCl-treated columns give considerable blank values for hich substantial corrections must be made.

The separation of dicarboxylic amino acids from the others by using HCl-pretreated alumina was also carried out by Darling. The solution is neutralized with KOH (phenolphthalein) and the filtrate contains the neutral and basic amino acids ; glutamic and aspartic acids can be eluted from the column with 3 *N*-KOH.

Bendall, Partridge and Westall have shown that the Tiselius-Claesson displacement principle can be applied to the separation of amino acids on cation exchangers. For example, glycine and creatinine could be fractionally displaced from Zeo-karb by 0·043 *M*-ammonia.

Estimation of Basic, Neutral, and Acid Amino Acids in Protein Hydrolysates (Th. Wieland 2). In hydrolysates of casein, ovalbumin, globin, gelatin or zein the sums of the different types of amino acids were estimated after the separation on basic and acid alumina columns. The van Slyke amino-nitrogen values as obtained with the substances adsorbed on the acid column check with the literature data concerning the aspartic acid + glutamic acid + cystine content ; the corresponding N-values obtained on the basic column check with those for arginine + lysine. The starting material required is 100 mg. of protein or less.

The neutralized solution of the HCl-hydrolysate of 80 mg. of casein (Hammarsten) in 10 ml. was filtered through 6 g. of acid alumina into a 25 ml. flask and the column was washed with water until the mark was reached. Of this 4 ml. was used for the amino-N estimation, while 20 ml. were adjusted to pH 7·0 with a few drops of 0·5 N-alkali and poured on to 100 g. of basic alumina. The first 25 ml. of the filtrate did not show the ninhydrin reaction and was discarded. The column was then washed with 100 ml. of (preboiled) water to obtain all neutral amino acids (+ histidine). After the addition of a little acetic acid, this filtrate was evaporated to 20 ml. in vacuo and used for a van Slyke analysis.

From the basic column the basic amino acids (without histidine) could be eluted with 2*N*-acetic acid ; the analysis took place after evaporation to 15 ml. The acid column was eluted with 0·5 *N*-NaOH until the volume of the filtrate was 12 ml. ; it was then brought to 15 ml. with glacial acetic acid and analysed.

Quantitative chromatography of silk hydrolysates was carried out by Polson, Mosley and Wyckoff, who also used electron diffraction methods with an RCA electron microscope for the identification of spot extracts.

Detection of methionine sulphone and cysteic acid in hydrolysates of oxidized caseine : Dent and Rimington.

Separation of the Basic Diamino Acids (Arginine, Lysine, Histidine) from other Amino Acids and from each other on Filtrol and Floridin (Turba ; Turba and Richter). This can be carried out on neutrol filtrol in a quantitative manner. The adsorbed basic amino acids are best eluted with a pyridine-sulphuric acid mixture. The same adsorbent separates arginine from lysine quantitatively when the column is washed with monopotassium phosphate ; lysine is then precipitated with alcohol. For a separation of histidine from arginine (or lysine) floridin XXF extra was found to be satisfactory ; histidine passed into the filtrate. By combination of these methods a mixture of the three diamino-acids and of monoamino acids was also differentiated. Since the earths mentioned have the tendency to swell, all operations must be carried out rapidly, and thin adsorbent layers on fritted glass are used instead of a column.

Quantitative Separation of Histidine from a Monoamino Acid. An aqueous suspension of 3 g. of neutrol filtrol was placed on fritted glass and the pump was gently set into action 5 min. later. A solution containing 5–10 mg. of each compound (2 ml., pH 7) was then introduced with a pipette,

without stirring the adsorbent, and washed with 30–60 ml. of water, until the ninhydrin reaction became negative in the filtrate (monoamino acid). The histidine can then be eluted quantitatively with 80 ml. of a *N*-sulphuric acid + pyridine + water mixture (5 : 1 : 4); the diazo reaction of this filtrate becomes gradually negative. After elimination of the acid with $BaCO_3$ and centrifuging, the eluate is evaporated and analysed.

Separation of Arginine and Histidine. For the separation of 20 mg. of each compound in 10 ml. of water 12 g. of floridin can be used. The histidine is quantitatively eluted by washing with 200 ml. of water within an hour. After the diazo reaction becomes negative, the arginine is washed down with 100 ml. of pyridine-sulphuric acid.

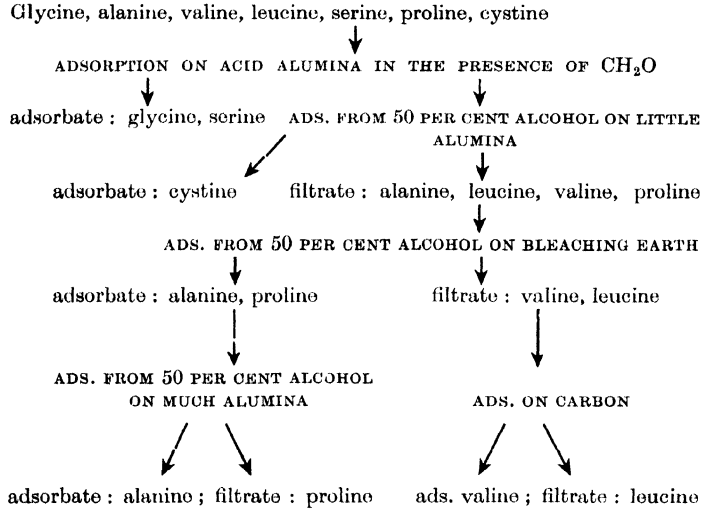
Separation of Arginine, Lysine, Histidine and a Monoamino Acid (Leucine) (25 mg. of each). From a mixed adsorbate on 12 g. of floridin only histidine + leucine were washed down with 200 ml. of water. This solution was evaporated to 15 ml. and sucked through 5 g. of neutral filtrol; 100 ml. of water eliminated leucine, and 100 ml. of pyridine-sulphuric acid the histidine from this adsorbent. The arginine-lysine mixture which remained on floridin was eluted with 100 ml. of pyridine-sulphuric acid, whereupon the acid was eliminated with $BaCO_3$ and the pyridine by repeated evaporations. (The receivers must be carefully rinsed with hot water.) In order to separate arginine from lysine, 25 g. of neutrol filtrol were suspended in $M/6$-KH_2PO_4 and formed into a compact layer by leaving it for $\frac{1}{2}$ hour on fritted glass with gentle suction. The amino acids were then introduced and washed with the phosphate until the filtrate became ninhydrin positive. The receiver was then changed and with 200 ml. of the phosphate solution all lysine was eluted. An elution of the arginine with 100 ml. of the pyridine-sulphuric acid followed.

Resolution of Aliphatic Monoamino-Monocarboxylic Acid Mixtures. Turba *et al.* propose tentatively the following

scheme, which, however, they do not consider yet as generally applicable in the present form (Table 12).

TABLE 12

SCHEME FOR THE SEPARATION OF AMINO ACIDS (Turba *et al.*)



Separation of Amino Acid Hydrochlorides in the presence of HCl. Recently, Koschara (9) in a one-page article outlined the following procedure for the separation of the hydrochlorides contained in protein hydrolysates, in the presence of 2 *N*-HCl. The adsorbent used is carbon (activated with H_2S) ; the ratio, adsorbent : substance, is only 4 : 1. The introduction and the first development take place at $-10°$ to $0°$. The strongly fixed amino acids are eluted with NH_3 (or aqueous acetone-alkali) while the other hydrochlorides can be rechromatographed in an alcohol-acetone medium. All acidic chromatographic filtrates should be collected in fractions which are evaporated and treated with alcohol. Hydrochlorides of glutamic acid, lysine or histidine then crystallize directly or on addition of acetone. (Arginine dihydrochloride is sticky, but it can be converted into the

crystalline monochlorohydrate with aniline in alcohol solution.) Hydrochlorides of, e.g., leucine, isoleucine or norleucine are obtained in crystals upon the addition of much acetone or they can be precipitated with acetone from their alcohol solutions as free amino acids.

Separation of Amino Acid Salts on precipitated Silver Sulphide or Barium Sulphate. Amino acid mixtures were recently chromatographed by Hamoir (1, 2) from aqueous solutions on silver sulphide. The precipitate is formed from silver nitrate (equivalent to 12·5 g. of the sulphide) in 2 *l.* of solution. This precipitate is left in the presence of excess H_2S for several hours, then the latter is completely removed and the Ag_2S is pulverized (CO_2-free water should be used in the following operations). This sulphide is activated by a treatment for 3 hours with 1–2 per cent silver nitrate and washed until the washings give no precipitate with NaCl. (If the silver sulphide is activated with 0·1 *M*-nitric acid for several hours, the adsorbing power is doubled without affecting the selectivity.)

Most amino acids can be classified into four groups: Group 4: Not adsorbed at pH 6–7: glycine, alanine, serine, valine, threonine, leucine, proline, hydroxyproline, lysine and arginine. Group 3: adsorbed at pH 6 with easy elution; in order of decreasing adsorbability: glutamic acid, aspartic acid, tryptophan, histidine, phenylalanine and tryosine. Group 2: Greater stability of the adsorbate at acid pH: methionine. Group 1: cystine and cysteine, adsorbed from *M*-acetic acid (not recovered by elution).

A mixture of the amino acids (10 mg. each) of Group 3 (except phenylalanine) as well as alanine, serine, valine, leucine and arginine (Group 4) were chromatographed in 200 ml. of an aqueous solution adjusted to pH 6. When 58 per cent of the amino-N had passed into the filtrate, the latter contained the members of Group 4. The others could be quantitatively eluted with 0·1 *M*-acetic acid. The total amino-N recovered was almost 100 per cent. All adsorption

experiments were followed by van Slyke estimations and by checking the diazo reaction in fractions of filtrate or eluate.

The separation of individual amino acids is not described in this paper, except for methionine, which forms a group for itself (see above). While the elution of glutamic acid from a 12·5 g. column is complete after washing with 50 ml. of 0·1 *M*-acetic acid, this washing does not elute any methionine which in turn can be carried into the filtrate with 150 ml. of *M*-acetic acid.

In a somewhat earlier investigation, which has several points in common with Hamoir's experiments, Tarte showed that the solubilities of barium salts of amino acids and their adsorbabilities on barium sulphate from water are parallel. If a few milligrams of monocarboxylic amino acids and aspartic acid are adsorbed, the former can be removed by washing with double distilled water, while the elution of the aspartic acid requires *N*/50-acetic acid. Even glutamic and aspartic acids can be separated ; e.g., the elution of the former was carried out with 400 ml. of *N*/1000-acetic acid, and then that of the aspartic acid with 300 ml. of *N*/50-acid.

Some Biological Applications of Paper Chromatography. The direct detection and differentiation of free amino acids in plant tissues was first attempted by La Cour and Drew, who applied one dimensional chromatography on smears placed on filter paper strips ; a few spots could be located. Dent, Stepka and Steward extracted potato tubers with 70–80 per cent alcohol, evaporated and dissolved the dry residue in water. With quantities corresponding to 50 μg. of N a 30 hours' development, first with aqueous phenol and then with aqueous collidine-lutidine, was carried out, and resulted in 24 ninhydrin-positive spots.

Dent studied the amino acids excreted in the urine by cases of Fanconi syndrome. His recent paper includes a number of technical improvements on the micro scale and will certainly stimulate the investigation of constituents other than the amino acids of normal and pathological urines.

In a study of the creatinine metabolism of the rat, this compound was identified by paper chromatography (Maw). Amino acids of cholera vibrios : Blass and Macheboeuf.

Separation of Monoamino-Monocarboxylic Acids in the presence of Formaldehyde (Schramm and Primosigh 1). As is well known from the formol titration (cf. p. 150), neutral amino acids change their behaviour to the acid side in the presence of formaldehyde. The extent of the pK change is different with the individual amino acids, and in particular glycine and serine can be separated from alanine, leucine, valine and isoleucine. Under suitable conditions formaldehyde carries the latter amino acids into the filtrate, from which the aldehyde can then be eliminated by distillation. When 1 mg. of each amino acid is used, a 20 g. column of alumina (pre-treated with HCl according to Wieland ; tube, 30 × 1·7 cm.) is washed with 100 ml. of 10 per cent formaldehyde (faint pink with phenolphthalein ; neutralized with *N*-KOH). The mixture of the amino acids is introduced in 5 ml. of the formaldehyde and the column is washed with the same solution until the filtrate amounts to 100 ml. The glycine-serine mixture can then be eluted with 50 ml. of *N*/2-KOH, cf. also Kibrick.

Micro-estimation of Glycocyamine (Guanido-Acetic Acid) and Arginine in Biological Fluids. According to Dubnoff and Borsook, the adsorption method using Lloyd's reagent (Davenport, Fisher and Wilhelmi) can be improved by using permutit. Urine (diluted 5–10 times) or deproteinized blood or a tissue extract can be handled in this manner. The tissue suspension (40 ml./g., pH 6) is immersed in boiling water for 10 min., cooled and filtered. Arginine is then removed by filtration through permutit contained in the stem of a funnel made of two glass tubings (upper, 10 × 1·5 cm. ; lower, 10 × 0·7 cm. ; internal diam., 0·5 cm.). After 5 ml. of the solution have passed through a 8·5 cm. high column (0·9 g.), 5 ml. of 0·3 per cent NaCl are introduced and the filtrate is made up to 10 ml. in order to determine the arginine-free glycocyamine (Sakaguchi reaction). The arginine can be eluted quantitatively with 10 ml. of 3 per cent NaCl (Dubnoff).

There are considerable differences between permutit batches, and Sims was unable to find a satisfactory one. He solved the problem by using Amberlite IR-100-H. Urine

samples were diluted 1 : 6 and serum ultrafiltrates with 2 parts of water. In order to convert the resin entirely into the Na-form, it should be treated in a column with 10 ml./g. of 5 per cent NaCl, allowing a 10 hours' flow. After washing with water, the resin is stored in 0·3 per cent NaCl, in a refrigerator. During the adsorption experiment the flow should be so retarded that 5 ml. require 20 min. to pass through (column, 7·5 cm. ; internal diameter, 0·4 cm.). The resin is introduced in 0·3 per cent NaCl. The column is washed with the NaCl solution ; the filtrate is made up to 15 ml. and is then available for a colorimetric glycocyamine estimation.

Estimation of Citrulline in the Plasma (Archibald). The urea is removed by urease and KCN and the dialysate is filtered through Amberlite IR-100 which retains citrulline but does not adsorb allantoin. The citrulline content is determined by subtracting the colour equivalent (diacetyl-monoxime + sulphuric acid) of the chromatographic filtrate from that of the untreated dialysate. (For further details see the original.) The column (24 × 0·8 cm.) is pretreated by successive washings with 10 ml. of 10 per cent NaCl ; 5 ml. of water ; 10 ml. of 12 *N*-HCl ; 25 ml. of water ; 5 ml. of alcohol ; and 5 ml. of ether ; it is dried by an air current. Zeo-karb H (Permutit Co.) may be also used after a treatment with conc. HCl.

The *total base of the serum* can be estimated by filtration through the cation adsorbing resin, Amberlite IR-100 and titration of the eluted anion (Polis and Reinhold).

Separation of Coloured Amino Acid Derivatives. N-*p*-phenyl-azobenzoyl-amino acid-methylesters (Karrer, Keller and Szönyi) are of reddish or brownish-yellow colour and can be adsorbed on basic zinc carbonate in the following sequence : glycine > alanine > leucine > valine. A mixture of such derivatives of glycine, *l*-alanine, *l*-valine and *d*, *l*-leucine (50 mg. each) in 10 ml. of benzene was poured on to a column (50 × 2·6 cm., washed with 70 ml. of benzene) and developed with 430 ml. of light petroleum (b.p. 70-80°) containing 5 per cent benzene ; four pigmented zones appeared :

Zone I (35 mm.) : small amounts of phenyl-azobenzoyl-glycine (eluted with alkali).

Zone II (100 mm.) : eluted with benzene ; the evaporation residue boiled with light petroleum. Pure N-*p*-phenyl-azobenzoyl-glycine methylester.

Zone III (85 mm.) : treated like II ; pure N-*p*-phenyl-azobenzoyl-*l*-alanine-ester.

Zone IV (130 mm.) : the upper half was treated like II and gave N-*p*-phenyl-azobenzoyl-*d*, *l*-leucine-ester. The lower half contained a mixture which yielded on rechromatography (beside a pale top zone) the expected *l*-valine derivative.

It seems that this method could well be developed in the direction of quantitative determinations.

For the COLORIMETRIC ESTIMATION OF HISTIDINE OR HISTAMINE according to Pauly's method, the products are first coupled with diazotised *p*-nitro- or 2 : 4-dichloro-phenylaniline. Chromatography on Brockmann's alumina from alcohol indicates that the products are complicated mixtures. However, if the reaction is carried out in a dilute solution of sodium carbonate, only two pigment zones appear (Diemair and H. Fox).

TISELIUS–CLAESSON ANALYSIS OF AMINO ACID MIXTURES

Some experiments for such separations were published recently by Tiselius (13). As Table 13 shows, the individual specific retention volumes vary a good deal. Fig. 22 illustrates a frontal analysis of alanine + leucine on carbon ; the first step corresponds to pure alanine. The displacement analysis of a valine and leucine mixture is shown in Fig. 23.

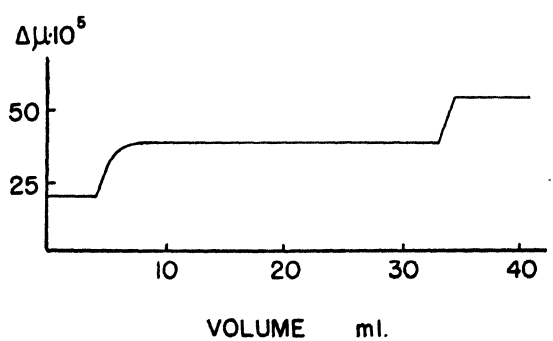


FIG. 22.—Frontal analysis of a mixture of alanine (0·159 mg. N/ml.) and leucine (0·104 mg. N/ml.) in water, on 1·5 g. of " Norit P 3 Special ". (The ordinate denotes the difference in refractive index between the solution and the solvent) (Tiselius).

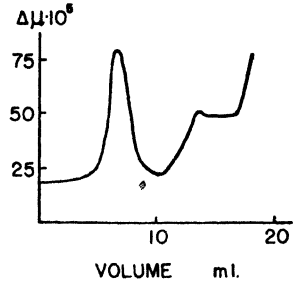


FIG. 23.—Displacement analysis of valine (1.28 mg. N) and leucine 0·96 mg. N) on 1·5 g. of " Carboraffin C ", displaced with a 5% phenol solution. (Found, 1·35 mg. and 0·88 mg. N) (Tiselius).

TABLE 13

RETENTION VOLUMES IN THE ADSORPTION OF SOME AMINO ACIDS ON CARBO ACTIV (SCHERING); 0·5 PER CENT AQUEOUS SOLUTIONS (TISELIUS).

Amino Acid	Retention Vol. ml./g. adsorbent
Alanine	0·3
Hydroxyproline	2·0
Proline	2·5
Valine	3·2
Leucine	7·7
Isoleucine	9·2
Methionine	12·4
Histidine	15·0
Arginine	40·4
Phenylalanine	62·5
Tryptophan	76·5

SEPARATION OF AMINO ACIDS BY PARTITION CHROMATOGRAPHY

As has been pointed out in the General Section (p. 38) the most important application of partition chromatography is the separation of some amino acids, especially as occurring in acetylated protein hydrolysates. *R* values for a number of such acids are summarized in Table 14. While silica gel was used in these experiments, free amino acids can be separated on starch, as discussed below (Synge 1). More detailed information can be found in the papers published by Gordon,

Martin and Synge (1–7), Martin and Synge (1, 2) and Consden, Gordon and Martin. Review: Martin and Synge (3); Martin (2).

Obviously, many variations of the described procedures are still possible. According to Sanger, methylsulphonyl amino acids migrate very similarly to the acetyl derivatives. Gordon, Martin and Synge (5) found that the products of repeated treatment of silk fibroin with alkali and dimethyl sulphate can be partition chromatographed, and the presence of etherified hydroxyamino acids can be demonstrated.

TABLE 14

R-VALUES OF ACETAMINO ACIDS (GORDON, MARTIN and SYNGE).

	Butanol-chloroform			Propanol-cyclohexane		Ethyl acetate
	1%	3%	17%	5%	30%	
Acetylphenylalanine	0·5	0·9	Fast	0·3	Fast	Fast
Acetylnorleucine	0·4	—	Fast	—	—	—
Acetylleucine, Acetylisoleucine	0·3	0 6	Fast	0·3	Fast	Fast
Acetyltryptophan	0·3	0·6	Fast	0·15	Fast	—
Acetylvaline, Acetylnorvaline	0·15	0·3	Fast	0·15	Fast	0·9
Acetylmethionine	0·15	0·3	Fast	0·09	0·7	0·8
Acetylproline	0 15	0·3	Fast	0·04	0·3	0·2
α-Acetaminobutyric acid	0·07	—	—	—	—	0·5
Acetylalanine	0·025	0·04	0·35	0·04	0·3	0·2
N-Acetyltyrosine	0·02	0·04	0·7	—	0·6	Fast
Acetylglycine	Slow	Slow	0·15	Slow	0·15	0·1
Acetylaspartic acid, Acetylglutamic acid, NN′-Diacetylcystine, NN′-Diacetyllysine	Slow	Slow	0·1–0·2	—	—	—
N-Acetylhydroxyproline	Slow	Slow	0·07	Slow	0·1	0·06

The techniques for the separation of the amino acid constituents of acetylated protein hydrolysates were given by Gordon, Martin and Synge (2) as follows: Three grams of silica gel are saturated with methyl orange solution and suspended in 3 per cent butanol-chloroform. As much as possible of the acetylated mixture is transferred to the column

(internal diam., 1 cm.) by hot extraction with 5 × 1 ml. (or less) of the solvent mixture. Each portion should drain into the gel before the next one is added. (The flask is put aside.) While developing with 3 per cent butanol-chloroform, the following zones are allowed to run through and are collected in pyrex flasks : (I) phenylalanine ; (II) leucine + isoleucine ; (III) proline + valine + methionine. The surface of the developer is then allowed to sink to the top of the adsorbent and the acetylated material that remained in the flask is dissolved in 1 ml. of boiling 17 per cent butanol-chloroform and is run on the column. After rinsing out three times as before, development is continued with the new solvent. Consequently, tyrosine and alanine migrate faster than earlier and also separate from each other. The tyrosine (IV) runs twice as fast as alanine (V). All fractions are freed from the solvent in vacuo. Fractions I and II are rechromatographed (2 g. of silica, 5 per cent propanol-cyclohexane) and Fraction III also (3 g. of silica). The first zone collected from the latter corresponds to valine. When 30 per cent propanol-cyclohexane is employed as a developer, methionine and proline are collected separately. Fractions IV and V are rechromatographed on 2 g. of silica (30 per cent propanol-cyclohexane). Minor contaminants are discarded each time.

Preparation of Acetylated Protein Hydrolysates (Martin and Synge 2 ; Gordon, Martin and Synge 1, 6). After refluxing 100 mg. of protein in 6*N*-HCl for 6 hours and removing the acid by repeated evaporations with water in vacuo, the solution is made alkaline with 6*N*-NaOH (thymolphthalein), concentrated to a thin syrup, and acetylated with 10 ml. of 2 *N*-NaOH + 1 ml. of acetic anhydride in five equal portions in the course of 15 min. After each addition the liquid is shaken and cooled with ice-water. After it has been allowed to remain alkaline to thymolphthalein for 10 min., 1 ml. of 10*N*-sulphuric acid is added, the solution is concentrated in vacuo below 40° to 5 ml. and is then acidified with 10*N*-sulphuric acid (strong red colour with thymolblue). The solution is transferred to a separating funnel at a volume of

10 ml. with water and is extracted with 5 × 50 ml. of chloroform. The combined extracts are filtered through paper, evaporated in vacuo and the residue is made up with alcohol to 10 ml. Three ml. of this solution (= 30 mg. of proteins) are evaporated in vacuo. The free acetic acid is removed by standing overnight over sulphuric acid and soda-lime in vacuo (cf. Wachtel and Cassidy). It is then ready for chromatography in chloroform-butanol. As the main zones pass out of the bottom of the tube, the receiver is changed. The solution is evaporated in vacuo, and the solution of the residue in a little water is titrated with 0·01 *N*-barium hydroxide in the absence of CO_2 (phenolphthalein).

As pointed out by Gordon, Martin and Synge (1, 7), the following difficulties may arise : 1. Acetyltryptophan is very sensitive to the conditions of hydrolysis and acetylation and cannot be recovered in any satisfactory way. 2. As shown by the treatment of artificial mixtures of acetamino acids, several artifact bands are formed in small quantities. This difficulty seems to have been overcome by rechromatographing with propanol-cyclohexane as mentioned.

From the HYDROLYSATE of crystalline TYROCIDINE hydrochloride Gordon, Martin and Synge (5) isolated phenylalanine (mainly *d*), leucine, proline, valine, tyrosine, ornithine and glutamic acid. The chromatographic separation of the acetylated glutamic and aspartic acids was not satisfactory and these components were determined by other methods. The tryptophan values were found to be much too low. As stated later by Gordon, Martin and Synge (7) this was caused by the procedure employed, viz., direct adsorption of the acetylated mixture on silica gel, which resulted in the presence of free mineral acid, to which acetyltryptophan is especially sensitive.

Some features of the procedure employed for the analysis were as follows : hydrolysis as described by Gordon, Martin and Synge (1) for gramicidine ; acetylation in the presence of 5 ml. of 4*N*-NaOH ; after acidification, immediate adsorption on 16 g. of silica gel ; after mixing this was made to a slurry with 17 per cent butanol-chloroform and a column was prepared without the addition of an indicator. The vessels were washed three times with the solvent and the fractions were allowed to drain separately into the column, followed by 1 l. of fresh solvent. The initial use of 17 per cent butanol-chloroform secured the complete solution of the material. The silica was saturated with 0·05 per cent aqueous pelargonin chloride (indicator).

Coloured Nitro Derivatives of Amino Acids. For the determination of free amino groups in peptides and proteins

Sanger devised a method based on the separation of the coloured dinitrophenyl derivatives which can be obtained at room temperature by using 2 : 4-dinitrofluoro-benzene. The method was applied to the estimation of the terminal residues in insulin by partition chromatography. The behaviour of individual amino acid derivatives and artificial mixtures was also studied. The general procedure of Gordon, Martin and Synge was used except that the indicator was eliminated and the type of solvents was somewhat modified :

" Ethanol-ligroin column " : shake 1 vol. of ethanol, 1 vol. of water and 10 vol. of light petroleum (b.p. 80–100°) together ; from the lower phase 1 ml. is added to each 2 g. of dry silica while the upper one is used as the moving phase in the column. In a similar manner are used : 1 vol. of methanol, 1 vol. of water and 15 vol. of carbon tetrachloride ; or 1 vol. of acetone, 1 vol. of water and 10 vol. of cyclohexane. Glycol and benzene are shaken together and 1 ml. of the glycol layer is added to each 1 g. of dry silica.

The reaction product of *insulin* and dinitro-fluorobenzene (1 g.) was refluxed with 100 ml. of 20 per cent HCl for 4 hours and the whole extracted with ether. The evaporation residue of the ether was first passed through an ether column and then fractionated on a chloroform column (40 g. silica). The coloured phenylalanine and glycine derivatives (identified by mixed chromatograms) were run through separately. The dry residue of the N-2 : 4-dinitrophenyl-*l*-phenylalanine fraction was passed through a 20 g. acetone-cyclohexane column. When its filtrate was evaporated, dissolved in a minimum of warm dilute bicarbonate and acidified, crystals appeared within a few days (14 mg.). The corresponding glycine derivative was similarly treated and crystallized from hot water (16 mg.).

The aqueous solution (after the ether extraction, see above) was evaporated and hydrolysed with 20 per cent HCl for 20 hours. After several operations 24 mg. of ε-dinitrophenyl-lysine hydrochloride were isolated.

Copper Compounds of Neutral Amino Acids (Th. Wieland and Fremerey). Partition chromatography can be applied to these well-crystallized, dark blue complexes. Their

relative adsorption affinities are : serine or glycine > hydroxyproline > alanine > valine, proline, leucine, methionine.

One gram of silica gel was moistened with 1 ml. of an aqueous, saturated phenol solution, then suspended in phenol + chloroform (1 : 1, saturated with water) and filled into a tube. A mixture of 8 mg. of valine- and 8 mg. of alanine-copper in 0·6 ml. of phenol + chloroform (2 : 1) was then introduced and developed with phenol + chloroform (1 : 1, saturated with water). Two blue zones migrated down ; they were collected separately and titrated. Iodometrically found : 7·9 mg. of valine-copper (first filtrate), and 7·6 mg. of alanine-copper (second filtrate).

TWO-DIMENSIONAL CHROMATOGRAMS : p. 42.

PEPTIDES

The desired development in this field should include the separation of peptides from amino acids as well as the resolution of peptide mixtures. Relatively few attempts have been made in either direction. Short review ; Jutisz and Lederer.

A mixture of leucyl-glycine and leucine (10 mg. of each) was adsorbed by Turba, Richter and Kuchar on 0·7 g. of active carbon (pre-treated with 1 per cent HCN). Washing with 100 ml. of water carried leucine quantitatively into the filtrate ; the peptide could be eluted with pyridine + glacial acetic acid. Synge (1) observed in partition chromatographic experiments that valylglycine travelled on starch as hydrochloride at twice the rate of alanine (which emerged almost HCl-free) while in the free state the rates are closely similar in butanol-water systems. Evidently, the acid has a differentiating effect in this case. On silica, in chloroform-butanol-water or cyclohexane-propanol-water or ethyl acetate-water systems acetyl-dipeptides travel at rates lying between those of their acetylated constituents (Gordon, Martin and Synge 1). R_F values for some peptides in filter paper strips were given by Synge (1).

Stronger heating than for amino acids is required in order to produce a colour with ninhydrin.

Recently, Consden, Gordon and Martin (2) published a much improved method for the identification of the lower peptides

that occur in partial hydrolysates of proteins. Their procedure includes ionophoresis and then partition chromatography on paper. The spots are washed off the paper and the amino acid constituents are identified after hydrolysis or deamination and hydrolysis.

The isomers, leucyl-glycine and glycyl-leucine can be separated on starch (S. Moore and Stein).

Peptide Mixtures obtained from Clupeane and Clupein (Waldschmidt-Leitz and Turba ; Waldschmidt-Leitz, Ratzer and Turba). The zones could be directly located on the neutrol filtrol column because they were of lighter shade than the empty sections. The peptides were characterized by the ratio, total nitrogen/amino nitrogen. Such ratios observed were not altered by rechromatography ; nevertheless, it is possible that the zones contained mixtures of very similar peptides. Arginine-poor fractions showed the weakest adsorbabilities. A limited fractionation was reached with clupeane hydrolysates. When a pancreatic hydrolysate of clupein itself was adsorbed, it yielded three fractions (= 99 per cent.) with the chromatographically constant ratios, $N/NH_2 = 17$, 9, and 5 (in order of decreasing adsorbabilities). The authors suggest that these fractions could contain four, two, and one arginine building stones respectively.

A solution containing 672 mg. N in 92 ml. ($N/NH_2 = 8{\cdot}5$) was adsorbed on neutrol filtrol ($7{\cdot}3 \times 2{\cdot}4$ cm.), washed several times with some water, then with 200 ml. of $M/15$-phosphate buffer pH 5·6 (fraction IV) and finally with 350 ml. of $M/3$-buffer (fraction III). The column was then divided into two parts making use of a good boundary line (lower, fraction II ; and upper, fraction I), and both were eluted with a mixture of 3 parts of pyridine and 7 parts of $2N$-sulphuric acid (pH 4). Kjeldahl and van Slyke estimates gave : Fraction I (28·8 per cent), $N/NH_2 = 16{\cdot}4$; II (33·6 per cent), 8·6 ; III (23·1 per cent), 9·0 ; and IV (13·6 per cent), 4·9.

Peptides from Cowhide Gelatin. Several lower peptides were isolated from a partial hydrolysate by Gordon, Martin and Synge (4). These hydrolysates were obtained on using $10N$-HCl at 37° for 4 or, preferably, 19 days. Basic and acidic materials were removed by electrodialysis and the " neutral " fraction was partition chromatographed on an

ethyl acetate-water column. The faster moving fractions were run out as usual, but the slower moving ones were cut out after extrusion and eluted with alcohol on a Buchner filter. Each fraction was then rechromatographed, using first a 1 per cent and then a 17 per cent butanol-chloroform-water system. The final products were analysed for component amino acids. Only a few fractions could be definitely identified, viz., a tripeptide (proline-alanine-glycine), a dipeptide (proline-alanine), and a fraction containing mainly proline-glycine but also proline-alanine.

Gramicidin. A chromatographic purification of a gramicidin sample has not yet been successful (Gordon, Martin and Synge 2). For the resolution of the acetylated gramicidin hydrolysate by partition chromatography, the procedure outlined by Gordon, Martin and Synge (1) was followed. First, a 5 per cent propanol-cyclohexane fraction was run out (chiefly leucine), then four fractions with 30 per cent propanol-cyclohexane (tryptophan + valine ; tryptophan degradation products ; alanine ; glycine). The two first fractions required rechromatography on a 3 per cent butanol-chloroform column. Valuable control experiments showed that corresponding artificial acetylamino acid mixtures yield chromatograms practically identical with those from the hydrolysate.

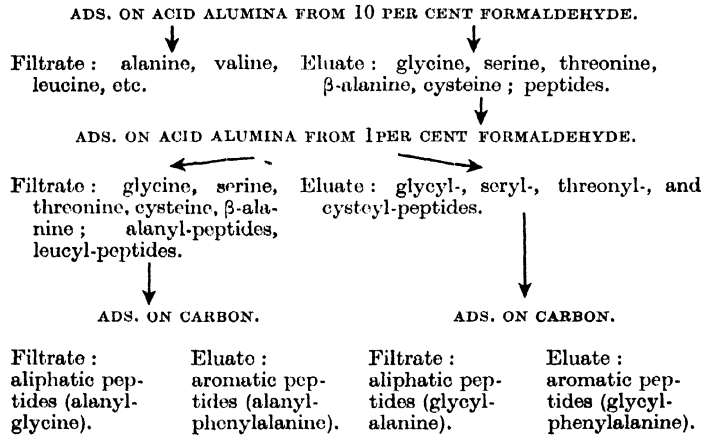
Some progress in the resolution of partial hydrolysates has been made with gramicidin by Synge (1) who prepared a sample by treatment at 37° for 10 days with 10*N*-HCl and acetic acid (1 : 1 v/v). About half of the peptide bonds were opened in this manner. Crude potato starch was adequate as column material on which the development was carried out by using *n*-butanol (saturated with water). Amino acid zones were indicated after development by passing through an ethereal solution of ninhydrin. In contrast to amino acids, peptide zones became coloured upon heating only. As the main part of a fraction, the presence of *l*-valylglycine was proved.

The partial hydrolysate of 0·25 g. of gramicidin (which became bluish purple) required 150 g. of air-dried starch ; by

washing with wet butanol 40 fractions were collected in 48 hours. A drop of each fraction was tested with ninhydrin on filter paper by heating.

In a recent detailed study Consden, Gordon, Martin and Synge investigated partial hydrolysates of gramicidin S in order to establish the sequence of the amino acid units. Among others, use has been made of the method of Sanger, who introduced the 2:4-dinitrophenyl residue into basic groups (p. 160). For many technical improvements, see the original paper.

Separation of Peptide and Amino Acid Mixtures into Groups, in the Presence of Formaldehyde. Lederer and Kiun observed that from a 10 per cent formaldehyde solution neutral di- and tripeptides are adsorbed on acid alumina and so are glycine and serine. They can be eluted with alkaline media. Making practical use of this behaviour, Jutisz and Lederer recently recommended the following scheme for the resolution of synthetic mixtures :



Separation of Peptides by the Tiselius-Claesson method. As Table 15 shows, the peptides studied were generally more strongly adsorbed on active carbon than were the amino acids. A separation was obtained, e.g., by displacing

a mixture of leucyl-glycyl-glycine and leucyl-glycine on carboraffin C with a 0·25 per cent phenol solution. In such experiments considerable losses may occur, especially with peptides showing strong adsorption. For peptides containing diamino-monocarboxylic residues the use of exchange adsorbents seems to be more effective than the method just mentioned.

TABLE 15

RETENTION VOLUMES IN THE ADSORPTION OF SOME PEPTIDES ON CARBO ACTIV (SCHERING); 0·5 PER CENT AQUEOUS SOLUTIONS (TISELIUS 13)

Peptide	Retention vol. ml./g. adsorbent
Glycyl-glycine	3·5
Leucyl-glycine	18·2
Leucyl-glycyl-glycine	29·8
Glycyl-alanine	4·0
Valyl-alanine	22·0
Alanyl-leucyl-glycine	34·4
Glycyl-leucyl-alanine	42·5
Glycyl-leucyl-glycine	38·0

Fractionation of thyroglobulin: Rivière, Gautron and Thély.

NUCLEOPROTEINS

The behaviour of a nucleoprotein (from calf thymus; 0·06–0·09 per cent solution) in the Tiselius analysis was studied by von Euler and Hahn (1, 2). They found that this nucleoprotein was adsorbed as such and that no differentiation was possible between its nucleic acid and the protein component in the frontal analysis on carbon, floridin or alumina. This is contrasted with the markedly salt-like behaviour of some model substances, e.g., piperidine-nucleate, of which the acid component was more strongly adsorbed on alumina than the piperidine, while the sequence was inverted on carboraffin. Compounds like scombrin-nucleate, clupein-chloroacetate, etc., also behaved like salts. There is no difference in the adsorption behaviour of nucleoproteins obtained from normal and irradiated cell nuclei.

CHAPTER XIII

STEROLS AND SOME STEROIDS INCLUDING SEX HORMONES

(Cf. Table 16, p. 176)

No detailed study is yet available of the dependence of adsorption affinity on certain details of steroid structure. However, the influence of double bonds, hydroxyl groups and some steric configurations can be demonstrated on simple examples chosen more or less at random :

Upon adsorption on alumina from benzene, cholestanone is carried into the filtrate by means of benzene + light petroleum, while cholestenone requires ether (Bretschneider). Esterified cholesterol of the blood serum is eluted from alumina by CCl_4 but free cholesterol only with $CHCl_3$ (Trappe 5 ; cf. Hess). The separation of cholic from desoxycholic acid, and dehydrocholic from dehydrodesoxycholic acid can be effected by gradual development with methanol on alumina (Crippa and Maffei). Coprosterol is much more weakly held by alumina than is cholesterol (Christiani and Eck). 3(β)-Acetoxycholestanol-7(α) is washed into the filtrate by benzene + hexane (1 : 1), whereas 3(β)-acetoxycholestanol-7(β) requires benzene + ether (1 : 1) (Wintersteiner and Moore). Some deuterio-derivatives : Anker and Bloch.

In this important field the *liquid chromatogram method* was applied with success in many instances, for example, by Ruzicka and, especially, by Reichstein and their collaborators. The characteristic feature of the method, which is illustrated below by a few examples, is the use of a relatively small alumina column (Merck, standardized according to Brockmann) which is systematically washed by a series of different solvents and solvent mixtures. The residue left on evaporation of each filtrate fraction is examined ; identical fractions are combined and rechromatographed, if necessary. Some fractions can usually be neglected owing to their smallness or to the presence of contaminants. The method may require unusually large volumes of solvents and considerable time ; however, it has proved of great value also for the resolution of crude reaction mixtures.

In some complicated cases, e.g., when commercial suprarenal gland concentrates were investigated, as many as seven chromatographic steps, all based on the above fractionation

principle, were necessary (Reichstein and von Euw). Similarly, an elaborate resolution of pig testes extracts was carried out by Ruzicka and Prelog.

We list below some series of solvents that were successfully used with alumina, in the sequence given.

Benzene + light petroleum (1 : 4), (7 : 3), (1 : 1); benzene; benzene + ether (9 : 1), (4 : 1); ether; ether + methanol (1 : 1).

Light petroleum; benzene + light petroleum (1 : 9), (1 : 4), (3 : 7), (2 : 3); benzene; benzene + ether.

Pentane; benzene + pentane (1 : 1); benzene; benzene + 1 per cent to 50 per cent ether; ether; ether + acetone (1 : 1); acetone.

Benzene + light petroleum (1 : 4), (1 : 1); benzene; benzene + ether (20 : 1), (10 : 1), (5 : 1), (3 : 1), (1 : 1); ether; ether + chloroform (1 : 1); chloroform; chloroform + methanol + ethyl acetate (2 : 1 : 1).

Benzene + light petroleum (1 : 8), (1 : 4), (1 : 1); benzene; benzene + ether (400 : 1), (80 : 1), (8 : 1), (4 : 1), (1 : 1); ether; ether + chloroform (100 : 1); (40 : 1), (20 : 1), (7 : 1), (3 : 1), (1 : 1); chloroform; chloroform + methanol + ethyl acetate (2 : 1 : 1).

Resolution of Mother-liquors containing 15-β-Hydroxy-progesterone. The liquors were obtained from suprarenal gland extracts after previous separations and acetylation (von Euw and Reichstein 3); 3·3 grams of the material were adsorbed on 90 g. of alumina. Each of the following numbers denotes a fraction obtained after washing the column with 400 ml. of solvent.

No. 1.	30%	benzene,	70%	light petroleum		
„ 2.	45%	„	55%	„	„	allo-pregnanolone acetate (5 mg.).
„ 3.	60%	„	40%	„	„	
„ 4.	75%	„	25%	„	„	
„ 5.	100%	„				progesterone (15 mg.).
„ 6.	100%	„				
„ 7.	100%	„				
„ 8.	100%	„				androstene-dione (5 mg.).
„ 9.	100%	„				
„ 10.	100%	„				
„ 11.	100%	„				hexagonal leaflets, m.p. 277–8° (2 mg.).
„ 12.	100%	„				
„ 13.	100%	„				
„ 14.	100%	„				
„ 15.	99%	„	1%	ether		rods, m.p. 234–6° (3 mg.).
„ 16.	99%	„	1%	„		
„ 17.	98%	„	2%	„		
„ 18.	98%	„	2%	„		crystals, m.p. 242–5° (2 mg.).

(continued on page 168)

No. 19.	97% benzene,	3% ether	}	allo-pregnane-diol-3 : 11-one-17
,, 20.	96% ,,	4% ,,	{	acetate-3 (15 mg.).
,, 21.	94% ,,	6% ,,	}	andrenosterone (40 mg.).
,, 22.	92% ,,	8% ,,	{	
,, 23.	88% ,,	12%		leaflets, m.p 259–261° (6 mg.).
,, 24.	85% ,,	15% ,,	}	17 β-hydroxy-progesterone (60 mg.).
,, 25.	80% ,,	20% ,,	{	
,, 26.	70% ,,	30% ,,	}	17a-methyl-D-homo-androstene-
,, 27.	50% ,,	50% ,,	{	(4)-ol-(17a)-dione-(3 : 17) (80 mg.)
,, 28–36.	ether, then ether with 1% to 60% acetone.			.. high melting crystals.

Isolation of Cholesterol Derivatives and Some Other Compounds from Extracts of Spleen. A further good example of this kind of chromatographic work was given by Prelog, Ruzicka and Stein, who worked with fractions of pigs' spleen (1,500 kg.). After a number of preliminary operations, the non-ketonic fraction, which weighed 82 g. and gave a negative reaction with Girard reagent T, was separated from the unsaponifiable part (from which the main bulk of cholesterol had been eliminated). This was chromatographed on 2 kg. of alumina (Grade II) by washing it with the following solvents, the filtrate being collected in 800-ml. portions :

Light petroleum, 16 portions : 12·3 g. hydrocarbons.
Benzene, 3 portions : 6·8 g.
,, 1 portion : 2·55 g. of $\triangle^{3:5}$-cholestadiene-one-(7).
,, 19 portions : 4·4 g.
Ether, 11 portions : 3·7 g.
Ether, 4 portions : 2·3 g.
Ether + methanol (99 : 1), 11 portions : 23 g. } Chromatogram
,, ,, (99 : 1), 5 portions : 3·3 g. } " B ".
Ether + methanol (9 : 1), 5 portions : 2·5 g. of $\triangle^{5}$-cholestene-diol (3β, $7a$).
Ether + methanol (9 : 1), 13 portions : Chromatogram " D ".

Chromatogram B. From the 2·3-g. and 23-g. fractions, cholesterol (9·4 g.) was eliminated by fractional crystallization from acetone ; the mother liquors were combined with the 3·3-g. fraction and chromatographed on 500 g. of alumina (Grade III). The liquid chromatogram was prepared by using 300-ml. portions : The first 2·4 *l.* of benzene washed a mixture into the filtrate while the next 2·1 *l.* eluted 5·4 g. of $\triangle^{4}$-cholestene-diol-(3:5)(?).

Chromatogram D. This fraction (see above) was chromatographed on 300 g. of alumina (Grade III) ; after some additional purifications, the following compounds were obtained :

Benzene, 2,550 ml. : 5 g.
Benzene + ether (9 : 1), 280 ml. : 1·8 g.
„ „ (9 : 1), 150 ml. : 1·3 g. of batyl alcohol.
Ether, 150 ml. : 0 8 g. batyl alcohol and palmityl-sphingosin.
„ 300 ml. : 0·9 g. $\triangle^4$-cholestene-diol-(3β : 6) and another compound.
Ether, 450 ml. : 0·4 g. $\triangle^4$-cholestene-diol-(3β : 6).

The ketonic fraction (1·4 g.) of the starting material was dissolved in light petroleum + benzene (2:1) and chromatographed on 55 g. of alumina (Grade II) :

Light petroleum + benzene (2 : 1), (150 ml.) : 70 mg.
Light petroleum + benzene (2 : 1), (150 ml.) : 100 mg. friedelin and $\triangle^{3:5}$-cholestadiene-one-(7).
Light petroleum + benzene (2 : 1), (50 ml.) : 20 mg. of $\triangle^{3:5}$-cholestadiene-one-(7).
Light petroleum + benzene (2 : 1), (100 ml.) : 55 mg.
Benzene (150 ml.), 180 mg. of $\triangle^{4\cdot6}$-cholestadiene-one-(3).
Benzene (150 ml.), then benzene + ether (500 ml.), then ether (350 ml.), then ether + methanol (99 : 1), (350 ml.) : in all 335 mg. substance.
Ether + methanol (98 : 2), (150 ml.) : 95 mg. of cholestanol-(3β)-one-(6).

An investigation of arteriosclerotic aorta extracts was made along similar lines by Hardegger, Ruzicka and Tagmann. In addition to unidentified substances the following compounds were isolated : $\triangle^{3\cdot5}$-cholestadiene-one-(7) ; $\triangle^{4:6}$-cholestadiene-one-(3) ; cholestane-triol-(3β : 5:6 *trans*) ; 7 (β)-hydroxycholesterol ; batyl alcohol ; friedelin. (The fact that the friedelin originated from the cork used emphasizes the sensivity of the method.)

Suint. The unsaponifiable residue of suint was fractionated by Daniel, Lederer and Velluz by adsorbing 9 g. of the material, dissolved in 200 ml. of light petroleum, on 200 g. of alumina. The following constituents were successively eluted by washing the column with different solvents ; 200 ml. of light petroleum : hydrocarbons ; 400 ml. of light petroleum, then 200 ml. of light petroleum + benzine (9 : 1) : mixture ; 200 ml. (9 : 1) : unsaturated ketone ; similar fraction :

mixture; 200 ml. (2 : 1): trace of $\Delta^{3:5}$-cholestadiene-one-(7); 200 ml. of light petroleum + benzene (1 : 1), then 200 ml. of benzene: *iso*cholesterol; 500 ml. of benzene: mixture; and 500 ml. of ether, then 500 ml. of acetone: *iso*cholesterol and cholesterol.

Separation of Some Products Obtained by Treating Cholesterol Dibromide with Silver Nitrate in Pyridine (Spring and Swain). The brown, resinous crude product (15 g.) was dissolved in 100 ml. of light petroleum (b.p. 40–60°) and adsorbed on alumina (25 × 2·5 cm.), which was then washed with the following solvents:

No. 1 light petroleum (100 ml.): 5·1 g. (after evaporation).
„ 2 „ „ (200 ml.): 0·95 g.
„ 3 „ „ (300 ml.): 0·40 g.
„ 4 „ „ (600 ml.): 0·55 g.
„ 5 benzene + light petroleum (1 : 3) (100 ml.): 0·05 g.
„ 6 „ „ „ (600 ml.): 1·55 g.
„ 7 benzene (200 ml.): 0·7 g.

Fractions 1 + 2 yielded, upon recrystallization from acetone + methanol, Δ^4-cholestenone. Fraction 3 gave, after crystallization from light petroleum, 3 : 6-diketo-Δ^4-cholestene. Fraction 4 contained mainly the same compound in an impure state. Fractions 6 and 7 (the latter after rechromatography) were combined and acetylated, giving $\Delta^{4:6}$- cholestadienyl acetate.

Cellobioside-heptaacetate of Cholestanol (Plattner and Uffer). To a boiling solution containing 3 g. of cholestanol, 1·47 g. of mercuric-acetamide and 1 g. of calcium sulphate in 150 ml. of absolute toluene, 6·5 g. of acetobromocellobiose in 150 ml. of toluene were added with stirring. After 5 hours boiling, filtration through sodium sulphate and vacuum evaporation, this residue was treated for 2 days with 30 ml. of pyridine and 20 ml. of acetic anhydride. The residue left on evaporation was adsorbed on 250 g. of neutral alumina. The first benzene washings yielded 1·57 g. of cholestanol acetate, and further washings with benzene and benzene + ether gave 3·22 g. of the desired heptaacetyl-cellobioside.

Coprostanone -(3), the main ketone of "gray amber" (ex *Physeter macrocephalus*) was isolated by chromatography on alumina by Lederer, Marx, Mercier and Pérot.

Urinary Steroids. Chromatography is gaining importance in the study of steroids, including sex hormones that occur in normal and pathological urines of man or animals. The method consists of fractional desorption by washing the column with different solvents, and, in many instances, it follows other methods of general fractionation; for example, the separation of ketonic from non-ketonic material by means of Girard's reagent, and the fractionation of non-ketonic hydroxy-compounds with digitonin or with succinic acid.

An early method for the fractionation of urinary steroids was devised by Callow (1, 2) and applied by Callow and Callow (1, 2). Crude ketonic material (0·5 g.) originating from the neutral fraction of urinary extracts, was adsorbed from CCl_4 on Brockmann's alumina (Merck). Washing the column with the same solvent eliminated gummy substances, and further washing with 150 to 300-ml. portions of CCl_4 containing 0·1–0·2 per cent (or more) alcohol carried *trans*-dehydroandrosterone, androsterone, and *aetio*-cholane-3(α)-ol-17-one successively into the filtrate.

This method has been applied, with some modifications, by a number of authors. Wolfe, Fieser and Friedgood isolated, besides the compounds mentioned, $\triangle^{3:5}$-androstadiene-17-one, 3-chloro-androstene-17-one and a 3(α)-hydroxy-androsterone-17-one. Dorfman obtained *aetio-allo*cholane-3(α)-ol-17-one. The following provide further illustrations in this field: androsterone, androstenone-17 and 3(α)-hydroxy-*aetio*-cholanone-17 (Hirschmann 1); dehydro-*iso*androsterone (a 17 × 1 cm. column takes 40 mg.; Talbot, Wolfe, MacLachlan and Berman; Hirschmann 1; Mason and Kepler); *aetio*-cholane-3(α)-ol-17-one (Mason and Kepler; Engel, Thorn and Lewis); pregnanediol-3(α) : 20(α) (Fish, Dorfman and Young); pregnanediol-3(α) : 17-one-20 (Lieberman and Dobriner; 11(β)-hydroxy-androsterone (?) (Mason).

By gradually increasing the ethyl alcohol content of CCl_4 Mason and Kepler, using alumina, isolated the following compounds from some pathological urines (the percentages refer to the alcohol content of the developer in the liquid chromatogram). Ketonic fraction: dehydro*iso*androsterone (0·1%); androsterone (0·1%); *aetio*cholane-3(α)-ol-17-one (0·1–0·2%); and androstane-3(α):11-diol-17-one (0·4–0·5%). In the non-ketonic fraction, benzene was used instead of CCl_4 and gave: pregnane-3(β):20(α)-diol (0·1–0·2% alcohol); Δ^5-androstene-3(β):17(α)-diol (0·2–0·4%), pregnane-3(α):20(α)-diol (0·3–0·5%); $C_{19}H_{32}O_2$ (0·3–0·5%); pregnane-3(α):17:20-triol (0·3–0·5%); and Δ^5-androstene-3(β):16:17-triol (acetic acid + pyridine + ether 2:20:78).

In the following references only those developers are included that carry the compound mentioned from an alumina column into the filtrate: α-oestradiol with ligroin + 10% acetone (Heard and Hoffmann 1); oestrone with acetone + 2·5% alcohol (Fish and Dorfman); pregnanediol-3(α):20(α) with benzene + 1% methanol (Horwitt, Dorfman, Shipley and Fish); pregnanol-3(α) with ether (Marker and Lawson 1); Δ^5-androstene-triol-3(β):16:17 with benzene + 25% ether (Hirschmann 3). Mixtures of benzene and aliphatic hydrocarbons in different proportions were also found to be useful, e.g., androstenone-one-17 with benzene + pentane (1:1) (Venning, Hoffman and Browne); benzoate of $\Delta^{5,7,9}$-oestratrienol-3(β)-one-17 with benzene + ligroin (1:4) (Heard and Hoffman 1). From a non-ketonic, digitonin-precipitable fraction, after acetylation, Hirschmann and Hirschmann, by washing the column with a (1:4) benzene + petroleum ether mixture, obtained Δ^5-pregnenediol-3(β):20(α) diacetate, followed by a monoacetate, and then, on washing with a (1:1) mixture, Δ^5-androstenediol-3(β):17(α) diacetate.

Isolation of 3-Desoxy-equilenin from the Urine of Pregnant Mares (Prelog and Führer). The starting material was 1 kg. of neutral by-products obtained during the industrial isolation of oestrone. The non-saponifiable fraction (corresponding to 14,000 l. of urine) was subjected to molecular distillation and the ketonic fraction was separated by means of Girard reagent T. A 10·8-g. sample, dissolved in 100 ml. of benzene, was adsorbed on 324 g. of alumina (activity II-III). The column was washed with six 100-ml. portions of benzene, giving fractions 1 to 6. Fraction 1, after evaporation and

trituration with light petroleum, yielded 15 mg. of crude 3-desoxy-equilenin. The mother liquor, combined with the oily residue left on evaporation of fraction 2 (1·28 g.), was adsorbed on alumina (activity I-II) and the column developed with 60 ml. of benzene; the filtrate (0·29 g.) yielded 3-desoxy-equilenin. The next three 100-ml. portions of benzene eluted 0·4 g. material, from which, by washing with ether, a crystalline ketone, $C_{13}H_{18}O$, was obtained.

Plant Sterols and Some Related Compounds

Sitosterols. Barton and E. R. H. Jones (cf. Jones, Wilkinson and Kerlogue) suggest that oxidation with the Oppenauer reagent, followed by chromatography, is of value in testing the homogeneity of sitosterol preparations. A sample of Tall-öl *sitosterol* yielded pure $\triangle^4$-β-sitostenone; wheat germ sitosterol, however, gave a heterogeneous product. The experiments were carried out with 10 to 30 g. of starting material. The crude product was adsorbed from light petroleum on "Birlec" alumina and the column was washed with benzene, benzene + 2·5 per cent ether, benzene + 5 per cent ether, etc. The fractions obtained from wheat germ sitosterol showed very varied melting points.

"*Helisterol*" isolated earlier from sunflower by Zechmeister and Tuzson was resolved by Zimmermann (2) and characterised as arnidiol containing faradiol, by chromatographic filtration of the benzene solution through alumina.

i-Stigmasteryl methylether can be purified by adsorption from benzene + hexane on alumina, being washed down the column with hexane, whilst stigmasterol remains adsorbed. *i-Brassicasteryl methylether* was purified in like manner (Fernholz and Ruigh).

Taraxasterol, $C_{30}H_{50}O$, was isolated from chamomile flowers (*Anthemis nobilis*) by Burrows and Simpson, who adsorbed the unsaponifiable fraction of the CCl_4 extract on Merck's alumina from light petroleum. Impurities were washed out from the column with light petroleum + chloroform (2 : 1) and the taraxasterol was then eluted with light petroleum + methanol.

From the complex mixture contained in the unsaponifiable matter of *Taraxacum* root, after acetylation, taraxasterol and other related compounds were isolated (alumina, benzene, liquid chromatogram). Taraxasterol (= α-lactucerol) has also been isolated from the " resin " of the arrow poison of *Calotropis procera* by saponification of the crude product and filtration of the light petroleum solution through alumina. When the column was washed with absolute (!) ether, all esters migrated into the filtrate and the main product could be washed down the column with ether + 2 per cent alcohol (Hesse, Eilbracht and Reicheneder).

Some cleavage products of the arrow poison, calotropin, were purified on alumina by Hesse, Reicheneder and Eysenbach.

Closely related to digitalis is a new heart-active glycoside, *somalin*, $C_{30}H_{46}O_7$, obtained from the arrow poison, *Adenium somalense*. The homogeneity of this substance was tested on alumina (chloroform) by Hartmann and Schlittler. After hydrolysis, the corresponding genin was purified by adsorption on alumina from chloroform + benzene (1 : 1).

Cafestol acetate can be purified on alumina or floridin using light petroleum + benzene (1 : 1) (Wettstein, Spillmann and Miescher). For the chromatography of some derivatives of nor-cafestane cf. Wettstein, Hunziker and Miescher; Wettstein and Miescher.

Peanut oil was freed from such sterol fractions as had growth promoting activity on rice-moth larvae, by passing the light petroleum solution through several alumina columns (DeSouza and Sreenivasaya).

Leaf sterols : Wall and Kelley.

For the sterols of *algae* cf. Carter, Heilbron and Lythgoe.

Ergosterol can be easily eliminated from crude sitosterol by washing the alumina column with light petroleum + 10 per cent alcohol (Lobert). For the separation of acetates of ergostanol and ergostenol derivatives, cf. Stavely and Bollenback (1, 2). Benzene solutions of *zymosterol* and *β-zymostenol* were percolated thrice through alumina to achieve purification (Heath-Brown, Heilbron and E. R. H. Jones). Cryptosterol acetate, purified by filtration through alumina

and development with benzene + petroleum ether (1 : 4), was found to be identical with lanosterol acetate (Ruzicka, Denss and Jeger).

Isolation of Ergosterol, Ergosterol Palmitate and Ergosterol Peroxide from Aspergillus fumigatus (P. Wieland and Prelog). The mycelium as obtained from 30 l. of culture was exhaustively extracted at pH2 with ether, the extract was repeatedly washed with soda, dried and evaporated. The resulting oil (6 g.) was adsorbed from 100 ml. of benzene on 180 g. of alumina (Grade IV). When washed with benzene, the first fractions removed almost 4 g. of material from this column and yielded, after recrystallization from chloroform + alcohol, 1·3 g. of colourless crystals. These were dissolved in benzene + light petroleum (1 : 1) and rechromatographed on 40 g. of the adsorbent. The first fractions yielded 45 mg. of crude ergosterol palmitate (purified by repeated crystallization from chloroform + alcohol). Subsequent fractions from this second chromatogram yielded some ergosterol, while from later benzene fractions from the first chromatogram 120 mg. of ergosterol peroxide could be isolated.

Coloured Sterol Derivatives

There are scattered references to the conversion of sterols into coloured derivatives and chromatography of the latter.

Azobenzene-p-carboxylic esters have been used by Ladenburg, Fernholz and Wallis to separate (I) cholesterol, (II) β-sitosterol, (III) stigmaterol, and (IV) ergosterol.

The method has limited applicability, since the mixtures I + II, II + III, II + IV, and II + III + IV do not form sharply separated zones. However, I + III, III + IV, I + IV or I + III + IV separate on alumina (60 × 1·4 cm.). The resolution of a mixture of cholesteryl and ergosteryl esters (50 mg. each) took 4 hours. The solution was washed into the column with some benzene and developed with light petroleum.

Cholic and desoxycholic acids were separated after reaction of their Na-salts with ω-bromo-*p*-methylazobenzene. The coloured ester of cholic acid showed the stronger adsorbability when developed with benzene + light petroleum on magnesium carbonate (Silberman and Silberman-Martyncewa).

Steroids with phenolic properties can be *coupled* with phenyl-diazonium chloride and then adsorbed on alumina from light petroleum (Inhoffen and Zühlsdorff). Oestrone and equilenin were separated on alumina in the form of their 2 : 4-*dinitrophenyl-hydrazones*. When developed with light petroleum + 1% alcohol, the oestrone derivative migrated into the filtrate while equilenin-dinitrophenylhydrazone required a higher proportion of alcohol in the developer (Veitch and Milone). A similar method was used by Heywood, Kon and Ware ; and for androgens by Johnston.

TABLE 16

CHROMATOGRAPHY OF SEX HORMONES AND OTHER STEROIDS ON ALUMINA (THE SOLVENTS MENTIONED ARE MAINLY THOSE THAT CARRY THE COMPOUND INTO THE FILTRATE). *trans*-Androsterone (benzene ; Shoppee and Prins 1 ; Reichstein and Meystre). $\triangle^{1\,2\,4\,5}$-Androstadiene-ol-(17)-one-(3) acetate (benzene + light petroleum ; Inhoffen, Zühlsdorff and Huang-Minlon). $\triangle^{5}$-Androstene-6 : 17-dione-5-ol (benzene ; Ruzicka, Grob and Raschka). Benzal-17-chloro-$\triangle^{5}$-androstenol-(3) acetate and 17-chloro-$\triangle^{5}$-androstene-ol-(3) acetate (benzene ; Westphal, Wang and Hellmann). Androstane-17-one-3(β) : 5 : 6 (*trans*)-triol-3 : 5 diacetate (benzene + ether ; Ehrenstein 1). $\triangle^{1}$-Androstenol-(17)-one-(3) acetate (benzene + hexane ; Butenandt and Dannenberg). $\triangle^{16}$-Androstene-one-(3) (benzene + light petroleum ; Prelog, Ruzicka and P. Wieland 1). $\triangle^{4}$-Androstene-dione-3 : 17 (benzene ; Pfiffner and North ; Reichstein). $\triangle^{4}$-3-Keto-17-[17^{2}-oxopropyl]-androstene (benzene + hexane ; Plattner and Schreck 2). Androstane-5 : 6-(α)-epoxy-17-one-3β)-olacetate (benzene + light petroleum ; Ehrenstein and Decker). Androstanone-(17) (benzene + hexane) and 3(β)-acetoxy-β-5 : 6-epoxy-androstanone-(17) (benzene ; Ruzicka and Muhr). 2 : 3-Androstane-2//3-dicarboxylic acid dimethyl ester (light petroleum) and A-nor-androstane-one-(2) (light petroleum) and D-*homo*-androstane-ol-(3β) (benzene ; Ruzicka, Prelog and Meister). 3 : 17a-Diketo-D-*homo*-androstane (benzene ; Goldberg and Wydler). 3(β) : 17(α)-Diacetoxy-17a-methyl-D-*homo*-androstane (light petroleum ; Hardegger and Scholz). 3-epi-Hydroxy-D-*homo*-androstanone-(17a) (benzene + light petroleum, then benzene + ether ; Goldberg and Monnier). 17(α)-Methyl-D-*homo*-androstane (hexane) and $\triangle^{5\ 17}$-3-*trans*-Hydroxy-17a-methyl-D-*homo*-androstadiene (benzene + ether ; Ruzicka and Meldahl 3). 17a-Methyl-D-*homo*-androstane (hexane ; Ruzicka and Meldahl 2 ; cf. also Hirschmann and Hirschmann ; Clark, Kochakian and Lobotsky ; Heer and Miescher 2 ; Johnson, Petersen and Gutsche). Oestrone (benzene + methanol 9 : 1) and α-oestradiol (benzene + methanol 9 : 1) and oestriol (benzene + methanol 4 : 1 ; Stimmel 13), 15-Methyl-15-dehydro-x-*nor*-oestrone-methyl ether (benzene + light

petroleum 1 : 1 ; Goldberg and P. Muller). D-*homo*-oestrone acetate (benzene + ether 1 : 1 ; Goldberg and Studer 2). D-*homo*-oestradiol (benzene + ether 1 : 9 ; Goldberg and Studer 1). $\Delta^{1\ 3\ 5\cdot 16}$-Oestratetraene-ol-(3) (benzene, ether) and the benzoate (benzene ; Prelog, Ruzicka and P. Wieland 2). *iso*-Equilin A (benzene + acetone 10 : 1 ; Hirschmann and Winterstemer). *trans*-Androsterone (benzene ; Shoppee and Prins 3). Testosterone (ether + benzene 1 : 1, then ether ; Marker). 17(α)-Allyl-testosterone (benzene ; Euw and Reichstein 1). 6-Dehydrotestosterone benzoate (benzene + ether ; Wettstein 1). A-*homo*Dihydrotestosterone (benzene ; Goldberg and Kirchenstemer). 6-Dehydrotestosterone benzoate (benzene + ether ; Wettstem 1). *cis*-Testosterone (Clark and Kochakian).

Pregnanol-20(β) acetate and *allo*-pregnanol-20(α) acetate (benzene + light petroleum ; Marker and Lawson 3). Pregnane-diol-3(α) : 20(α) (benzene + 1% methanol ; Horwitt, Dorfman, Shipley and Fish). Pregnanediol acetates (benzene + light petroleum ; Heard, Hoffman and Mack). 3(β) : 21-Dihydroxy-*allo*-pregnane diacetate (benzene ; Plattner, Bucher and Hardegger). Pregnane-triol-3(α) : 17 : 20 diacetate (benzene + light petroleum 1 : 10 ; Hirschmann 2). *allo*-Pregnanetetrol (3β : 17α : 20α : 21) triacetate-(3 : 17 : 21) (benzene + ether ; Prins and Reichstein 2). α-*allo*-Pregnane-tetrol-(3 : 17 : 20 : 21) triacetate-(3 : 20 : 21) (benzene + pentane ; Serini, Logemann and Hildebrand). 3(β)-Acetoxy-17(α) : 20-dihydroxy-20-methyl-*allo*-pregnane (benzene + ether ; Shoppee and Prins 3). 6-Methylpregnane-3(β) : 5 : 20 : 21-tetraol 21-monoacetate (chloroform, methanol), and the 3 : 21-diacetate (benzene + ether ; Ehrenstein 2).

epi-Pregnanol-(3)-one-(20) (acetone + light petroleum 1 : 5), and *epi-allo*-pregnanolone-(3 : 20) (benzene ; Butenandt and G. Muller). Pregnane-3(α)-ol-20-one (benzene + ether) and 17-*iso*-pregnane-3(α)-ol-20-one (benzene ; Moffett and Hoehn). 21-Chloro-pregnane-ol-(3β)-one-(20) (benzene) and the dimethylacetal of *allo*-pregnane-ol-(3β)-one-(20)-al-(21) (benzene ; Ruzicka, Prelog and P. Wieland). *allo*-Pregnanedione (benzene) and *epi-allo*-pregnanolone-(3 : 20) (benzene ; Butenandt and Heusner). Pregnanol-(12β)-dione-(3 : 20) benzoate (benzene ; Hegner and Reichstein 3). *allo*-Pregnane-diol-(3 : 21)-one-(20) diacetate (benzene + pentane 1 : 1 ; Reichstein and Montigel). Pregnane-diol-3(β) : 21-one-20 diacetate (benzene + isopentane ; Fried, Linville and Elderfield). 3(α) : 21-Diacetoxy-pregnanone-(20) (benzene + light petroleum ; Ruzicka, Plattner and Balla). Pregnane-triol-(3α : 12β : 21)-one-(20) monoacetate-(21) and pregnane-diol-(12β : 21)-dione-(3 : 20) monoacetate-(21) (ether + chloroform ; Fuchs and Reichstein 2). Pregnane-diol-(3α : 21)-dione-(11 : 20) monoacetate-(21) (methanol + chloroform + ethyl acetate 1 : 1 : 1) and pregnane-triol-(3β : 11α : 21)-one-(20) monoacetate-(21) (benzene + ether ; Euw, Lardon and Reichstein 2). Pregnane-20-one-3(β) : 5 : 6 (*trans*)-triol 6-monoacetate and the 3 : 6-diacetate and the free triol (chloroform + methanol ; Ehrenstein and Stevens 3). *allo*-Pregnane-triol-(3β : 17α : 21)-one-(20) triacetate (benzene) and the 3 : 21-diacetate (benzene + ether ; Prins and Reichstein 2). *allo*-Pregnane-tetrol-(3α : 11? : 17β : 21)-one-(20)

(benzene + ether; Euw and Reichstein 4). Progesterone (ether + benzene 1 : 1; Marker). 7 : 12-Diacetoxy-progesterone (benzene + light petroleum 4 : 1; Ehrenstein and Stevens 2). 17(β)-Hydroxy-progesterone (Prins and Reichstein 1). 11-Hydroxy-progesterone (benzene + ether; Reichstein and Fuchs 2). 21-Methyl-progesterone (Wettstein 2). 12(β)-Benzoxy-progesterone (benzene; Hegner and Reichstein 2). 16-Methyl-16-dehydroprogesterone and 16-methyl-progesterone (benzene + hexane; Wettstein 4). 10-*nor*-Progesterone (benzene + light petroleum; Ehrenstein 3).

Corticosterone acetate (benzene + ∠ 8% ether; Euw, Lardon and Reichstein 2). Desoxycorticosterone acetate (benzene + ether; Reichstein and Euw 1). 17-*iso*-Desoxy-corticosterone acetate (acetone + ether 1 : 1; Shoppee 1). Anhydro-corticosterone acetate (benzene + 1% ether; Shoppee and Reichstein 4). Substance "P" acetates (Reichstein and Gätzi). 10-*nor*-11-Desoxy-corticosterone acetate (Ehrenstein 3). Heptaacetyl-maltoside of desoxycorticosterone (ether); and 6-[β-lactosido-]-*d*-glucoside of desoxycorticosterone (acetone; Miescher and Meystre). Pregnane-5 : 6-epoxy-3(β) : 20 : 21-triol (chloroform + methanol; Ehrenstein 2). 3(β)-Hydroxy-*allo*-pregnane-21-carboxylic acid methylester (ether) and 3-keto-*allo*-pregnane-21-carboxylic acid methylester (benzene + ether; Plattner, Bucher and Hardegger).

Pregnadiene-ol-(3) (benzene + hexane 1 : 2; Ruzicka, Goldberg and Hardegger). 3(β)-Acetoxy-pregnadiene-(5 : 16)-one-(20) (benzene + ether; Shoppee and Prins 3). Pregnadiene-(4 : 20)-ol-(17α)-one-(3) acetate (benzene + pentane; Prins and Reichstein 2). Pregnadiene-(4 : 11) dione-(3 : 20) (benzene; Shoppee and Reichstein 2). 3-Acetoxy-pregnadiene-(5 : 17)-al-(21) (benzene + pentane 1 : 1) and pregnadiene-(4 : 17)-one-(3)-al-(21) (benzene; Reich 3). $\triangle^{4 \cdot 5; 17 \cdot 20}$-Pregnadiene-3-one-21-al (benzene + light petroleum; Miescher, Wettstein and Scholz). Pregnadiene-(5 : 17)-ol-(3β)-carboxylic acid-(20) methylester (benzene + ether; Lardon and Reichstein 1). *homo*-(ω)-Pregnadiene-(5 : 17)-triol-(3β : 21α : 22) (chloroform + 5 per cent methanol; Fuchs and Reichstein 1). 3(α),12(β)-Diacetoxy-20-bromopregnane (Brink, Clark and Wallis).

$\triangle^4$-Pregnenone-3 (benzene; Marker and Lawson 2). Pregnene-(11)-dione-(3 : 20) (benzene + light petroleum; Hegner and Reichstein 3). 4-Pregnene-3 : 20-dione-6 (α)-ol acetate (benzene + ether; Ehrenstein and Stevens 1). $\triangle^5$-3-*trans* : 17(α)-Diacetoxy-pregnene-one-(20) (benzene + pentane 1 : 1; Ruzicka and Meldahl 1). 3(β) : 17(α)-Diacetoxy-pregnene-(5)-one-(20) (benzene + pentane; Shoppee and Prins 2). Pregnene-5-diol-(3 : 21)-one-(20) acetate-(3)-tosylate (benzene + pentane 1 : 1; Reichstein and Schindler). Pregnene-(4)-diol-(12β : 21)-dione-(3 : 20) diacetate (benzene + ether; Fuchs and Reichstein 2). *allo-homo*-(ω)-Pregnene-(17)-triol (3β : 21α : 22) (ether + chloroform; Euw and Reichstein 2). 20-Methyl-pregnene-(5)-triol-(3β : 20α : 21) diacetate (3 : 21) (benzene + ether 50 : 1; Hegner and Reichstein 1). Pregnene-(5)-tetrol-(3β : 17α : 20α : 21) triacetate-(3 : 20 : 21) (benzene + ether) and 20 : 21-monoacetone-pregnene-(4)-triol-(17α : 20α : 21)-one-(3) (benzene + ether; Reich, Montigel and

Reichstein). Pregnene-(5)-diol-(3 : 20)-al-(21) dimethyl-acetal diacetate (benzene) and pregnene-(4)-(ol-)20-one-(3)-al-(21) dimethyl-acetal acetate-(2) (benzene) and pregnene-(4)-dione-(3 : 20)-al-(21) diethyl-mercaptal (benzene + light petroleum; Schindler, Frey and Reichstein). Pregnene-(4)-dione-(3 : 20)-al-(21) dimethylacetal (benzene + pentane; Reich and Reichstein 1). 17(α) : 22-Epoxy-*allo-homo*-(ω)-pregnene-(20)-ol-(3β) acetate (benzene + light petroleum; Wenner and Reichstein). $\triangle^5$-3-Acetoxy-pregnene-21-carboxylic acid methyl ester (benzene + hexane 1 : 4; Plattner and Schreck 1). $\triangle^5$-3(β)-Acetoxy-21-keto-21-diazomethyl-pregnene (benzene + ethyl acetate; Plattner, Hardegger and Bucher). Pregnane-3-(α)-ol-20-one (Sutherland and Marrian). Dehydro-corticosterone acetate (benzene + ether; Lardon and Reichstein 5). Dehydro-desoxy-corticosterone acetate (Wettstein 1).

Cholestane (light petroleum, Anker and Bloch; benzene + light petroleum; Ruzicka, Plattner and Furrer). 7(β)-Hydroxy-cholesterol (ether; Ruzicka, Prelog and Tagmann). Cholestanol-(4) and cholestanol-(1) (benzene + ether 4 : 1; Ruzicka, Plattner and Furrer). Acetoxy-cholestanol (ether + chloroform) and cholestane-diol (chloroform; Ruzicka, Plattner and Furrer). $\triangle^2$-2-Formyl-cholestene (benzene + ether; Plattner and Jampolsky). $\triangle^6$-Cholestene-diol-3(β) : 5 diacetate (benzene + light petroleum; Bergström and Wintersteiner 2). 3(β) : 6(α)-Diacetoxy-cholestane (benzene + ether) and 3(β)-Hydroxy-5-acetoxy-cholestane (ether + chloroform) and cholestane-triol-(3β : 5 : 6β) 3-monoacetate (ether + chloroform 1 : 1; Plattner and Lang). Cholestane-triol-3(β) : 7 : 8 diacetate (benzene + ether 1 : 1) and 3(β)-acetoxy-cholestadienone-7 (benzene; Wintersteiner and Moore 2). 7-Keto-$\triangle^{3\ 5}$-cholestadiene (benzene + pentane 1 : 4; Bergström and Wintersteiner 1). $\triangle^4$-Cholestenone (benzene; Barton and E. R. H. Jones 2). Cholestane-dione-(3 : 6) (benzene; Prelog and Tagmann), 3(β)-Acetoxy-5-hydroxy-cholestane (hexane) and α- and β-cholesterol-oxide acetates (benzene + light petroleum; Plattner, Petrzilka and Lang).

3(α) : 12(β)-Dihydroxy-*aetio*-cholanone-(17) (benzene + light petroleum; Reich and Reichstein 3). *aetio*-Cholane-trione-(3 : 12 : 17) and 12(β)-acetoxy-*aetio*-cholane-dione-(3 : 17) (benzene + ether; Reich 4). 3(α) : 7(α) : 12(β) : 25-Tetraacetoxy-24-keto-25-*homo*-cholane (benzene + light petroleum; Ruzicka, Plattner and Heusser 1). 17-Methyl-D-*homo-aetio*-cholane-one-(3) (benzene + light petroleum; Shoppee 2). $\triangle^{16}$-*aetio*-Cholene-one-(3) (light petroleum; Prelog, Ruzicka, Meister and P. Wieland). [3β-Acetoxy-11α-hydroxy-*aetio*-cholanyl-] methyl-diphenylethylene (benzene + light petroleum; Euw, Lardon and Reichstein 1). $\triangle^5$-3*t*-Acetoxy-*nor*-cholene-22-one and $\triangle^4$-*nor*-cholene-3 : 22-dione (benzene; Wettstein 3). $\triangle^{20\ :\ 22}$-3(α) : 12(β)-Dihydroxy-24 : 24-diphenyl-choladiene (benzene + hexane; Meystre, Ehmann, Neher and Miescher). 3-Acetoxy-20-*iso-ternor*-cholenyl-ethyl-ketone (benzene; Cole and Julian). 3-Hydroxy-5-*ternor*-cholenyl-dimethyl-carbinol (acetone; Julian, Cole, Meyer and Herness). 1'-Methyl-1'-[3α-acetoxy-12β-hydroxy-*aetio*-cholanyl-(17)]-2' : 2'-diphenylethylene (benzene + light petroleum 1 : 1; Sorkin and

Reichstein 3). 3 (α), 12 (β)-Diacetoxy-23-bromonorcholane (benzene + ether ; Brink, Clark and Wallis).

Lithocholic acid methyl ester (benzene + ether) and desoxycholic acid methyl ester (chloroform + methanol ; Press and Reichstein). Cholen-(11)-ic acid methyl ester (light petroleum) and 11 : 12-dihydroxy-cholanic acid methyl ester (benzene + light petroleum 1 : 4 ; Alther and Reichstein 1). 3(α) : 12(α)-Dihydroxy-cholanic acid methyl ester (benzene + light petroleum ; Koechlin and Reichstein 1). 12(β)-Benzoxy-cholanic acid methyl ester (benzene + light petroleum ; Lardon, Grandjean, Press, Reich and Reichstein). 3(α)-Acetoxy-cholene-(11)-carboxylic methyl ester (benzene + light petroleum) and the 3(β)-compound (benzene + ether), and 3(α)-acetoxy-11 : 12-dibromo-cholanic acid methyl ester (light petroleum ; Ott and Reichstein). 11-Keto-cholanic acid methyl ester (benzene + light petroleum) and 12-keto-cholen-(9)-ic acid methyl ester (benzene + light petroleum ; Reich and Reichstein 2). 3 : 11-Diketo-cholanic acid methyl ester (benzene) and 3 : 12-diketocholen-(9)-ic acid methyl ester (ether ; Lardon and Reichstein 2). 12-Keto-cholen-(9)-ic acid methyl ester (benzene ; Alther and Reichstein 2). 3-Keto-cholen-(11)-ic acid methyl ester and 3-keto-choladien-(4 : 11)-ic acid methyl ester (benzene + light petroleum 1 : 1 ; Burckhardt and Reichstein). 3 : 12-Diacetoxy-7-keto-cholanic acid methyl ester (ligroin ; Gallagher and Long). 3(α)-Acetoxy-12-keto-cholanic acid methyl ester (benzene + light petroleum ; Koechlin and Reichstein 1). 3(α)-Acetoxy-12-keto-cholen-(9)-ic acid methyl ester (benzene + light petroleum ; Seebeck and Reichstein). 3(β)-Acetoxy-11-keto-cholanic acid methyl ester (benzene + light petroleum ; Press, Grandjean and Reichstein). 3(α) : 12(β)-Diacetoxy-14 : 15-epoxy-cholanic acid methyl ester (benzene ; Plattner, Ruzicka and Holtermann). *aetio*-Cholic acid methyl ester triacetate (light petroleum-benzene) and 3 (α), 12 (α)-diacetoxy-7-keto-*aetio*-cholanic acid methyl ester (Lardon). 3(β)-Acetoxy-*aetio-allo*-cholanate and methyl-3(β)-acetoxy-*allo*-homobilianate (Prins and Shoppee). 3(α)-Acetoxy-11 : 12-epoxycholanate (Long and Gallagher). Methyl-3(α)-acetoxycholanate (Gallagher and Hollander). 3(α)-Acetoxy-11 (α)-bromo-12-ketocholanate and methyl-3 (α)-acetoxycholanate and methyl-3 (α)-acetoxy-11 : 12-epoxycholanate (Gallagher and Long 3). 3(α)-12-Dihydroxy-$\triangle^{9:11}$-cholenic acid (Gallagher and Long 2).

aetio-Cholanic acid methyl ester and *aetio*-lithocholic acid methyl ester (benzene + ether ; Reichstein and Fuchs 1). 17-*iso-aetio-allo*-cholanic acid methyl ester (light petroleum ; Euw and Reichstein 5). 3(β)-Acetoxy-*aetio*-cholen-(14)-ic acid methyl ester (benzene ; Hunziker and Reichstein). 3 : 7-Dihydroxy-*aetio-allo*-cholanic acid methyl ester (benzene + pentane 2 : 3 ; Reichstein and Fuchs 3). $\triangle^{5}$-3(β)-Acetoxy-16-methoxy-*aetio*-cholenic acid methyl ester (benzene + ether ; Ruzicka, Hardegger and Kauter). 3(α)-Acetoxy-12(β)-hydroxy-*aetio*-cholanic acid methyl ester (benzene + ether ; Wenner and Reichstein 2). 3(α)-Hydroxy-*aetio*-cholen-(11)-ic acid methyl ester (benzene + ether ; Lardon and Reichstein 3). 7-Keto-3(β)-acetoxy-*aetio*-cholen-(5)-ic acid methyl ester (benzene + ether) and 7-keto-3(β)-*aetio*-cholen-(5)-ic acid methyl

ester (benzene + ether; Reichstein and Fuchs 3). 3 : 11-Diketo-*aetio*-cholen-(4)-ic acid methyl ester and 3(α)-acetoxy-12-keto-*aetio*-cholen-(9)-ic acid methyl ester (benzene + ether; Lardon and Reichstein 4). β'-[$\triangle^{5}$-3β-Acetoxy-*aetio*-cholenyl-(17)]-α'-methyl-$\triangle^{\alpha':\beta'}$-butenolide (benzene, ether; Ruzicka, Plattner and Heusser 2). 17-Methyl-(3*a* : 12β)-diacetoxy-*aetio*-cholanic acid methyl ester (benzene + light petroleum; Koechlin and Reichstein 2). 3(α)-Acetoxy-*aetio*-cholen-(9)-ic acid methyl ester (benzene + light petroleum) and 3 : 12-diketo-*aetio*-cholen-(9)-ic acid methyl ester (benzene + ether; Lardon and Reichstein 7).

3(α)-Acetoxy-*nor*-cholen-(11)-ic acid methyl ester (benzene + light petroleum; Lardon and Reichstein 7). $\triangle^{5:6}$-3(β)-Acetoxy-*nor*-cholenic acid methyl ester (benzene + ether; Plattner and Pataki 1). $\triangle^{16:17;20:22}$-3(β)-Acetoxy-21-hydoxy-*nor-allo*-choladienic acid lactone (ether; Plattner and Pataki 1). β-Heptaacetyl-maltoside of $\triangle^{5\ 6,\ 20\ .\ 22}$-3 : 21-dihydroxy-*nor*-cholandienic lactone (benzene, ether, acetone; Meystre and Miescher). $\triangle^{20\ .\ 22}$-3(α)-Acetoxy-12(β) : 21-dihydroxy-*nor*-cholenic acid lactone-(23→12) (Plattner and Pataki 2). $\triangle^{20\ 22}$-3(β)-Acetoxy-5 : 6(β)-epoxy-21-hydroxy-*nor*-cholenic acid lactone-(23→21) (benzene + light petroleum, 3 : 1; Ruzicka, Plattner and Heusser 3). β-(*nor*-Cholanyl)-$\triangle^{\alpha,\beta}$-butenolide (benzene; Knowles, Fried and Elderfield). Lactone of $\triangle^{5\ 6,\ 20\ 22}$-3-acetoxy-21-hydroxy-*nor*-choladienic acid (ether) and lactone of $\triangle^{5\ 6}$-3-acetoxy-20 : 21-dihydroxy-*nor*-cholenic acid (acetone; Ruzicka, Reichstein and Fürst). 3(*a*)-Acetoxy-*bisnor*-cholen-(9)-ic acid methyl ester (benzene + light petroleum; Lardon and Reichstein 7). 3(α)-Acetoxy-*bisnor*-cholen-(11)-ic acid methyl ester (benzene + light petroleum; Grandjean and Reichstein). 3(α)-Acetoxy-12(β)-hydroxy-*bisnor*-cholanic acid methyl ester and 3-keto-12(β)-hydroxy-*bisnor*-20-*iso*cholanic acid methyl ester (benzene + light petroleum; Lardon and Reichstein 6). 3(α)-Hydroxy-12-keto-*bisnor*-cholanic acid methyl ester (benzene + ether; Sorkin and Reichstein 1).

12-*epi*-Desoxy-digoxigenin 3 : 12-diacetate (benzene + ether) and 12-*epi*-14-desoxy-digoxigenin (ethyl acetate + methanol; Ruzicka, Plattner and Pataki 1). 12-*epi*-20-*iso*-Tetrahydro-anhydro-digoxigenin 3 : 12-diacetate (benzene; Plattner, Ruzicka and Pataki). Tetrahydro-diginigenin diacetate (benzene + light petroleum; Shoppee 3). Digitoxigenin acetate (benzene + ether; Hunziker and Reichstein). Pentaacetyl-ascigenin (benzene + light petroleum; Ruzicka, Janett and Rey). Reaction products of tetraacetyl-dehydro-scilliroside and related compounds with alkali (ether, benzene; Stoll, Renz and Helfenstein). 14-Desoxy-thevetigenin acetate (benzene; Fried, Linville and Elderfield). Solanidane-one-(3) and solanidane-ol-(3*a*) (benzene; Prelog and Szpilfogel 1).

CHAPTER XIV

TERPENES AND SOME RELATED COMPOUNDS.

Both synthetic and natural terpenes and many derivatives have been purified on alumina. In many instances simple filtration of the light petroleum or benzene solution was satisfactory. In more complicated instances, however, the liquid chromatogram method had to be employed with a number of solvents and solvent mixtures, the eluting power of which increased only slightly from one step to the next. Such operations, which may require large volumes of solvents, were followed by evaporation of the solvent from the individual fractions.

Examples of *diterpenes:* Dehydro-abietic methyl ester (Ruzicka, Bacon, Sternbach and Waldmann); Tetrahydro-abietic methyl ester (Ruzicka and Sternbach 1); Ozonization products from *l*-pimaric acid and agathene-diacid (Ruzicka and Kaufmann 2; Ruzicka, Bernold and Tallichet).

The extensive literature on the *triterpenes* and related compounds has made it necessary to condense many of the data in Table 17, p. 184.

Separation of some Oxidation Products of Acetyl-lupenalic acid, $C_{32}H_{48}O_5$ (Ruzicka and Rey 3). The acidic oxidation products (1·4 g.) obtained from 1·8 g. of the substance by treatment with chromic acid were adsorbed on 30 g. of alkali-free alumina and washed with various volumes of solvents as follows:

Nos. 1–2: 100 ml. of benzene, then 100 ml. of ether: no eluate.

No. 3: 150 ml. of ether + methanol (10:1): 90 mg. of unchanged starting material.

Nos. 4–5: 110 ml. of ether + methanol (4:1), then 100 ml. of methanol: 580 mg. of crystals. After purification of the ethyl acetate solution: acetyl-lupenoldiacid, $C_{32}H_{48}O_6$.

Nos. 6–7: 100 ml. of methanol + glacial acetic acid (10:1), then 100 ml. (10:3): 840 mg. of oil (+ inorganic salts). After esterification with diazomethane in methanol, adsorption on 20 g. of alumina and elution with benzene + ether (1:1): acetoxy-*bisnor*-lupanedi-acid methylester, $C_{32}H_{50}O_6$.

Resolution of the Reduction Product of β-Amyradienonyl Acetate (Green, Mower, Picard and Spring). Instead

of the expected β-amyradienol-I, reduction of 1·3 g. of the substance with hydrazine hydrate (3·4 ml.) and sodium (1·2 g.) in alcohol (30 ml.) in a sealed tube at 200° for 12 hours gave a mixture. The residue left on evaporation of the ethereal extract was chromatographed from 50 ml. of light petroleum + 20 ml. of benzene on activated alumina (21 × 3 cm.). Washing of the column with 250 ml. of benzene yielded, after acetylation, *allo*-β-amyrin acetate, $C_{32}H_{52}O_{21}$; continued washing with the same solvent and crystallization gave an alcohol, $C_{30}H_{48}O$. By a subsequent washing with 250 ml. of ether and acetylation, *allo*-β-amyrenonyl acetate, $C_{32}H_{50}O_{31}$, was obtained.

Treatment of β-Amyradienyl-I Acetate with N-Bromosuccinimide (Newbold and Spring 2). The reaction product was washed with light petroleum + benzene (1:1) through Brockmann alumina. The first fractions of the filtrate yielded, after recrystallization from aqueous acetone, impure β-amyratrienyl acetate.

Oxidation Products obtained with SeO_2 from the 13:28-Lactone of 2-Acetoxy-x-keto-13-hydroxy-oleanan-28-ic acid (Ruzicka, Jeger, Grob and Hösli). The reaction product obtained by heating 1 g. of the substance at 200° overnight with 2 g. of SeO_2 in 20 ml. of dioxane, was divided by means of sodium carbonate into an acid and a neutral fraction. The latter was resolved as follows into a non-enolic component and an enolic one that gave a dark brown spot reaction with ferric chloride solution. The ether solution was filtered through 40 g. of alumina. The column was washed first with ether until the spot test became positive, then with methanol and then with methanol + glacial acetic acid (20:1), until the colour reaction became negative again. The enolic product was extracted, transferred into chloroform, and crystallized. It was the 13:28-lactone of the enol-2-acetoxy-7:8-diketo-13-hydroxy-oleanan-28-ic acid, $C_{32}H_{46}O_6$.

Euphol and α-Euphorbol from Euphorbium (Newbold and Spring 1, 2). From euphorbone, a material prepared by light petroleum extraction of the resinified latex of *Euphorbium*

spp., two crystalline monohydric alcohols, viz., euphol (probably, $C_{30}H_{50}O$) and *α*-euphorbol (probably, $C_{29}H_{48}O$) were obtained. The light petroleum extract (Soxhlet) of 20 g. of euphorbone, which was filtered after one day's standing, was separated on a 40 × 4 cm. column of activated alumina by washing with 5 *l.* of light petroleum (b.p. 60–80°). The first 1·5 *l.* contained a negligible amount of material, while the next 3 *l.* contained 7·8 g. of euphol (crystals from acetone). In the last 0·5 *l.*, a mixture (0·2 g.) was present. A further fraction (0·5 *l.* of benzene) contained 4·2 g. of *α*-euphorbol that crystallized from acetone.

Ambrein, $C_{30}H_{52}O$, an animal triterpene, was isolated from the non-ketonic constituents of "gray amber" (ex *Physeter macrocephalus*). A distilled crude product, when adsorbed on alumina, first yields a hydrocarbon ($C_{30}H_{50}$) with light petroleum, then with light petroleum + benzene 4:1, ambrein, and finally, with alcohol, *epi*coprosterol (Lederer, Marx, Mercier and Pérot).

Rubber. Levi and Cajelli fractionated benzene solutions of rubber on charcoal and found this method superior to others. However, no substantial progress can be reported in this field.

TABLE 17

EXAMPLES OF THE CHROMATOGRAPHY OF TRITERPENES AND RELATED COMPOUNDS ON ALUMINA

Compound	*Solvent (eluent)*	*Literature*
α-Amyrin	Light petroleum	Ruzicka and Wirz (1) cf. Ruzicka and Marxer.
β-Amyrin	Benzene	Ruzicka and Wirz (2, 3).
α-Amyrene	Light petroleum	Ruzicka, Müller and Schellenberg.
Hydroxy-*β*-amyrin		Ruzicka and Marxer (1).
Dehydro-*β*-amyrin acetate	Benzene	Ruzicka and Jeger (2).
δ-Amyrin acetate oxide	Benzene + light petroleum	Ruzicka, Jeger and Norymberski (1).
α-Amyradienone-II	Benzene + light petroleum	Ruzicka, Volli and Jeger.
α-Amyradienone-II oxide	Benzene + light petroleum	Ruzicka, Volli and Jeger.
Triketo compound $C_{30}H_{46}O_3$	Benzene + light petroleum	Ruzicka, Volli and Jeger.

TABLE 17—(*continued*)

EXAMPLES OF THE CHROMATOGRAPHY OF TRITERPENES AND RELATED COMPOUNDS ON ALUMINA

Compound	*Solvent (eluent)*	*Literature*
α-Amyradienol benzoate	Light petroleum + benzene 3:1 (light petroleum)	Ruzicka, Jeger and Redel.
enol-β-Amyranonol diacetate	Benzene	Ruzicka and Jeger (1).
2-Acetoxy-6:7-epoxy-α-amyrane	Light petroleum (acetone + methanol)	Ruzicka, Jeger, Redel and Volli.
enol-2-Acetoxy-7:8-dioxo-α-amyrane	Benzene	Ruzicka, Jeger, Redel and Volli.
$\triangle^{6\cdot7\cdot8\cdot9}$-23-*nor*-α-Amyradiene	Light petroleum	Ruzicka, Jeger and Ingold.
Acetyl-*nor*-β-amyrenonol	Light petroleum	Ruzicka, Jeger and Winter.
Acetyl-*nor*-β-amyradienol	Light petroleum	Ruzicka, Jeger and Winter.
Reaction product of $\triangle^{6:7}$-23-*nor*-α-amyrene with N-bromosuccinimide	Light petroleum	Ruzicka, Jeger and Ingold.
Amidiol	Benzene	Zimmermann (1).
Dihydrobetulin (to free it from Raney nickel)	Alcohol	Ruzicka, Brenner and Rey (1).
Acetyl-dihydrobetulinic acid	Benzene (methanol)	Ruzicka, Brenner and Rey (1).
Betulinaldehyde	Benzene + light petroleum (same 1:9)	Ruzicka and Rey (1).
Acetyl-betulinaldehyde	Hexane (benzene)	Ruzicka and Brenner (1).
Betulone aldehyde	Benzene	Ruzicka and Rey (1).
Betulonic acid methyl ester	Benzene + light petroleum 1:9 (same 1:4)	Ruzicka and Rey (1).
2-Desoxybetulin (Lupeol)	Benzene + hexane 1:1 (benzene + ether 1:1)	Ruzicka and Heineman; Ruzicka and Brenner (1)
2-Desoxy-betulinic acid methyl ester	Benzene	Ruzicka and Rey (1).
Tetrahydroxy-lupane	Acetone (methanol + acetone)	Ruzicka and Brenner (2).
α-Lupene (from betulone aldehyde by reduction)	Benzene + light petroleum 1 : 2) (light petroleum)	Ruzicka and Rey (1).
Lupenol-2-one	Benzene	Ruzicka and Rey (1).
Diacetoxy-lupenal	Benzene	Ruzicka, Brenner and Rey (2).

TABLE 17—(*continued*)

EXAMPLES OF THE CHROMATOGRAPHY OF TRITERPENES AND RELATED COMPOUNDS ON ALUMINA

Compound	*Solvent (eluent)*	*Literature*
Diacetoxy-lupenic nitrile	Light petroleum + benzene 4 : 1 (1 : 1)	Ruzicka, Brenner and Rey (2).
Lupenalol acetate	Benzene + hexane (1 : 5) (same 1 : 2)	Ruzicka and Rosenkranz.
Lupenalol oxime	Benzene + hexane 1 : 1 (benzene + ether)	Ruzicka and Rosenkranz.
Acetyl-lupenalolic acid	Benzene (benzene + methanol 5 : 1)	Ruzicka and Rey (3).
Diol $C_{30}H_{52}O_2$ (from oxidation of γ-lupene)	Benzene	Ruzicka, Jeger and Huber.
nor-Lupanol	Benzene (ether)	Jones and Mackins.
nor-Lupantriol	Ether	Ruzicka and Brenner (2).
Dihydroxy-*nor*-lupanone	Ether	Ruzicka and Brenner (2); Ruzicka and Rey (2).
Diacetoxy-*nor*-lupanone	Benzene (benzene + ether)	Ruzicka and Brenner (2).
Formyl-diacetoxy-*nor*-lupanol	Benzene	Ruzicka and Brenner (2).
trisnor-Lupanone	Light petroleum, benzene	Ruzicka, Huber and Jeger.
Elemene	Light petroleum	Ruzicka, Rey, Spillmann and Baumgartner (2).
Elemenal	Benzene + light petroleum 1 : 5 (1 : 1)	Ruzicka, Rey, Spillmann and Baumgartner (2).
iso-Elemenal	Benzene + light petroleum 1 : 5 (1 : 1)	Ruzicka, Rey, Spillmann and Baumgartner (2).
Methyl-*iso*-elemenate	Light petroleum	Bilham and Kon (2).
α-Elemolic acid	Benzene + ether 1 : 1 (ether)	Ruzicka, Rey and Spillmann.
Elemedienonic acid	Benzene, benzene + ether (ethyl acetate + methanol)	Ruzicka, Rey, Spillmann and Baumgartner (1).
Dihydro-β-tritelemol	Benzene (benzene + ether 1 : 1)	Ruzicka and Häusermann.
Dihydro-β-tritelemone	Light petroleum	Ruzicka and Häusermann.
Dihydro-*nor*siaresinol	Light petroleum	Bilham, Kon and Ross (3).

TABLE 17—(*continued*)

EXAMPLES OF THE CHROMATOGRAPHY OF TRITERPENES AND RELATED COMPOUNDS ON ALUMINA

Compound	*Solvent (eluent)*	*Literature*
Triterpenoids from Manila elemi resin	Benzene	Morice and Simpson (1).
γ-Lanosteryl acetate	Hexane + benzene 1 : 4	Ruzicka, Rey and Muhr.
keto-Dihydro-lanosteryl acetate	Hexane + benzene 1 : 1	Ruzicka, Rey and Muhr.
γ-Lanostene	Hexane	Ruzicka, Rey and Muhr.
γ-Lanostenone	Hexane	Ruzicka, Rey and Muhr.
Acetyl-*trisnor*-lanosteric methyl ester	Benzene + hexane 1 : 1	Ruzicka, Rey and Muhr.
Breieneonol A and B (from breienedione by reduction)	Benzene	Morice and Simpson (2).
Hederadiol (from diacetyl-hederaldehyde semicarbazone)	Benzene (acetone)	Ruzicka and Marxer (2).
Reaction products of hederagenin lactone	Several solvents	Ruzicka, Norymberski and Jeger
C_{27}-Oxy-tetraacid trimethyl ester-lactone	Benzene (benzene + ether)	Ruzicka, Norymberski and Jeger (1).
Methyl-glycyrrhetate	Ether	Bilham, Kon and Ross (2).
Acetyl-dehydro-desoxo-glycyrrhetic methyl ester	Benzene	Ruzicka and Jeger (3).
Acetyl-desoxy-glycyrrhetinaldehyde	Benzene	Ruzicka and Marxer (1).
enol-23-*nor*-2 : 3-Dioxo-13-hydroxy-oleanane-28-acid lactone (diosphenol)	Several solvents	Ruzicka, Jeger and Norymberski (2).
$\triangle^{10:11,13:18}$-2 : x-Diacetoxy-12 : 19-diketo-oleadien-28-ic acid methyl ester.	Benzene	Ruzicka, Jeger, Grob and Hösli.
Reduction products of *nor*-chinova-dienolal-semicarbazone	Benzene, benzene + ether	Ruzicka and Marxer (3).
Nova-aldehyde-semicarbazone (from novaic acid chloride)	Several solvents	Ruzicka and Marxer (3).

Miscellaneous Hydroaromatic Compounds

trans-Cyclohexanediol-(1 : 3) benzoate-[3 : 5-dinitrobenzoate] can be adsorbed on alumina and eluted with ethyl acetate (Dimroth and Resin). A filtration through the same adsorbent of 1-hydroxy-2-benzoxymethylene-cyclohexyl-1-acetic acid ethyl ester in methanol constitutes a satisfactory method of purification (Plattner, Treadwell and Scholz). Bicyclo-undecanol-1 was adsorbed on alumina from hexane and fractionally eluted with hexane + benzene (Plattner). Hydnocarpyl-sulfonamide, $C_5H_7 \cdot (CH_2)_{11} \cdot SO_2 \cdot NH_2$ in benzene can be purified on alumina and elution with hot alcohol (Arnold, Helmert, Möbus, Prigge, Rauen and Wagner-Jauregg).

The following compounds were purified by adsorption on or filtration through alumina: β-(*trans*-β-hydroxy-cyclohexyl)-$\triangle^{\alpha\beta}$-butenolide (light petroleum; Hardegger, Plattner and Blank). Methyl-1-hydroxy-6-methoxy-2-carbomethoxy-2 : 5-dimethyl-tetralin 1-acetate (Martin and Robinson 1). 3-(6′-Methoxy-2′-naphthyl)-2-methylcyclopentane-1-one (benzene; Koebner and Robinson). 3-Keto-4-acetoxy-7-methoxy-1 : 2-cyclopenteno-naphthalene (benzene; Martin and Robinson 2). Methyl-4 : 7-dimethoxy-phenanthrene-1 (β)-propionate-2-carboxylate (benzene; Robinson and Rydon). 2-Methyl-1-$\triangle^{\alpha,\gamma}$-butenyl- 3 :4-dihydrophenanthrene (light petroleum; Burnop, Elliott and Linstead). 2 : 13-Dimethyl-perhydro-phenanthrene-dione-(1 : 7) and 2 : 13-dimethyl-1 : 4 : 5 : 6 : 7 : 8 : 9 : 10 : 11 : 12 : 13 : 14-dodecahydrophenanthrene-(1 : 7) (benzene + light petroleum; Reich). 7-Methyl- and 4-methyl-3′ : 7-dimethyl- and 3′ : 7-dimethyl-1 : 2-cyclo-pentenophenanthrene (light petroleum; Kon and Woolman).

CHAPTER XV

BENZENE, NAPHTHALENE, DIPHENYL, DIPHENYLALKYL AND INDENE SERIES

A systematic exploration of the chromatographic behaviour of simple aromatic substances is yet to be made. A study of some compounds in benzene on alumina was carried out by Sinomiya, the details of which were not available to the author. The effect of functional groups on adsorbability was as follows : COOH or OH > NH_2 > NO_2 > CH_3 > H.

Nitrogen-free Compounds

An earlier observation that *phenols* can be differentiated on alumina after treatment with ferric chloride in aqueous solution was not confirmed by Bielenberg *et al.*, and a separation after coupling with diazonium solutions was tentatively proposed by them. Catechol diphenylmethylene ether can be purified by filtering the ether solution through alumina (H. S. Mason 1), and the acid phthalate of phenyl-ethinylcarbinol using benzene solution (E. R. H. Jones and McCombie). For some monoalkyl ethers of pseudo-cumohydroquinone benzene +light petroleum was used (Werder, Moll and Jung).

Gentisyl alcohol : Engel and Brzeski ; derivatives of veratroin : Kubiczek ; 2-hydroxy-4-*n*-pentadecyl-isophthalic acid : Prelog, Metzler and Jeger.

From " *Castoreum* ", the glandular excretions of a beaver (*Castor fiber*), Lederer and Polonsky separated a viscous *phenolic* portion, b.p. about 240°. The pyrocatechin content of 1 g. was adsorbed from 5 ml. of toluene on alumina (25 g., " Prolabo ") and the column was washed with 100 ml. of toluene. The pyrocatechin was then washed through the column with 75 ml. of toluene + 25 ml. of ether and purified by vacuum sublimation. Similarly, hydroquinone was isolated from a fraction (b.p. 240–50°) of the oily phenol mixture by chromatography and demethylation with HI. From a nearly solid fraction (b.p. 184–190° under 1 mm.) 2 : 4-dihydroxy-diphenylmethane was obtained. From the ketonic fractions of " Castoreum ", Lederer isolated *p*-methoxyacetophenone.

(Some other ketonic compounds were obtained by chromatographing their 2 : 4-dinitrophenylhydrazones from benzene on alumina.)

A filtration of the benzene or benzene-petroleum solution through alumina was found effective in the following instances : 4 : 4′-diacetylbenzil, 4 : 4′-diacetyl-α, β-diethylstilbene and 4 : 4′ : 4′-triacetyltriphenylethylene (Ross) : 2-methoxy (or 4-methoxy)-3′ : 4′-methylenedioxy-*diphenyl* and its 6′- aldehyde ; 2-methoxy-2′ : 3′-methylenedioxy-diphenyl and its 5′- aldehyde (Fujise ; Uyeo) ; 2-chloro-2′-methyl-diphenyl (Orchin and Woolfolk) ; α, β-di-(5 : 6-dimethylnaphthyl-1)-ethane or α-(7-methyl-naphthyl-1)-β-(5 : 6-dimethylnaphthyl-1)-ethane (Ruzicka and Hofmann). 8-Benzoyl-1-ethylnaphthoate when adsorbed from benzene-light petroleum forms a grey-violet fluorescent zone, which can be eluted with alcohol (French and Kircher).

Diethylstilbestrol (4 : 4′-dihydroxy-α : β-diethylstilbene) was separated by Dingemanse from natural oestrogens ; the latter were eluted from alumina with chloroform containing 0·2 per cent of alcohol, whilst stilbestrol required 0·5 per cent of alcohol.

Compounds like 2-(*p*-methoxyphenyl)-3-ethyl-6-methoxy-*indene* (and some *indanones*) can be purified by developing on alumina with light petroleum + benzene (2 : 1). The main zone (blue fluorescence) is preceded by various impurities which show stronger adsorption affinities and a green fluorescence. In other instances development with acetone + benzene was required (Solmssen).

Amino Compounds

A labile hydrogenation product obtained from *o*-cyanoacetophenone was purified on alumina from ether solution (Helberger and Rebay). The adsorbability of p-aminobenzoic acid was compared with that of sulphanilamide and sulphathiazole on different adsorbents by Hartmann and Druey. In some instances the adsorbent retained more sulphathiazole than *p*-aminobenzoic acid at an alkaline pH but less at an acid pH. From active carbon, *p*-aminobenzoic acid is displaced both by sulphanilamide and sulphathiazole. Adsorption of 3-hydroxy-sulphanilamide (ex urine) on alumina : R.T. Williams.

The adsorption of procaine on charcoal was studied by Régnier, David, and Jean. Diaminobiphenyls : Carlin.

An ethanol solution of d_1l-α-benzamido-β-[4-(2′,6′-diiodo-4′-aminophenoxy)-phenyl]-propionic acid can be purified by filtration through alumina (Niemann and McCasland).

Nitro Compounds

According to Semtin, the intensely coloured zones of 2 : 4-dinitrotoluene and 2 : 4 : 6-trinitrotoluene can be separated from benzene + light petroleum solutions on a mixture of magnesium oxide and calcium hydroxide (5 : 8). Schuler and Yang separated isomeric nitro-compounds (and indeed, homologous quinones) on alumina, calcium carbonate or magnesia. Crude Schiff bases obtained, e.g., from *p*-nitrotoluene or 2 : 6-dinitrololuene and *p*-nitrosodimethylaniline were purified by Chardonnens and Heinrich (1) (benzene, Brockmann alumina). The homogeneity of a tetranitro-2 : 2′ : 4 : 6′-tetramethyldiphenyl was tested by developing with benzene + light petroleum (1 : 1) on activated alumina (Carlin).

Nitro derivatives of *dinaphthylene-dioxide* (Pummerer, Buchta, Gündel, Kiessling, Pfeiffer, Rath, Schuler and Stinzendörfer). When 1 g. of the crude nitration product of dinaphthylene-dioxide is adsorbed from 800 ml. of chlorobenzene on 1 kg. of activated alumina, the following top to bottom sequence is observed :

Brick red : dinitro-compound ; red-violet : another dinitro compound ; blue : a third dinitro-compound ; red-violet : mononitro compound ; bluish violet : another mononitro compound ; and yellow : unchanged dinaphthylene-dioxide.

The two lowest zones are separately washed into the filtrate with chlorobenzene and then the column is washed with absolute alcohol and dried in absence of moisture. The red-violet mononitro zone is cut out and eluted with hot chlorobenzene. The dinitro zones can be separated in a similar manner.

For 4-methyl-2-amino-5-nitrobenzophenone cf. Chardonnens and Perriard.

Oximes

p-Benzoquinone monoxime and dioxime can be separated by adsorbing a mixture of the two substances on activated alumina from acetone and developing with a solution of dilute

acetic acid in acetone, then with acetone + 5 per cent methanol. The upper zone (monoxime, green) can be eluted with aqueous NaOH. The dioxime forms a yellow zone below the monoxime (Gullstrom, Burchfield and Judy).

Anils

Some *p*-dimethylamino derivatives of aromatic aldehydes (3- or 4-nitrophenylglyoxal, 2-nitro-4-cinnamoyl-benzaldehyde) and also some anilides of acids in benzene can be purified on alumina (Chardonnens and Venetz 2; Chardonnens and Heinrich; cf. also p. 191).

Azo Compounds

Derivatives, such as 4′-nitro-4-hydroxy-2-methyl*azobenzene* (and its acetate), 4′-nitro-4-hydroxy-2 : 5-dimethylazobenzene or 2 : 3 : 6-trimethylazobenzene, etc., were purified by chromatographing on alumina from ethyl acetate or alcohol solution (Smith and Irwin). For 3 : 5 : 3′ : 5′-tetramethyl-4,4′-dihydroxyazobenzene (obtained by electrochemical oxidation), dioxane should be used (Fichter and Gunst). Azobenzene-2 : 2′-di-(methylsulphide) forms a red zone below the orange one of the corresponding azoxy compound (Simons and Ratner).

Condensation Product of 4-Methyl-3-nitro-azobenzene and p-Nitroso-dimethylaniline (Chardonnens and Heinrich 2). The crude product (24 g.) obtained by refluxing in alcohol with sodium carbonate, was chromatographed from benzene on alumina. Besides minor pigments, an orange brown and a violet brown zone appeared, whilst the yellow zone of the unchanged azo compound was washed by benzene into the filtrate (17 g.). The orange-brown zone yielded, upon elution with acetone and recrystallisation from benzene, 3 g. of the *p*-dimethylamino-anilide of the 3-nitro-azobenzene-4-carboxylic acid; and the violet-brown zone yielded 2 g. of the *p*-dimethylamino-anil of 2-nitro-4-(benzene-azo)-benzaldehyde.

Sulphones

The crude bis-(*p*-dimethylaminoanil) of 3 : 3′-dinitro-4 : 4′-diformyl-diphenylsulphone, $C_{30}H_{28}O_6N_6S$, can be developed

with pyridine on alumina where it forms a dark red zone that passes into the filtrate (Chardonnens and Venetz 1).

Separation of Aromatic cis-trans Isomers

(*a*) **Azo Compounds.** The extensive data published by A. H. Cook (3) refer mainly to azobenzene and the following derivatives which, before irradiation, had been purified on alumina : *m*, *m'*-azotoluene ; *p*, *p'*-azotoluene ; benzeneazo-*p*-toluene ; *p*-benzeneazophenetole ; *p*-azoanisole ; *m*-methyl-, *p*-nitro-, *m*-nitro-, *m*, *m'*-dinitro-, 2 : 4-dimethoxy-, 2 : 6-dimethoxy-, *p*-chloro-, *p*-bromo- and *p*-iodo-azobenzene ; benzeneazo-α-naphthylmethyl ether ; bisbenzene-azobenzene ; and bisbenzene-azodiphenyl (cf. p. 13).

In order to obtain *trans*→*cis* rearrangement, the solutions in light petroleum (+ benzene) were irradiated in Pyrex containers with a Hg-vapour lamp at a distance of 30 cm., and the solution was chromatographed on active alumina (Merck). Usually the *trans* form could easily be washed into the filtrate ; and the *cis*-compound was then eluted by adding a little alcohol to the suspension of the adsorbate zone in the solvent originally used. After rapid removal of the alcohol by washing, the dried solution was evaporated in vacuo at 20° and the *cis*-compound was recrystallized from light petroleum or benzene + light petroleum at low temperature. (These operations should be carried out in the absence of light.)

cis- and trans-Azobenzenes. The irradiation of 1 g. of *trans*-(ordinary) azobenzene in 50 ml. of light petroleum for ½ hour changed the colour to red. The solution was developed on alumina (20 × 2 cm.) with 100 ml. of light petroleum. Only the *cis* form remained on the column, where it formed a sharp 4-cm. zone beginning 1 cm. from the top. After elution and recrystallization from a little cold light petroleum, the orange-red plates melted at 71° (cf. also author, Frehden and Fischer-Jörgensen).

Bisbenzene-azobenzenes (Cook). A benzene+light petroleum (1 : 4) solution (0·5 g. in 100 ml.), after a 45-min. irradiation,

was adsorbed on alumina (15–20 × 1 cm.). Upon washing with 200 ml. of the mixture mentioned and then with a (1 : 1) mixture, the sequence was : 2 cm. broad, deep orange (top) ; 1 cm., light orange ; and a bottom zone of unchanged all-*trans* compound.

The *α*- and *β*-forms of p-azophenol (green, and brick-red) were not accepted by Lauer, Klug and Harrison as *cis-trans* isomers, because they did not separate on any column tested. Zones of both samples in ethyl acetate solution (1 per cent.) migrated slowly downwards on calcium carbonate.

(*b*) **cis- and trans-Benzoin and Anisoin Oximes.** As is well known, *trans*-benzoin oxime gives with ammoniacal copper sulphate a deep-green complex (0·15 mole of sulphate and 4 moles of ammonia per litre), whilst the corresponding colour from the *cis* form is brown. This difference can be utilised for the location of each stereoisomer. On a 40 to 200 mg. scale, 1–2% of either form can be detected in the other. On neutral filtrol the two zones are well separated, that of the *trans* isomer occupying the upper layer. If the rate of flow is low, the otherwise empty interzone may contain small amounts of the *trans* compound formed on the column. After several minutes the whole streak takes on a slightly greenish tint. In contrast, the reaction in the main zones is instantaneous.

From neutral filtrol a trace of oil was removed by repeated extractions with benzene and alcohol + ether (1 : 1) at 20° with mechanical shaking. The adsorbent was dried at 90° for 4 hours. This increased the adsorptive power but decreased the rate of flow, and 17 per cent of celite (No. 535) was therefore used as a filter aid.

For the preparation of artificial mixtures of *cis*- and *trans*-benzoin oxime, each isomer had first to be purified chromatographically. A mixture of equal parts (75 mg. each) of the two purified isomers in 20 ml. of chloroform+benzene (2 : 1) was developed with 40 ml. of benzene containing 2 per cent ethanol on a column (17·5 × 1·7 cm.) within 20 min. The streak showed the following sections (the figures indicate width of zones, in mm.) :

2 sky blue (empty).
17 dark green (*trans*).
42 blue (trace of *trans*).
26 dark brown (*cis*).
88 sky blue (empty).

The *trans*-zone and the 72-mm. interzone were each eluted with 50 ml. of dry alcohol + ether (1 : 1), whilst the *cis* compound required 40 ml. of ether. After evaporation, 74·5 mg. and 1·7 mg. of *trans*- and 70·1 mg. of *cis*- benzoin oximes, with the correct melting points, were recovered. A mixture of *cis*- and *trans*-anisoin oxime (75 mg. of each) in 35 ml. of benzene was developed with 50 ml. of benzene containing 3 per cent of absolute alcohol on a 14 × 1·7 cm. column. The recovery was 69·2 mg. of *trans*- and 68·3 mg. of *cis*-oxime. The interzone yielded 0·8 mg. of the *trans* form (author, McNeely and Sólyom).

(*c*) **cis- and trans-Stilbenes.** The two forms of stilbene, *p*-methyl- or *p*-methoxy-stilbene, etc., can be located on alumina by drawing a streak of 1 per cent permanganate along the column. Each *trans* form was adsorbed above the corresponding *cis* form and the two were separated by a broad interzone. The reagent rapidly turns brown when it crosses a zone, whilst the empty sections change it only after several minutes. The experiment takes about an hour on a 10–500 mg. scale, the limit of detection being 1–2 per cent for each stereoisomer in the other.

The use of chromatography also revealed the presence of impurities even in the best commercial stilbenes and laboratory products. From stilbene itself a strongly adsorbed fluorescent contaminant was eliminated by filtering the benzene + light petroleum solution (1 : 1) through activated alumina. The adsorbate of stilbene did not then fluoresce. The melting point remained unchanged. A crude sample of *p*-methylstilbene in benzene + light petroleum (1 : 3) was purified, instead of by high vacuum sublimation, by filtration through super filtrol, whereby a number of coloured zones could be removed. When crude methoxystilbene in benzene passes through alumina, a strongly fluorescent by-product of its synthesis separates out.

A *cis*- and *trans*-stilbene mixture (obtained by quartz lamp illumination; 160 mg.) in 25 ml. of light petroleum (b.p. 60–70°) was developed on "Alorco" alumina (150–200 mesh or −80 mesh; 17·5 × 1·7 cm.) with 90 ml. of the same solvent. A yellow contaminant and a minor fluorescent zone were retained near the top, whilst the main section on streaking showed a 42 mm. zone (*trans*), separated by a 6 mm. empty

section from a 48 mm. zone (*cis*). Upon elution with dry ether and evaporation, the yields were 99 mg. of *trans*- and 52 mg. of *cis*-stilbene (recovery, 94 per cent). Before analysis, the oily *cis* form was rechromatographed. On a similar column it was possible to detect 2 mg. of *cis*- in admixture with 75 mg. of *trans*-stilbene or 0·5 mg. of *trans*- in 30 mg. of *cis*-stilbene (column, 10·5 × 0·9 cm.).

Analogous results were obtained in the two following examples. A mixture of 450 mg. of *trans*- and *cis*-*p*-methylstilbene in 90 ml. of light petroleum was developed on alumina (22·5 × 4·3 cm.) with 700 ml. of light petroleum; and a mixture of the two stereoisomeric *p*-methoxystilbenes (161 mg.) in 5 ml. of chloroform + light petroleum (1 : 1) was developed on 17·5 × 1·7 cm. alumina with 70 ml. of benzene + light petroleum 1 : 3 (author and McNeely).

(*d*) **cis-cis-, cis-trans- and trans-trans-Diphenylbutadienes** : p. 15.

(*e*) **Stereoisomeric Diphenyloctatetraenes.** A solution of 1 mg. in 1–2 ml. of benzene, after irradiation in a quartz tube for 12 hours with a mercury quartz lamp, was developed in darkness with benzene + light petroleum (1 : 10) on calcium hydroxide (25 × 1·7 cm.) and inspected with an ultraviolet lamp (author and LeRosen 2; stereoisomerization on the column : p. 35).

10 stongly fluorescent, all-*trans*.
5 dark interzone.
20 weaker fluorescence, *trans-cis-trans-trans* (probably).
5 dark interzone.
2·5 similar to the above, *trans-cis-cis-trans* (probably).
2 dark interzone.
2 two small zones, perhaps sterically "hindered" isomers.
The zones can be eluted with benzene + methanol (3 : 1).

Polycyclic Aromatic Compounds
(Cf. Table 18, p. 200)

Many compounds belonging to this class were successfully purified on alumina as shown by Table 18. The fluorescence of certain zones may offer the necessary guidance. In some

instances, diffused daylight may induce chemical alterations (Levy and Campbell, cf. p. 20).

Some molecular compounds are split during their passage through the alumina column, and this may be used for preparative purposes. The polycyclic component passes into the filtrate, whilst the other remains adsorbed (p. 19). This has been reported, e.g., by Kondo, for the nitrobenzolate of anthracene, the picrates of anthracene, phenanthrene or γ-methyl-cyclopentenophenanthrene. For the trinitrobenzolate of tetrahydro-1 : 7 : 8-trimethyl-phenanthrene, cf. Ruzicka and Dalma.

In order to separate 9 : 10-*dihydro*- and 1 : 2 : 3 : 4-*tetrahydro*-anthracene, a light petroleum solution, containing 100 mg. of each compound, was adsorbed on activated alumina (23 cm. long, 65 g.) and the column was washed with 125 ml. of the same solvent and then with petroleum ether + benzene (4 : 1). The first 150 ml. of the filtrate gave no residue, the next 50 ml. contained a mixture, and the last yielded 25 mg. of 9 : 10-dihydroanthracene (Orchin).

The products formed by the interaction of 1-diazo-*anthraquinone*-2-sulphonate (or derivatives) with bases, if necessary in the presence of copper bronze, were resolved by Lynas-Gray and Simonsen. After the evolution of nitrogen had ceased, and the excess base had been eliminated in the presence of strong alkali, a mixture of salts was precipitated with brine, dissolved in alcohol and developed with the same solvent on alumina. The zones were then eluted with water. For example, a mixture obtained from aniline and 1-diazo-4-bromoanthraquinone-2-sulphonate gave four zones, viz. : (*a*) rose-violet (sodium 1-anilinoanthraquinone-3-sulphonate) (top) ; (*b*) blue (sodium 1-amino-4-anilinoanthraquinone-2-sulphonate) ; (*c*) red (sodium 4-bromo-1-aminoanthraquinone-2-sulphonate) ; and (*d*) green (sodium 1:4-dianilinoanthraquinone-2-sulphonate).

Adsorptions on Japanese acid earths : Yukawa.

Isolation of Chrysene from Garden Soil (and from wood tar) (Kern). The benzene extract (Soxhlet) of 3·5 kg. of dry

soil was concentrated to 100 ml., adsorbed on a 20×5 cm. activated alumina column and washed with 1 *l.* of benzene. After strong concentration of this filtrate the substance was rechromatographed (70×6·5 cm.). The two blue and yellow fluorescent main bottom zones were eluted with light petroleum. The residue (6 g.) left on evaporation of the latter was dissolved in a little benzene; this solution was diluted with light petroleum and rechromatographed. The 70×6·5 cm.-column was washed with benzene + light petroleum, with a gradually increasing benzene content, and 0·25 to 0·5 *l.*-portions were taken. From the first eluate 55 mg. of chrysene was isolated, after recrystallization from a little benzene and light petroleum. From other fractions two unidentified yellow hydrocarbons were obtained.

Similar operations gave 250 mg. of chrysene from 100 g. of wood tar.

3 : 4-Benzpyrene. Chalmers and Kirby (*cf.* also Chalmers), after having injected benzpyrene into rats, separated small portions of the unchanged compound from a derivative by chromatographing benzene extracts of the urine or feces on alumina. The benzpyrene was rapidly washed into the filtrate with ether, but fluorescent, photolabile compounds remained on the column. Similar fluorescent products of unknown structure, obtained from the intestinal tract and the bile of mice that had been given benzpyrene, were separated by Weigert and Mottram on alumina (benzene).

After intravenous injection of carcinogenic and non-carcinogenic polycyclic hydrocarbons into fowl, the investigation of the bile (which became fluorescent) was carried out by filtering a benzene extract through alumina. In most instances a fluorescent derivative was strongly adsorbed near the top. Elution was carried out with benzene + methanol (Chalmers and Peacock).

The estimation of benzpyrene in tissue extracts of whole animals can be carried out as follows (Weil-Malherbe 2). The light petroleum solution (from one mouse) was passed through

a column of 1·5 g. of silica gel, which was washed with three 2-ml. portions of light petroleum and a 25-ml. portion of light petroleum ether + benzene (4 : 1). The entire filtrate was adsorbed on a second column of alumina (2 g.), and developed by 20 ml. of the petroleum + benzene mixture. The combined filtrates were rejected. Finally, the compound was washed into the filtrate with 15 ml. of benzene and was ready for fluorometric estimation (cf. Berenblum and Schoental).

Some products of the action of lead tetraacetate on 1′ : 2′ : 3′ : 4′-*tetrahydro*-3 : 4-*benzpyrene* were separated by repeated chromatography on activated alumina and washing of the column with light petroleum. One of the main products was a fluorescent hydrocarbon, $C_{20}H_{14}$ (Kon and Roe).

Pycylene-ketone, $C_{21}H_{12}O$. (J. W. Cook) the purification of which by vacuum sublimation was incomplete, was treated on alumina by the liquid chromatogram method. As developers, light petroleum + benzene mixtures of increasing benzene content were used, and, finally, pure benzene. The first fractions contained colourless material while the main coloured zone yielded golden-orange leaflets.

Toxic Principles of Phenolic Character in Anacardiaceae. An oily fraction, obtained from poison ivy, when chromatographed under nitrogen by Mason and Schwartz on barium carbonate + hyflo supercel, separated into an unsaturated acid and a phenolic oil. If alumina "Alorco" (de-activated with 90% alcohol) was used, six zones appeared of which the first and the last were coloured and the others showed bluish-white fluorescence. Three of these were found to be toxic.

A related substance, bhilawanol, an allergenic oil that occurs in the Indian marking-nut tree was converted into its diphenylmethylene ether by Mason, dissolved in light petroleum + methanol and passed through alumina (Aluminum Ore Co.; minus 80 mesh). A broad red zone (which turned purple upon brushing with alcoholic $FeCl_3$ solution) remained on the column, whilst the filtrate was pale yellow. A further fractionation of the latter could be obtained by chromatographing 2·5 g. in light petroleum on alumina (15 × 2·5 cm.). Several fluorescent zones remained on the column; the main product, a colourless and odourless oil (2·1 g.) was collected in the filtrate.

TABLE 18

EXAMPLES OF POLYCYCLIC AROMATIC COMPOUNDS PURIFIED ON ALUMINA MOSTLY FROM BENZENE OR LIGHT PETROLEUM SOLUTION

o-Phenyl-biphenyl and *o,o'*-diphenyl-biphenyl	Morton, Massengale and Richardson
α - Naphthaquinone diallyl ether	Fieser, Campbell and Fry
4 : 7-Dimethyl-6-methoxy-tetralone-(1)	Ruzicka and Sternbach
2-(2'-Naphthyl)-phenyl-acetamide	Levy and Campbell
Anthracene	Levy and Campbell
1 : 2 : 10-Trimethyl-anthracene	Fieser and Webber
2 : 9 : 10-Trimethyl-anthracene	Fieser and Heymann
2 : 3 : 9 : 10-Tetramethyl-anthracene	Fieser and Webber
2 : 3 : 9 : 10-Tetramethyl-1 : 4-dihydro-anthracene	Fieser and Webber
1 : 2-Dimethyl-anthraquinone	Fieser and Webber
5-Chloro-1-*p*-anisidino-anthraquinone	Cook and Waddington
1 : 2-Benzanthracene	Levy and Campbell
5-Methyl-1 : 2-benzanthracene	Fieser and Johnson (2)
8-Methyl-1 : 2-benzanthracene	Fieser and Cason (1)
8-Methyl-1 : 2-benzanthracene (picrate, trinitrobenzene derivative)	Fieser and Johnson (2)
3 : 9-Dimethyl-1 : 2-benzanthracene	Fieser and Seligman (2)
4 : 9-Dimethyl-1 : 2-benzanthracene (trinitrobenzene derivative)	Fieser and Jones
5 : 8-Dimethyl-1 : 2-benzanthracene	Fieser and Johnson (2)
1' : 10-Dimethyl-2 : 3-benzanthracene	Fieser and Hershberg (10)
1' : 9-Methylene-1 : 2-benzanthracene	Fieser and Cason (3)
3 : 10-Dimethoxy-1 : 2-benzanthracene	Fieser and Hershberg (7)
10-Methyl-1' : 9-methylene-1 : 2-benzanthracene	Fieser and Cason (2)
5-Ethyl-9 : 10-dimethyl-benzanthracene	Bachmann and Chemerda (2)
5 : 6 : 9 : 10-Tetramethyl-1 : 2-benzanthracene (picrate)	Badger, Cook and Goulden
8-Methyl-1 : 2-benzanthraquinone	Fieser and Johnson (1)
5-*p*-Propyl-9 : 10-dimethyl-1 : 2-benzanthracene	Bachmann and Chemerda (2)
6-Chloro-10-methyl-1 : 2-benzanthracene (picrate)	Fieser and Cason (3)
1 : 2-Benzanthracene-10-aldehyde	Fieser and Hartwell
8-Methyl-1 : 2-benzanthracene-10-aldehyde	Fieser and Johnson (2)
6-Cyano-10-methyl-1 : 2-benzanthracene	Newman and Orchin
1' : 2' : 3' : 4'-Tetrahydro-4 : 9-dimethyl-1 : 2-benzanthracene	Fieser and Jones
7-Methyl-7-carbomethoxy-8-keto-3 : 4 : 5 : 6 : 7 : 8-hexahydro-1 : 2-benzanthracene	Bachmann and Chemerda (3)

TABLE 18—(*continued*)

EXAMPLES OF POLYCYCLIC AROMATIC COMPOUNDS PURIFIED ON ALUMINA MOSTLY FROM BENZENE OR LIGHT PETROLEUM SOLUTION

3 : 10-Dimethyl-1 : 2 : 5 : 6-dibenzanthracene	Bachmann and Chemerda
Diphenyl-tetrahydro-naphthacene	Allen and Bell
1 : 2-Tetral-9 : 10-diphenylanthracene	Allen and Bell
1 : 2 : 3-Trimethyl-phenanthrene	Fieser and Daudt
1 : 7 : 8-Trimethyl-phenanthrene	Ruzicka and Sternbach (2)
1 : 7 : 8-Trimethyl-phenanthrene	Ruzicka and Dalma
1 : 2 : 3 : 4-Tetramethyl-phenanthrene	Hewett and Martin
2 : 3 : 4 : 7-Tetramethoxy-phenanthraquinone	Barton, Cook and Loudon
1-Methyl-7-ethyl-phenanthrene	Ruzicka and Kaufmann (3)
β-Ethyl-retene	Ruzicka and Kaufmann (3)
1-Phenetyl-phenanthrene	Drake and McVey
1-Methyl-3 : 3-benzphenanthrene (picrate)	Hewett
2-Methyl-3 : 4-benzphenanthrene	Newman and Joshel
2-Ethyl-3 : 4-benzphenanthrene (picrate)	Everett and Hewett
2-Isopropenyl-benzphenanthrene	Hewett
9-Acetoxy-acetylphenanthrene	Ross (2)
3′-Methyl (or 9-methyl-)-cyclopentenophenanthrene (picrate)	Butenandt and Surányi
7-Methoxy-1 : 2 : 3 : 4-tetrahydrophenanthrene-2-carboxylic acid methyl ester	Heer and Miescher
2-Methyl-*meso*benzanthrone	Hey, Nicholls and Pritchett
3-Bromo-2-methyl-benzanthrone	Hey, Nicholls and Pritchett
3-Hydroxy-2-methyl-*meso*benzanthrone	Hey, Nicholls and Pritchett
Pyrene	Levy and Campbell
1 : 2 : 3 : 10-Tetrahydroperinaphthanone-7	Johnson, Johnson and Petersen
Dihydroperinaphthanone	Johnson, Johnson and Petersen
3′-Methyl-3 : 4-benzpyrene	Fieser and Hershberg (6)
5-Methyl-3 : 4-benzpyrene	Fieser and Hershberg (6)
3 : 4-Benzpyrene (trinitrobenzene derivative)	Fieser and Hershberg (6)
5-Methoxy-3 : 4-benzpyrene	Fieser and Hershberg (9)
5-Methoxy-6 : 7 : 7a : 8 : 9 : 10-hexahydro-3 : 4-benzpyrene	Fieser and Hershberg (9)
Methyl-3 : 4-benzpyrenyl-5-carbinol	Fieser and Hershberg (9)
3-Chloro-3 : 4-benzpyrene	Windaus and Raichle
5-Nitro-3 : 4-benzpyrene	Fieser and Hershberg (9)
5-Nitro-3 : 4-benzpyrene (oxidation products of)	Eckhardt
10-Amino-3 : 4-benzpyrene	Windaus and Raichle

TABLE 18—(*continued*)

EXAMPLES OF POLYCYCLIC AROMATIC COMPOUNDS PURIFIED ON ALUMINA MOSTLY FROM BENZENE OR LIGHT PETROLEUM SOLUTION

10-Acetyl-3 : 4-benzpyrene	Fieser and Hershberg (9)
Diacetyl-3 : 4-benzpyrene	Windaus and Raichle
3 : 4-Benzpyrene-5 : 8-quinone	Windaus and Raichle
Benzpyrene-5-nitrile	Windaus and Raichle
Cholanthrene	Fieser and Kilmer (1, 2)
7-Methyl-cholanthrene	Bachmann and Safir
Methyl-7-cholanthroate	Fieser and Kilmer (1, 2)
2-Methoxy-20-methyl-cholanthrene	Fieser and Desreux
6-Chloro-20-methyl-cholanthrene	Fieser and Desreux
Chrysene	Levy and Campbell; Kern
6-Methylchrysene	Fieser, Joshel and Seligman
5-Methylchrysene	Newman (3); Fieser and Joshel (2)
5-Ethylchrysene	Newman (3)
4 : 5-Methylenechrysene	Fieser and Cason (3)
5 : 6-Dimethylchrysene	Newman (3)
1 : 2-Naphtho-(2′ : 3′)-chrysene	Beyer and Richter
4-Methoxychrysene	Cook and Schoental
4-Methoxy-1 : 2-chrysaquinone	Cook and Schoental
6-Nitrochrysene	Newman and Cathcart
1-Acetamido-chrysene	Cook and Schoental
Methyl-perinaphthene	Craig, Jacobs and Lavin
Perinaphthanone-7	Fieser and Gates
1- and 3-(*o*-Cyanophenyl)-perinaphthene	Fieser and Gates
2 : 3-Trimethyl-triphenylene	Fieser and Daudt
2 : 3-Dimethyl-1-hydroxy-triphenylene	Fieser and Daudt
4-Methyl-fluorene	Orchin and Woolfolk
4-Methyl-fluorenone	Orchin and Woolfolk
2 : 3-Dimethyl-fluorene	Fujise
2 : 3-Dimethyl-fluorenone	Fujise
2-Methoxy-6′ : 7′ -methylenedioxyfluorenone	Uyeo
4-Methoxy-6′ : 7′-methylenedioxy-fluorenone	Uyeo
9-Methyl-3 : 4-benzfluorene	Fieser and Joshel (1)
2 : 7-Bis-acetoxyacetylfluorene	Ross (2)
2 : 3 : 5 : 6-Dibenzofluorene	R. H. Martin
2 : 3 : 5 : 6-Dibenzofluorenone	R. H. Martin
2 : 3 : 6 : 7-Dibenzofluorenone	R. H. Martin
4 : 5-Benzhydrindone-1	Fieser and Gates

CHAPTER XVI

HETEROCYCLIC COMPOUNDS

COMPOUNDS WITH HETEROCYCLIC NITROGEN

(Cf. also Table 19, p. 209)

Nicotinic Acid was estimated by Roggen in urine or faeces, by a method involving adsorption on frankonite KL from nitric acid solution and elution with baryta. A micro-chromatographic method could probably be based on these observations.

Estimation of Nicotinamide Methochloride (N'-Methyl-nicotinamide chloride) in Urine (Coulson, Ellinger, Glock and Platt; Coulson, Ellinger and Holden). This compound is converted by alkali into a strongly fluorescent substance, which is adsorbed on Decalso, 1 g. adsorbing up to 1 mg. from 10 ml. of urine. The column is washed with water and eluted with 25% KCl solution. The adsorption is not affected by urea between pH3 and 8.

Recently, Hochberg, Melnick and Oser worked out the following procedure. A six-minute urine aliquot is adsorbed at pH 4·5 on a synthetic zeolite column (11 × 0·8 cm.). The amide is eluted with 25% KCl solution and, after making alkaline and extracting with *n*-butanol, the solution is compared fluorometrically with a standard carried through all operations. A correction should be made for butanol-soluble fluorescent compounds in the eluate prior to alkalinization. Contact with rubber must be avoided. The actual determination is made as follows: The zeolite (50 mesh) is pre-treated by stirring twice with 3% acetic acid for 10 min., once with 5 vol. of neutral 25% KCl solution for 15 min., twice again with the acetic acid, and then with water, alcohol and ether; finally, it is dried in air and should be kept in sealed bottles. After the sample has been adsorbed on the column, a condenser is attached above the adsorption tube and steam is passed through the jacket. Water (30 ml.) is poured through the hot condenser in order to heat the adsorbate, and then the eluent, 15 ml. of the KCl-solution, is run through the column.

Quinoline Derivatives. The following compounds were purified by filtering their benzene solutions through alumina : 8-α-pyridylquinoline, 4 : 5′-methyl-2′-thiazylquinoline, 5 : 5′-methyl-2′-thiazylquinoline, 8 : 2′-thiazylquinoline, and some quinolyl-dicarbethoxy-dimethylpyrroles (Coates, Cook, Heilbron, Hey, Lambert and Lewis ; Coates, Cook, Heilbron and Lewis). Ethyl 4 : 8′-quinolyl-2 : 6-dimethyl-dihydropyribine-3 : 5-dicarboxylate was adsorbed as a yellow zone and eluted with benzene + alcohol (Cook, Heilbron and Steger).

Xanthurenic acid (4 : 8-dihydroxyquinoline-2-carboxylic acid, $C_{10}H\ O_4N$) was isolated from the urine of pyridoxine-deficient rats (Lepkovsky and Nielsen ; Lepkovsky, Roboz and Haagen-Smit). The yellow compound was adsorbed from the NaCl-saturated urine on a column composed of cotton or paper pulp. The other urine constituents were first washed out with saturated NaCl solution ; finally, the pigment appeared in the filtrate and, upon concentration, sodium xanthurenate crystallized out. This salt was separated from NaCl by recrystallization. It forms a green iron complex which is more readily adsorbed on paper columns than the xanthurenate.

4 : 6-Diaminopyrimidine (Kenner, Lythgoe, Todd and Topham). An ice-cold solution of 38 g. of Na in 500 ml. of absolute methanol was added to 140 g. of malondiamidine dihydrochloride. The NaCl was filtered off, the solution was evaporated in vacuo, and the residue was washed by decantation with 150 ml. of dry benzene. Ethyl formate (500 ml.) was then added ; the mixture was refluxed for several hours and evaporated. The crude product (88 g.) could not be purified by crystallization or sublimation. Its alcoholic solution was filtered through activated alumina and the adsorbate was then washed well with alcohol. The residue (left on evaporation of the combined liquids) yielded upon recrystallization from alcohol 39 g. of colourless needles, m.p. 267–8°.

6-Amino-4-*d*-xylosidamino-pyrimidine-I from 4 : 6-Diaminopyrimidine and *d*-Xylose (Kenner, Lythgoe and Todd). A solution of 40 g. of diaminopyrimidine and 20 g. of xylose in 1 *l.* of absolute alcohol was refluxed for 22 hours with

8 ml. of saturated alcoholic HCl solution. Absolute benzene was added at intervals and water removed through a 50-cm. Fenske column. The solution was chromatographed on 1·6 kg. of activated alumina and the unchanged diaminopyrimidine was washed quantitatively into the filtrate with 8 *l.* of absolute alcohol. The condensation product was eluted by washing with 9 *l.* of water until the Molisch reaction became faint. The solution was evaporated in vacuo to 100 ml. The product separated overnight in colourless prisms (3·5 g.), which after recrystallization from water, had the m.p. 207° (decomp.); $[\alpha]_D^{19} = +154°$.

The crude condensation product can be coupled with diazotised 2 : 5-dichloroaniline, acetylated in pyridine, and adsorbed from ethyl acetate on alumina. Development with ethyl acetate differentiates a top zone (tarry), a middle yellow zone (sugar free), and a yellow bottom zone of acetylated azoglycosides. Elution of the latter with ethyl acetate and recrystallization from benzene yielded 6-amino-4-triacetyl-*d*-xylosidamino - 5 - (2′ : 5′ - dichlorobenzeneazo) - pyrimidine - II (yellow needles). After hydrolysis, the resulting pyrimidine derivative may be purified chromatographically.

For a similar treatment of the condensation product of 4 : 6-diamino-2-methyl-pyrimidine and *d*-xylose, see Baddiley, Lythgoe and Todd.

4-Amino-6-*d*-ribosidamino-pyrimidine (Baddiley, Kenner, Lythgoe and Todd). The reaction product from 30 g. of 4 : 6-diaminopyrimidine and 15 g. of ribose was adsorbed on activated alumina (1·8 kg.); the unchanged starting material was removed with 7 *l.* of absolute alcohol, and then the riboside with 10 *l.* of water. In order to demonstrate the structure of this glucoside, one can, after acid hydrolysis, neutralization, evaporation and dissolution in alcohol, chromatograph on alumina. Washing with alcohol removes the pyrimidine, and the sugar can then be eluted with water and identified as *d*-ribosazone.

4 - Amino - 6 - triacetyl - *d* - ribosidamino-5-(2′ : 5′-dichlorobenzene-azo)-pyrimidine was purified by developing with ethyl acetate on alumina.

Purification of **6-amino-4-triacetyl-*d*-xylopyranosidamino-5-thioformamido-pyrimidine** (Kenner and Todd). The crude product was adsorbed from 25 ml. of ethyl acetate on neutral alumina (30 g., diam., 2·5 cm.) and, after impurities were washed down with ethyl acetate (50 ml.) and with chloroform (50 ml.), the pure thioformamido derivative was eluted with 50 ml. of pyridine. It crystallized from alcohol (500 mg.). The same procedure was applied to other acetylated thioformamido-glycosides.

The separation of minute amounts of **purines** present in nucleic acid hydrolysates was carried out (Vischer and Chargaff) by using one-dimensional partition chromatography on 50 cm. long paper strips on which the guanine, adenine and xanthine spots were located as follows. The ether washed paper was sprayed with *M*/4-mercuric nitrate in *N*/2-nitric acid, washed with the acid and with water and finally treated with ammonium sulphide. Black HgS-spots were formed.

For uncharacterized condensation products of 1 : 4-naphthaquinone and *o*-phenylene-diamine, cf. M. Koller (3).

Pyridyl-acridines were synthesized by Cook, Heilbron and Spinks by cyclizing pyridyl-diphenylamine-carboxylic acids and reducing the acridones formed. For example, the crude reduction product from 3 g. of 3-*a*-pyridylacridone was chromatographed in benzene on alumina (28 × 1·3 cm.). The lower yellow zone was eluted with benzene containing 10 per cent ether and yielded upon evaporation 0·8 g. of the corresponding acridine (m.p. 140°). For some *bromo*-acridines and bromo-9-succinimidyl acridines, see Schmid and Leutenegger.

Resolution of Tröger's Base into its Antipodes (Prelog and P. Wieland, p. 11). For this purpose, lactose-hydrate, $C_{12}H_{22}O_{11}\cdot H_2O$, was used as adsorbent. It can be prepared as follows: 5 kg. of a finely divided crystalline sample was extracted with two 3-*l.* portions of freshly distilled chloroform for 5 minutes. The hot mixture was filtered on a Buchner funnel and washed with 4 *l.* of hot chloroform. After drying in vacuo at 85° for 15 hours, this sugar was found to be inactive as an adsorbent but after 80 hours' milling in an iron ball mill,

rubbing in a mortar, sifting through a 250-mesh sieve, and drying in vacuo at 75° for 8 hours, the sample became highly active. A suspension of the material (2·7 kg.) in light petroleum was used for the preparation of a column, 88 × 7·6 cm. After washing with light petroleum (distilled over sodium), the solution of 6 g. of Tröger's base in 0·5 *l.* of light petroleum was introduced and the chromatogram was developed with the same solvent at the rate of 300 ml. per hour. The rotations observed in the fractions of the filtrate were :

Fraction (ml.) :
250 150 150 150 150 300 450 600 1200 4100 7350 17750
$[\alpha]_D$:
+75° +41° +26° +7° +1·5° −2·5° −8° −17° −25° −30° −35° −52°

The same column can be used several times.

The fractions with a high optical rotation were systematically recrystallized from the mother liquors of the fractions with a low rotation, whereby the least soluble racemate crystals appeared first. Thin needles of the racemate could be easily differentiated from the stout prisms of the two antipodes.

The final values obtained were : + 279° and −272°. Structure : 1,2'-methylene-3-ρ-tolyl-6-methyl-1 : 2 : 3 : 4-tetrahydroquinaxoline.

Separation of two Diastereoisomeric N-Benzoylcycloheptano-2:3-pyrrolidines (Prelog and Geyer 1; p. 11). A separation of the two isomers (α, m.p. 109° and β, m.p. 86–87°) could be achieved only by chromatography of the mixture of their N-benzoyl derivatives (3·6 g.) on alumina (activity I-II ; 110 g.). The following liquid chromatogram fractions were obtained (each number designates 150 ml. of solvent) :

No. 1–6	Benzene :	2·0 g.,	m.p. 107 –108·5°	: α-isomer
7		0·09	104 –105°	: mixture
8		0·08	100 –102°	,,
9		0·07	89 – 95°	,,
10		0·06	87 – 89°	,,
11		0·05	80 – 86°	,,
12		0·05	87·5– 88·5°	,,
13	Ether :	0·17	71 – 77°	,,
14		0·21	71 – 73°	,,
15		0·16	72 – 80°	,,
16–19		0·36	82 – 86°	β-isomer
20–25		0·19	73 – 78°	mixture

Isolation of 1 : 3 : 5-Trimethyl-4-carbethoxy-2-bromopyrrole, after bromination of the corresponding di-N-methylmethane (Brunings and Corwin). The bromination product from 3g. was dissolved in 300 ml. of dry CCl_4, chromatographed by pressure on activated alumina (— 80 mesh ; 15 × 1·2 cm.) and washed with 100-ml. portions of the solvent. Fraction I contained pure, crystallizable bromopyrrole (290 mg.), II contained 300 mg. of a less pure sample, and III an impure aldehyde (150 mg.).

Separation of 1-Phenyl-4-(*p*-methylbenzeneazo)-5-(*o*-hydroxyphenyl)-3-pyrazolone from the corresponding 5-Pyrazolone (Huebner and Link). A 2 per cent benzene solution was adsorbed on alumina and developed with benzene containing 2 per cent ethanol. The red zone of the 3-compound occupied the top position and could be eluted with benzene + ethanol (1 : 1). 1-Phenyl-3-(*o*-hydroxyphenyl)-4-benzeneazo-5-pyrazolone and its O-monomethyl ether were separated in a similar manner.

Indole from the Alkali Fusion Mixture of Erythratine (Folkers, Koniuszy and Shavel). 8·3 g. of erythratine was slowly added to 50 g. of stirred, molten KOH ; the melt was heated for another 10 min. and then poured on to 300 g. of ice. The solution was extracted continuously with ether for 10 hours and the extract was concentrated to a small volume by distillation through a Widmer column to minimize losses of indole. After a concentration at 10° at a somewhat reduced pressure, the solution of the gummy residue in 5 ml. of ether was filtered through Brockmann's alumina (3 × 1 cm.) and developed with 25 ml. of ether. Brown decomposition products were adsorbed at the top (2 mm.). After the addition of 5 ml. of light petroleum and some concentration, the colourless filtrate deposited 163 mg. of pure indole.

3-Carbethoxy-3-(3'-methyl-2'-quinoxalyl)-indole or a similar compound can be purified by adsorbing the impurities on alumina from an alcohol solution (A. H. Cook and Naylor).

TABLE 19

EXAMPLES OF HETEROCYCLIC NITROGEN COMPOUNDS PURIFIED ON ALUMINA, MOSTLY FROM BENZENE OR LIGHT PETROLEUM SOLUTION

Compound	*Literature*
2-Pyridyl-naphthalene . . .	Elks and Hey
Pyridyl-diphenyls (3-β-, 3-γ-, 4-α-, 4-β-, 4-γ-)	Heilbron, Hey and Lambert
6-γ-Pyridyl-quinaldine . . .	Coates, Cook, Heilbron, Hey, Lambert and Lewis
4'-Aza-1 : 2-benzanthracene . .	Fieser and Hershberg (11)
Aza-retene	Ruzicka and Sternbach (4); Ruzicka, Sternbach and Jeger
20-Methyl-4-azacholanthrene . .	Fieser and Hershberg (11)
9 : 10-Dihydro-3 : 4-benz-5 : 7-diazaphenanthrene	J. W. Cook and Thomson
Triazene (from 5-amino-6-methoxyquinoline)	Coates, Cook, Heilbron, Hey, Lambert and Lewis
1-β-Naphthyl-3 : 3-dimethyltriazene .	Elks and Hey
4 : 6-Dihydroxy-cyclopentadeceno-2 : 3-pyridine-5-carboxylic acid ethyl ester	Prelog and Geyer (2)
6-Hydroxy-5-cyano-cyclopentadeceno-2 : 3-pyridine	Prelog and Geyer (2)
8-γ-Chloropropylamino-6-methoxyquinoline	Crum and Robinson
8-γ-Ethylaminopropylamino-6-methoxyquinoline	Crum and Robinson
8-γ-Propylaminopropylamino-6-methoxyquinoline	Crum and Robinson
8-γ-*n*-Heptylaminopropylamino-6-methoxyquinoline	Crum and Robinson
8-γ-Benzylaminopropylamino-6-methoxyquinoline	Crum and Robinson
8-β'-Phenylisopropyl-γ-aminopropylamino-6-methoxyquinoline	Crum and Robinson
8-γ-Cyclohexylaminopropylamino-6-methoxyquinoline	Crum and Robinson
8-β'-Aminoethyl-γ-aminopropylamino-6-methoxyquinoline	Crum and Robinson
8-γ'-Aminopropyl-γ-aminopropylamino-6-methoxyquinoline	Crum and Robinson
4-Veratryl-3' : 4' : 3'' : 4''-tetramethoxy-1 : 4 : 5 : 8-tetrahydro-[1' : 6' : 2 : 3 : 1''' : 6''-6 : 7-dibenzoquinolizine]	Sugasawa, Kakemi and Kazumi

TABLE 19—*continued*

EXAMPLES OF HETEROCYCLIC NITROGEN COMPOUNDS PURIFIED ON ALUMINA, MOSTLY FROM BENZENE OR LIGHT PETROLEUM SOLUTION

Compound	*Literature*
1-Nitrocarbazole	Preston, Tucker and Cameron
5- and 7-Bromo-tetrahydrocarbazole .	Barclay and Campbell
3-Cyano-1-methyl-4-pyrrolidone . .	A. H. Cook and Reed
3 : 4-Dichloro-3'-methyl-4'-ethyl-5-carbethoxy-pyrrolketone	Fischer and Gangl
1'-Hydroxy-1 : 3 : 6-trimethyl-2 : 5-diethyl-tripyrryl - (2' : α : 4 : β) - diene-4-propionic acid methyl ester	Plieninger and Lichtenwald
4 : 4'-Dimethyl-3 : 3'-diethyl-5 : 5'-diaminopyrromethene	Fischer and Guggemos
Coloured iminazolidines . . .	Todd and Whittaker

COMPOUNDS WITH HETEROCYCLIC OXYGEN

Caldwell and Jones chromatographed on alumina a benzene solution of Indian lime oil (*Citrus aurantifolia*). Washing with benzene first carried some gum into the filtrate and then *limettin*. Elution with benzene + chloroform resulted in the isolation of a little isopimpinellin. The sediment-free lime oil was freed from volatile components by distillation up to 88° (4 mm.). The non-volatile residue desposited from methanol crystals which, when chromatographed on alumina from benzene, yielded a bluish fluorescent main zone of 7-*methoxy*-5-*geranoxy-coumarin*, $C_{20}H_{24}O_4$.

The acetates of 4-methyl-5-hydroxy-7-methoxy-coumarin and of 4-methyl-7-hydroxy-5-methoxy-coumarin were purified by Schmid (Brockmann's alumina, liquid chromatogram with benzene + chloroform, ether + chloroform, and methanol containing mixtures).

The mother liquors from the preparation of 5-hydroxy-3-palmitoyl-4 : 6 : 7-trimethyl-isocoumaranone were chromatographed on alumina from light petroleum benzene (10 : 1) by Bergel, Jacob, Todd and Work. The main bluish fluorescent zone yielded, on elution with a 1:4 ether + methanol mixture, 5-hydroxy-4:6:7-trimethyl-2-*n*-pentadecyl-coumaranone.

5-Hydroxyflavone from the Natural Cooling of Primula imperialis (Karrer and Schwab). The flour-like powder was extracted with light petroleum and then with

ligroin ; concentration of the extract yielded impure crystals of flavone + 5-hydroxyflavone. The benzene solution of the mixture was chromatographed on alumina and the flavone was washed through. The hydroxy compound formed a yellow main zone (lake) which did not migrate upon development but could be eluted by boiling HCl in acetone. The residue left on evaporation was recrystallized from ligroin ; m.p. 157°.

3:5:7:3':4'-Pentahydroxyflavone-3-rutinoside was isolated from tomatin concentrates by Fontaine, Ma, Poole, Porter and Naghski (starch column, butanol saturated with water).

Active Principles of Marihuana. A short remark on the possibility of purifying active oils obtained from hemp was made by Powell, Salmon, Bembry and Walton ; however, more detailed data were published earlier by Work, Bergel, and Todd. These authors *p*-nitrobenzoylated a crude product obtained from Indian hashish (*Cannabis indica*). The non-crystallizable portion (8 g.) was adsorbed from light petroleum (b.p. 40–60°) on a 45 × 5 cm. activated alumina column (Merck) which had been previously washed with phenol (in light petroleum) in order to reduce its alkalinity. Upon developing with 4 *l.* of light petroleum, ultraviolet light revealed six zones some of which were coloured. Each was eluted with ether + methanol (4 : 1) and hydrolyzed. Zones 3 and 4 (from the top) showed the convulsant effect of cannabinol, while the substance producing corneal anaesthesia accumulated in the subsequent zones. The latter, like cannabinol, gave a positive Millon reaction.

It was also possible to hydrolyze the non-crystallizable nitrobenzoate mixture first and then chromatograph after high vacuum distillation. For example, 2 g. in 300 ml. of light petroleum (b.p. 60–80°) were developed with 750 ml. of the same solvent and then with 1 *l.* of light petroleum + ether (1 : 1). Of the four bands that appeared in ultraviolet light, only the bottom one gave a positive Gayer test. It was combined with the filtrate, and developed on a 30 × 2 cm. column with light petroleum ether + 5% ether. Five zones appeared. The oil from No. 4 (50 mg.) showed typical cannabinol action ;

No. 2 (60 mg.) caused the rabbits to die in sleep without convulsions, whilst zone No. 3 showed both effects.

Preparation of Cannabinol *p*-nitrobenzoate (Jacob and Todd). A crude, distilled resin (28·4 g.) was acylated in two portions with *p*-nitrobenzoyl chloride in pyridine; the product was dissolved in 300 ml. of light petroleum (b.p. 80–100°) and kept over night at 0°. The solution was decanted from a thick oil that deposited. The latter was chromatographed on alumina (Merck or Birmingham Electric Furnaces Co.) from benzene + light petroleum (b.p. 40–60°) (3 : 7), in two portions and washed with the same solvent until no further solid came through. The filtrate yielded pure *p*-nitrobenzoate upon repeated crystallization from alcohol (5 g.; m.p. 159–160°).

A highly potent **tetrahydro-cannabinol,** $C_{23}H_{32}O_3$, has been isolated by Wollner, Matchett, Levine and Loewe from "red oil" derived from charas of Indian origin. The starting material was obtained by alcoholic extraction, evaporation, treatment with light petroleum, filtration, vacuum-distillation, acetylation, and fractionation in a high vacuum. A 2 per cent benzene solution (1,250 ml.) was washed through silica gel (150–200 mesh; 50 × 3·5 cm.) with 700– 800 ml. of benzene. (The column retained cannabidiol.) There followed a chromatographic filtration through alumina (150 g. per 30 g. of material of alorco, Grade A, 150–200 mesh + hyflo supercel 2 : 1, washed with 700 ml. of carbon tetrachloride), and then a similar adsorption from light petroleum. After a fractional distillation in high vacuum, the best fractions were again filtered through alumina + supercel. The colourless oil showed the expected composition.

***d*-Tetrahydrocannabinol *l*-menthoxyacetate,** obtained by the menthoxyacetylation of a pulegone-olivetol condensation product, was purified by Leaf, Todd and Wilkinson by filtration of the light petroleum solution through activated alumina and evaporation. The originally reddish oil was thus converted into a colourless material which was dissolved in alcohol; this solution deposited crystals in the ice-chest.

Compounds with Heterocyclic Sulphur

***a*-Polythienyls.** C_4H_3S. $(C_4H_2S)_n$. C_4H_3S (Sease and author). The reaction product from 14·4 g. of *N*/2-iodothiophene and 9 g. of copper bronze was extracted with cold CS_2, and as much as possible of the evaporation residue was dissolved in light petroleum (b.p. 60–70°) (Fraction A). The remainder of the material contained mainly members with $n \geq 2$. Fraction A (62 ml.) was developed on a 21 × 7 cm. alumina column (Alorco, Grade F, 80 mesh, reground to 200 mesh + 10 per cent celite 535) with light petroleum + 3 per cent acetone (figures on the left denote height of zones in mm.) :

25 several brownish zones (no fluorescence).
20 faint yellow, bluish fl. : unknown.
10 colourless, no fl.
15 faint yellow, bluish fl. : unknown.
35 interzone.
25 faint yellow, bluish-green fl. : quaterthienyl.
40 interzone.
2 yellow-bluish fl. : terthienyl.
40 empty bottom zone.
Filtrate : colourless, no fl. : bithienyl.

The *quaterthienyl* was eluted with methanol containing a little CS_2, and the evaporation residue was crystallized from CS_2 and light petroleum. Yield, 10–11 mg. The *terthienyl* zone was eluted with ether and its residue crystallized from methanol ; yield, 400 mg. The main chromatographic filtrate was concentrated to 5 ml. and yielded, upon the addition of 2 vol. of methanol, 2·45 g. of *bithienyl*.

Similar methods can be applied to the mixture formed by the interaction of 5-iodo-5'-methyl-2,2'bithienyl and 2-iodothiophene. 1 : 8-Dibromoterthienyl can be purified by washing its adsorbate with benzene + light petroleum 10 : 1.

a-Terthienyl, as obtained from certain varieties of marigold (*Tagetes erecta*), was purified by developing on alumina with light petroleum and eluting the main fluorescent zone with ether. This natural product did not separate from synthetic *a*-terthienyl on the column (author and Sease) but was adsorbed below its dimethyl or monomethyl derivative.

A macrocyclic **dithiazolyl** compound was submitted to liquid chromatography on alumina by Erlenmeyer and Degen.

CHAPTER XVII

ALKALOIDS

(cf. also Drugs and Galenicals, p. 265)

The adsorption isotherms of a number of alkaloids on activated silica gel were determined by Gyani and Ganguly; some data concerning steam-activated charcoal were reported by Drevon. A brief reference was made by Catch, Cook and Heilbron to the separation of alkaloids by a kind of partition chromatography using silica gel with which is incorporated a solution of sodium hydrogen sulphate.

A method for the systematic separation of different types of alkaloids still has to be worked out. For the estimation of alkaloids, see also p. 265.

Cinchona Alkaloids. In a short communication Applezweig suggests the use of ion exchange adsorbents for the isolation of such alkaloids. When 0·033 *M*- and 0·0033 *M*-solutions of quinine in 1% sulphuric acid were run at rates of 5 and 50 ml./min. respectively through a 20-cm. "Zeo-Karb" column (The Permutit Co., N.Y.), it was found that the capacity of a 200-ml. bed was 7–8 g. before breakthrough occurred. Ammoniacal alcohol liberates the alkaloid from the column. After the exchanger has been used once or twice, the recovery becomes almost quantitative. Such a procedure could possibly be used for the recovery of other alkaloids which are contained in mother liquors, after precipitation with alkali.

Opium Alkaloids. Preliminary experiments by Levi and Castelli showed that a separation of some opium alkaloids in 90 per cent ethanol solution on calcium carbonate is possible (fluorescence). Relative adsorption affinities are: papaverine > narcotine; morphine > narcotine; morphine > codeine. Attempts to work out a more general method for the separation of all the main opium alkaloids were not successful.

2-Amino- or 1-bromocodeine were purified by filtration through activated alumina (Ochiai and Nakamura).

Strychnos Alkaloids. Isostrychnine and isovomicine can be purified by filtering the acetone solution through alumina (Wieland and Jennen ; Wieland and Huisgen). Some degradation products of strychnine were chromatographed on alumina by Prelog and Szpilfogel (2). For strychnine acetic acid cf. Pausacker and Robinson. The toxiferins contained in the bark of *Strychnos toxifera* were precipitated as reineckates and chromatographed on alumina from acetone in a similar manner to the calebasse alkaloids (see below, Wieland, Bahr and Witkop).

Calebasse Alkaloids. The calebasses are used in some areas of South America as sources of arrow poison. They contain water soluble, strongly curare-active alkaloids of quaternary character. A convenient method for the isolation is the precipitation of the reineckates and chromatography of their acetone solution on Brockmann's alumina (Wieland and Pistor ; Wieland, Pistor and Bähr ; Wieland, Bähr and Witkop). In this manner the curarins I, II and III were obtained. About 40 g. of the adsorbent and 100 ml. of acetone per gram of reineckate are necessary. Curarin I reaches the filtrate first. The quality and quantity of the alkaloids seems to vary considerably.

The reineckate method was further developed by Karrer and Schmid as well as by Schmid and Karrer (1, 2). They also found that the reineckates can be conveniently converted into the picrates. The latter yield the chlorides (or iodides) if an aqueous-acetone solution (1 : 1) is filtered through a Wofatit M column which has been loaded with chlorine (or iodine) ions. The main alkaloid was found to be identical with the curarin I of Wieland *et al.* For minor bases and further experimental details, see the original papers.

Ergot Alkaloids. *d-Lysergic acid d-propanolamide-*(2) *and d-Isolysergic acid d-propanolamide-*(2)(Stoll and Hofmann 2). After rearrangement by treatment with alkali, an amorphous condensation product of *d*-isolysergic acid and *d*-alaninol was developed with chloroform + 1% alcohol on Brockmann's alumina ; the

progress of the chromatogram was followed in ultraviolet light. The rapidly moving zone gave, after elution and evaporation, *d*-isolysergic acid *d*-propanolamide-(2) which crystallized from acetone directly, while the top zone was eluted with alcohol and could be crystallized in the form of its acid oxalate (*d*-lysergic acid *d*-propanolamide-(2)).

In a similar manner *d-lysergic acid d-nor-ω-ephedride* (top) and the corresponding iso-compound (bottom zone) were separated. The separation of a mixture of *d*-lysergic and *d*-isolysergic acid diethylamides, however, required repeated chromatographic procedures.

Cinchona Alkaloids. A metabolic quinine derivative, viz. laevorotatory 2′-hydroxy-6′-methoxy-3-vinylruban-9-ol, obtained by the action in vitro of rabbit liver on quinine, was adsorbed by Mead and Koepfli from chloroform on calcium carbonate (Merck, heavy powder). On developing with chloroform + 2% ethanol, a single zone showing fluorescence in ultraviolet light migrated slowly through the column.

The reaction products of *quinamine* and nitrous gases (in alcohol) were separated by Kirby into a nitro-nitroso-quinamine, the benzene solution of which passed through alumina and nitroquinamine which was retained (elution, benzene + methanol).

Colchicum Alkaloids. Colchicine, which mostly forms an amorphous yellow powder, can be readily obtained in pure crystals as follows (Ashley and Harris). A column (15 × 1 cm.) of B.D.H. " chromatographic alumina " was moistened with benzene. A solution of 5 g. of colchicine in 50 ml. of chloroform was washed down with 100 ml. of $CHCl_3$. After evaporation the residue was triturated with a little dry ether. Yield, 4·75 g. of practically colourless colchicine ; on recrystallization from ethyl acetate, it had m.p. 155°. The column retained some brown material.

Some oxidation products of deamino-colchinol methyl ether were purified by adsorption an alumina from benzene + ligroin (1 : 3). On developing with benzene, 2 : 3 : 4 : 7-tetramethoxy-phenanthraquinone was isolated from a red upper zone (Barton, Cook, and Loudon).

Astragalus Alkaloids. Two very similar bases, α- and β-earleine which occur in some " loco weeds ", were separated by Pease and Elderfield by chromatographing the picrates on alumina from 95% alcohol + benzene (1 : 2). The α-compound occupied the top position. The picrates were eluted with hot alcohol.

Belladonna Alkaloids. In selecting the adsorbent, the possibility of a hydrolysis of atropine should be considered; bolus alba or magnesia usta cannot be used (Schloss).

Lycoris Alkaloids. Kondo and Ikeda report that hydrolycorenine or acetyllycorenine in benzene can be purified by chromatography (the adsorbent is not mentioned).

Erythrophleum Alkaloids. Coumingin, an alkaloid of *Erythrophleum Couminga* bark cannot be freed from contaminants by recrystallization. However, according to Ruzicka, Dalma and Scott, chromatography of 11 g. of crude product in 44 ml. of benzene on 300 g. of activated alumina is satisfactory. The column was first washed with benzene which eliminated traces of impurities, whilst further development with benzene + ether (1 : 1) yielded 7·94 g. of the pure alkaloid. On the column remained 2·45 g. of a yellowish substance which could be eluted with acetone but not with ether.

The oxime of coumingic acid methyl ester was purified by filtration through alumina (benzene).

Erythrina Alkaloids, which are widely distributed in a number of species, can be best isolated by chromatography (Folkers and Shavel). They were able to resolve " erysocine " into erysodine and erysovine. For 1 g. of such a mixture, a 30 × 1·9 cm. Merck alumina (Brockmann) column should be used. Since the fluorescence is weak, the liquid chromatogram method was adopted. Chloroform solutions were generally used, but ethanol was a better developer in some instances, especially for erysodine. A certain amount of residual alkaloid can be recovered by extraction of the column with chloroform for several hours. However, most of the alkaloid remained in the adsorbent. Occasionally, the adsorption was so strong that very little material passed into the filtrate; and, in this event,

the column was cut empirically and each portion was eluted separately in the extractor. If water, ethanol or morpholine pass through the alumina, cloudy liquids may result. The residue left on evaporation is then taken up in chloroform and filtered through a 2–3-cm. alumina layer.

Resolution of "Erysocine". A 162-mg. sample of the mixture in 5 ml. of chloroform was developed on a 15 × 1 cm. column with 40 ml. of chloroform. The first fraction yielded 56 mg. of erysovine (after recrystallization from ether, m.p. 178–178·5°, $[\alpha]_D^{25} = +230°$). Elution of the alumina in the extractor yielded 108 mg. of alkaloids which gave, after recrystallization from alcohol, 12 mg. of erysodine; m.p. 200–1°, $[\alpha]_D^{25} = +248°$.

Isolation of Erysovine on a Preparative Scale. The crude mixture (43 g., m.p. 169–172°) was dissolved in 150 ml. of chloroform, and the solution was poured on to a 92 × 4 cm. column, which was developed with chloroform. The following yields refer to recrystallized products. First fraction (0·5 *l.*): 14 g.; second fraction (1 *l.*): 12 g.; and third fraction (2 *l.*): 4·5 g. of erysovine.

The purity of *erysonine* samples was checked by chromatographing on Brockmann's alumina from morpholine or methanol + chloroform (1 : 1) solution; this eliminated a minor top zone (decomposition product).

Erysodine and *erythratine* separate when the alumina column is washed with chloroform; erysodine appears in the filtrate first (Deulofeu, Labriola, Hug, Fondovila and Kaufmann).

Anabasis Alkaloids (Späth, Galinovsky and Mayer). A mixture of the high-boiling bases was adsorbed from light petroleum on alumina and the column was washed with benzene. These washings contained crude, crystallizable aphyllidin, which was purified by rechromatographing. Subsequent washing of the column with benzene + methanol removed an oil which contained aphyllin as well as some other compounds.

Sabadilla Alkaloids (Ikawa, Dicke, Allen and Link). The alkaloid mixture (3 g.) obtained from the insecticide seeds (*Schoenocaulon* sp.) was adsorbed from benzene on alumina (24 × 2·2 cm.). The column was exhaustively washed with benzene and then with chloroform. From the benzene filtrate cevadine and from the chloroform fraction veratidine were isolated in the form of their diliturates.

Aconite Alkaloids. Amorphous staphisine, contained in the mother liquors from delphinine, could be crystallized after it had been washed out from its alumina adsorbate with large volumes of benzene (Jacobs and Craig).

Dehydrogenation products of atisine, staphisine and hexahydrobenzoyl-oxodelphonine acetate: Huebner and Jacobs (1, 2); Jacobs and Huebner (1, 2).

Veratrine Alkaloids. Selenium dehydrogenation products of germine, jervine and cevine can be fractionated on alumina (isohexane) (Craig and Jacobs, 1–3; Jacobs, Craig and Lavin). Dihydro-jervine: Jacobs and Huebner (2).

Ipecacuanha Alkaloids. Emetamine, obtained from emetine by Ahl and Reichstein, was purified on alumina (benzene+ether 19 : 1).

Alstonia Alkaloids. For the separation of the bases obtained by alkaline fusion of alstonine or tetrahydro-alstonine on alumina, cf. Leonard and Elderfield.

Amanita Toxin. Phalloidin can be purified by filtering an absolute alcoholic solution through Brockmann's alumina and washing with the same solvent. Further portions are recovered by eluting the lower, uncoloured section of the column with 50 per cent aqueous methanol (Wieland and Witkop).

CHAPTER XVIII

VITAMINS

Vitamin A

Extensive activity is now being carried on in the field of this vitamin. As Karrer remarked in 1939, "in all newer investigations the chromatographic purification turned out to be far superior to that reached by crystallization." At present one can state that a combination of chromatography, crystallisation and high vacuum distillation seems to be most efficient for isolation purposes; cf. for example, Hickmann.

Early data were published by Willstaedt and Jensen and by Hoffman-La Roche & Co.

There are also observations available which "served as a warning against the blind use of adsorption columns" (Holmes and Corbet) in this field. When they developed a solution of 0·25 g. of crystalline vitamin A in 5 ml. of benzene + pentane (1 : 4) on activated alumina (100–150 mesh; 12 × 1·7 cm.) with a similar mixture, a yellow (10 mm.), a brown (2 mm.) and a reddish-brown (3 mm.) zone appeared. A calcium hydroxide suspension scarcely caused any change in benzene or chloroform solutions of vitamin A, but it induced a large increase in the intensity of the colour of pentane, cyclohexane, ether or methanol solutions, within 20 hours. Pure silica gel was inactive. A chemical interpretation of the formation of pigment was given by Meunier and Vinet (2, 4; cf. p. 18).

Müller observed that vitamin A, and especially its esters, were partially destroyed on an alumina somewhat more active than that needed for their adsorption (confirmed by Lederer, Tchen, Pénau and Hagemann; and by Reed, Wise and Frundt). Repeated chromatography of vitamin A, especially of small amounts, causes considerable losses, probably because of oxidation, as was pointed out by Karrer, Geiger and Bretscher.

That vitamin A (like the carotenoids) cannot be chromatographed on acid earths, follows from the observation of Kobayashi and Yamamoto as well as of Emmerie and Engel

(1) that vitamin A is adsorbed with the formation of a dark blue colour. A similar polyene reaction, which was confirmed by Mayer and Sobotka (1, 2), by Lowman (2) and by Kreider, and which was theoretically interpreted by Meunier, also occurs with diphenyloctatetraene, phytofluene etc. (author and Sandoval 1).

Estimation of Vitamin A by the Floridin Method (Awapara, Deuel, Mehl and Greenberg ; cf. Awapara, Mattson, Mehl and Deuel). This procedure is based on the fact that activated floridin removes vitamin A quantitatively from a benzene solution while other components of the non-saponifiable residue are not absorbed if the floridin layer is sufficiently thin. The difference in the extinction values at 332 mμ. of the initial solution and the filtrate is a measure of the A-content. The method can be applied (with some modifications) to margarine, butter, eggs, milk, poultry feed, etc., but not to materials rich in phytofluene or other compounds with a considerable extinction in the spectral region indicated. For carotene a correction should be taken.

About 5 g. of *margarine* are saponified for 30 min. with 5 ml. of 50 per cent aqueous KOH + 20 ml. of 95 per cent alcohol. The cooled mixture is made up to 80 ml. using water and alcohol (final ethanol concentration, 50 per cent) and is extracted with light petroleum (Skellysolve A) continuously for 4 hours. This extract is then washed 3 times with water, filtered through sodium sulphate (which is washed with light petroleum) and evaporated under nitrogen. The residue is taken up in 10 ml. of reagent grade benzene. A 4-ml. aliquot is filtered through a 4-mm. floridin layer and the column is washed with 15 ml. of benzene. The combined filtrates are made up to 25 ml. while a 4-ml. aliquot of the untreated solution is diluted directly to 25 ml. Vitamin A (I.U./g.) = $(E_{332} \times 62{\cdot}5) : (0{\cdot}0442 \times w)$ where w is the weight of the sample in grams and E_{332} the observed extinction difference at 332 mμ, as mentioned above.

For the estimation of vitamin A in fresh or dehydrated *egg yolk*, the method of Awapara, Deuel, Mehl and Greenberg

(see above), can be used, but it is necessary to correct for xanthophylls and β-carotene, by subtracting from the reading at 332 mμ 14 per cent of the value obtained in the untreated benzene solution at 455 mμ.

Quantitative Separation of Vitamin A Alcohol, Vitamin A Ester and β-Carotene (Müller 3). On the Tswett column, free vitamin A is easily separable from its esters, whereas the esters are only slightly more strongly adsorbed on alumina than β-carotene. An effective separation of β-carotene and the esters therefore requires a previous saponification. Impure commercial mixtures can best be handled as follows : A sample is first saponified and then chromatographed, giving the total vitamin A content (alcohol + esters) in the form of vitamin A alcohol and, in a second zone, β-carotene. Another sample is chromatographed directly, yielding a zone of the unesterified vitamin A alcohol, ready for estimation.

The columns used contained three sections, each consisting of alumina, activated in different ways and standardized by Müller's calorimetric method (p. 30). In the top section, which contains the weakest adsorbent of the three, those substances are retained that have higher adsorbability than the component to be estimated. The latter is quantitatively adsorbed in the middle section. The lowest section adsorbs the total remaining material and plays no part in the estimation. The following figures refer to Q=calories of heat production by which the three samples of alumina (from top to bottom) are characterized. For the separation of vitamin A alcohol : 5·0, 10·0, 83·5 (35 g. of each) ; for the estimation of vitamin A ester : 50·0, 56·5, 83·5 (20 g., 40 g., 40 g.) : and for β-carotene ; 50·0, 54·0, 83·5 cal. (20 g., 40 g., 40 g.). It is also possible to use columns composed of five different sections ; such sections should be separated by filter paper discs. The adsorption was carried out in a 30 × 2·5 cm. tube protected from air by N_2 during the experiment. The vitamin must be present in quantities of at least 150 I.U./g. and the carotene in quantities of 10 μg per g. of starting material. Maximum amounts : 30,000 I.U. and 600 μg.

Some of Müller's methods were also used by Lederer, Tchen, Pénau and Hagemann. According to these authors, if a light petroleum solution of *fish liver oil* is filtered through alumina, and the column washed with the same solvent, the esterified vitamin passes quantitatively into the filtrate, whilst free A-alcohol and some of its destruction products are adsorbed.

Reed, Wise and Frundt washed the vitamin A ester content of their sample through alumina with ethylene dichloride and deducted its Carr-Price value from the total value found before chromatography in order to estimate the vitamin A alcohol content (cf. Marcussen ; Heimann).

Similar estimations were carried out in *milk* or *colostrum* (Parrish, Wise and Hughes) and in blood serum (Hoch.) Glover, Goodwin and Morton give directions for separations on defatted bone meal. The vitamin A esters were washed through with light petroleum + chloroform (40 : 1), and the free vitamin was eluted with pure acetone. (Vitamin A alcohol is remarkably unstable when left adsorbed on bone meal for a longer period of time.)

The methods for vitamin A estimation in *margarine* were recently surveyed by Wilkie (cf. p. 221). For *butter* cf. also Ellenberger, Guerrant and Fardig (cf. p. 221).

Thompson, Ewan, Hauge, Bohren and Quackenbush developed the unsaponifiable residue of *dried whole eggs* with pure benzene + light petroleum (3 : 2) on calcium hydroxide and Hyflo Supercel (3 : 2) and allowed the β-carotene, cryptoxanthin, vitamin A, lutein and zeaxanthin zones to differentiate. The provitamins were cut out and estimated separately, after which the Carr-Price value of the combined provitamin A and vitamin A fractions was taken.

In the analysis of *mixed feeds* Brew and Scott filtered the saponified, dry light petroleum extract through dibasic calcium phosphate which retained vitamin A without destruction but allowed the carotenes to pass into the filtrate when washed with Skellysolve F; Elution with ether. cf. Cooley et al.

Synthetic Vitamin A. According to Karrer and Rüegger (1), the condensation product from β-ionylidene-acetaldehyde and β-methylcrotonaldehyde (Kuhn and Morris) yields a very complicated polyene mixture, which can be separated into its components from light petroleum on fibrous alumina. Possibly, vitamin A occurs in the chromatogram, but its zone may be obscured by another compound.

Partial purification of synthetic vitamin A acetate (Isler, Huber, Ronco and M. Kofler). The crude product (40 g.; degree of purity, 25 per cent) contained in 100 ml. of light petroleum, was filtered at −5° through 3·5 kg. of neutral, slightly activated alumina. Its zone was recognized by the intense yellow-green fluorescence in ultraviolet light. The best fractions of the liquid chromatogram gave a pure cornflower-blue Carr-Price reaction while the preceding and subsequent portions were not of a pure blue colour. Yield, 10 g. oil; degree of purity, 50 per cent. If converted into the anthraquinone-β-carboxylic ester, the crude product can be purified by filtration through deactivated alumina.

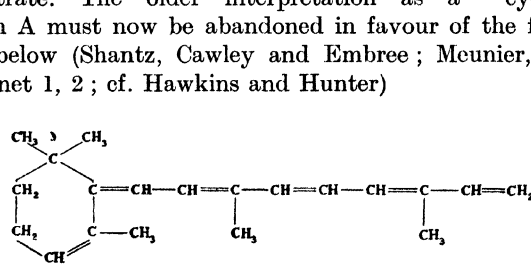
Neovitamin A. The vitamin A molecule possesses two sterically unhindered double bonds and may assume four *cis-trans* isomeric forms. One of these forms, termed neovitamin A, was recently isolated by Robeson and Baxter (2) from the " non-crystallizable " vitamin A fraction of some fish liver oils. Neovitamin A concentrates can be purified as follows:

11·2 g. of material in 100 ml. of light petroleum (b.p. 30–65°) were adsorbed on a 25 × 5 cm. Doucil column (225 g.; sodium aluminium silicate; American Doucil Co., Philadelphia, Pa.) and was developed with 1 *l.* of the same solvent. (The filtrate contained some impurities, including anhydrovitamin A.) The yellow zone, 15 cm. long, which was located at the top of the column was removed in sections. The ratio, neovitamin A : vitamin A, increased from the upper to the lower end of this zone, the bottom 2-cm. section of which yielded, upon elution with ether, 2·7 g. of a material in which the ratio mentioned was 88 : 12. From this product the *p*-phenylazobenzoate was

prepared, and from the latter, after recrystallization, the neovitamin A (m.p. 58–60°, $E^{1\%}_{1\,cm.}$ at 328 mμ = 1645. It seems to show lower adsorbability than ordinary vitamin A.

Vitamin A_1 aldehyde (Axerophthal), $C_{20}H_{28}O$. The preparation of this aldehyde was announced by Hunter and Hawkins and it was described by Hawkins and Hunter. Vitamin A concentrate (2 g.), aluminium isopropoxide (2 g.), acetaldehyde (15 ml.), and benzene (40 ml.) were kept in a sealed tube at 70° for 48 hours. The reaction mixture was treated with water and the crude aldehyde was transferred into benzene, dried and evaporated under reduced pressure in presence of H_2. A solution of the residue in 20 ml. of cyclohexane was adsorbed on partially inactivated " Mayfair " alumina under slight N_2 pressure. The main yellow zone contained the aldehyde + unchanged vitamin A ; the latter was eliminated by conversion to anhydro-vitamin A, by treatment with alcoholic HCl and rechromatography. The aldehyde exhibited maxima at 330, 350 and 368 mμ. A small amount of the aldehyde was also prepared by Morton and Goodwin by permanganate oxidation and chromatographic purification on alumina.

Anhydro-vitamin A_1, $C_{20}H_{28}$. This compound (maxima at 351, 371, 392 mμ. in alcohol), which can easily be obtained by leaving vitamin A in anhydrous alcoholic HCl at room temperature, was detected by Edisbury, Gillam, Heilbron and Morton. Castle, Gillam, Heilbron and Thompson found anhydro-vitamin A in the chromatogram of their vitamin A concentrate. The older interpretation as a " cyclized " vitamin A must now be abandoned in favour of the formula given below (Shantz, Cawley and Embree ; Meunier, Dulou and Vinet 1, 2 ; cf. Hawkins and Hunter)



On a calcium hydroxide column this anhydro-vitamin is adsorbed from light petroleum below vitamin A_1 but above the chemically uncharacterized isoanhydrovitamin A (Shantz, Cawley and Embree). The fluorescence in ultra-violet light is orange for both anhydrovitamin adsorbates as compared with the bluish fluorescence of vitamin A itself. (Phytofluene is located between vitamin A and anhydrovitamin A, and shows a greenish-grey fluorescence, cf. author and Sandoval 3.)

Vitamin A alcohol is kept in at least 100 parts of N/30 anhydrous alcoholic HCl at 20° for 15 min. After transfer into light petroleum, the solution is washed with the same solvent through an alumina, hydralo, doucil or calcium hydroxide column. It can be crystallized by keeping a 20% solution in low boiling light petroleum at −60° (Shantz, Cawley and Embree). If anhydrovitamin A_1 is kept for a prolonged time in contact with alcoholic HCl, isoanhydrovitamin is formed (330, 350, 370 mμ.).

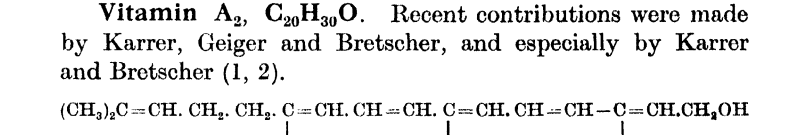
Vitamin A_1-epoxide (=Hepaxanthin=574–Chromogene), $C_{20}G_{30}O_2$, which occurs in some liver oils was obtained by Karrer and Jucker (12) synthetically as follows: 1 g. of crystalline vitamin in 300 ml. of absolute ether was kept in the presence of 1 mol. of perphthalic acid at 15°, in darkness, for about 40 hours. The reaction was followed by applying the Carr-Price reaction to small samples. The surplus acid was removed with very dilute NaOH, and the solution was washed, and dried over freshly heated sodium sulphate. After evaporation of the solvent in vacuo, the yellow residue was taken up with absolute, freshly distilled benzene and developed with the same solvent on a 20 × 2 cm. column of Brockmann's alumina (Merck) (height of the zones in mm.):

20 orange: traces.
50 yellow: vitamin A-epoxide.
30 yellowish orange: unidentified compound " Y ".
40 light yellow.

Elution with methanol-containing ether and evaporation yielded 400 mg. of vitamin A-epoxide and, after rechromatography, 300 mg. of pure substance (max. at 275 mμ.). This

epoxide can be converted with HCl in chloroform into other products which can be separated on alumina.

Vitamin A_2, $C_{20}H_{30}O$. Recent contributions were made by Karrer, Geiger and Bretscher, and especially by Karrer and Bretscher (1, 2).



Formulæ proposed by Karrer and Bretscher for vitamin A_2.

From pike liver oil (after saponification and freezing out of sterols) a concentrate was obtained with $E^{1\%}_{1cm.} = 220$ to 455. A solution of 6·6 g. in 50 ml. of light petroleum was adsorbed on calcium hydroxide (56 × 3 cm.), which had been washed with 200 ml. of the solvent. Upon developing with 220 ml. of light petroleum, the adsorbate zone was extended to a length of 46 cm. within 3½ hours. The greatest (top) part of this zone showed a continuous transition from colourless to dark yellow while several narrow rings were visible in the bottom part. The following sections were cut out and isolated by elution with methanol + light petroleum ($E_{693}=E^{1\%}_{1cm.}$ at 693 mμ. in the Carr-Price reaction ; $q=E_{693}/E_{620}$) :

(I) 170 orange oil+sterols, 0·6 g.
(II) 150 orange oil, 0·4 g., $E_{693} = 1320$, $q = 3·1$.
(III) 120 yellowish red oil, 0·4 g., $E_{693} = 1460$, $q = 2·6$.
(IV) 20 red, thin oil, 2·0 g., $E_{693} = 140$, $q = 2·2$.

Zones II + III were also eluted with chloroform, and yielded a further 0·3 g. of an oil from the concentrated methanol solution of which sterols were frozen out. The vitamin was then transferred into light petroleum. Finally, a solution of 2·3 g. of combined material in 30 ml. of light petroleum gave the following chromatogram on calcium hydroxide (32 × 2·5 cm., washed with 100 ml. of the solvent ; in N_2) :

30 red orange oil, 0·1 g., $E_{693} = 750$.
240 red orange oil, 0·6 g., $E_{693} = 2000$, $q = 2·0$.
50 red orange oil, 0·7 g., $E_{693} = 1200$, $q = 1·8$.

Some of the samples were submitted to molecular distillation, which increased the value of $E^{1\%}_{1cm}$ from 1000, for example, to 1450 at 345 mμ.

Anhydro-vitamin A_2 was obtained by Embree and Shantz (2) as well as by Hawkins and Hunter, as described above. It is more strongly adsorbed on alumina than the corresponding A_1-derivative.

Subvitamin A (formula unknown ; 290 mμ. in alcohol) was accumulated by Embree and Shantz (2) in the unsaponifiable residue of shark liver oil by chromatographing from benzene on Brockmann's alumina. It is more strongly adsorbed than vitamin A alcohol. Anhydro-subvitamin A (332, 348, 367 mμ.) is located well above anhydro-vitamin A, and a little above anhydrovitamin A_2. These observations were confirmed by Hawkins and Hunter (cf. also Lie and Kringstad).

Kitol, $C_{40}H_{58}(OH)_2$ (Embree and Shantz 3), a colourless compound, was accumulated from certain unsaponifiable fractions of whale oil by repeated adsorption on alumina. It is somewhat more strongly adsorbed than vitamin A_1.

The Vitamin B Complex

Adsorption of Vitamin B_1 (Thiamine, Aneurine) from Urine (Urban and Goldman). 30 ml. of urine and 10 ml. of glacial acetic acid were shaken with 30 ml. of isobutyl alcohol for 1 min. and centrifuged. The urine was filtered through paper previously washed with hot water. To 20 ml. of the filtrate were added 2 ml. of glacial acetic acid and 8 ml. of water, and the solution was adsorbed on 5–7 g. of decalso (activated by 50 washings with boiling water). After washing the column with three 10-ml. portions of 2 per cent acetic acid, the vitamin was eluted with three 10-ml. portions of 25 per cent KCl solution.

Another method is based on an investigation by Melnick and Field and was recorded by Hochberg and Melnick (cf. Emmett, Peacock and Brown). A one-hour aliquot of the urine (brought to pH 4·5 ; bromocresol green paper) is adsorbed in a 12 × 0·8 cm. tube containing 3·0 g. of Decalso (this

adsorbent had been stirred with four 10-vol. portions of 3 per cent acetic acid for 10 min.; between the second and third treatment a 15 min. treatment with 5 vol. of 25 per cent KCl solution was given). The top of the adsorption tube is connected with a condenser, into the jacket of which steam can be introduced. During the passage of the steam 30 ml. of water were poured on to the column. The water was allowed to heat up for ½ min., and was then drawn through the column by maximum suction. The thiamine adsorbed on the zeolite was eluted with 10 ml. of 25 per cent KCl solution, which had also been passed through the steam-heated condenser. Finally, 200 ml. of water were sucked through the column while steam was passing through the condenser. The filtrate was then ready for the estimation. The column can be used several times. It is advisable first to run a control with a standard solution of 25 μg. of thiamine in 50 ml. of acidified water.

An evident difficulty in using this method lies in the erratic behaviour of different samples of zeolite, which should always be carefully investigated. According to Brown, Hamm and Harrison some decalso preparations were not activated sufficiently to adsorb thiamine quantitatively from extracts, by washing successively with 5 per cent acetic acid, 25 per cent KCl solution and water. Some other samples caused a marked oxidation of the vitamin during adsorption. Upon drying, the adsorbents gradually lost their activity. The effect of several factors on the adsorption of vitamin B_1 by decalso powder was studied by Holman. Super filtrol was used for the estimation of aneurine in flour by Platt and Glock (2). For a combined estimation with riboflavin see below.

VITAMIN B_2 (riboflavin = lactoflavin). For the use of lead sulphide as an adsorbent cf. Emmerie. Conner and Straub recommend a method of estimating riboflavin and thiamine in the same sample, e.g., in food extracts, by extraction with hot N/25-sulphuric acid, addition of clarase and centrifuging. An aliquot of the liquid is then passed through an activated 60–80 mesh decalso column, which adsorbs thiamine, and then

through a 60–80 mesh supersorb column, which adsorbs riboflavin (cf. Ferrebee) attached below the first. The first column is eluted with 25 per cent KCl solution, and the second with a 20 per cent solution of pyridine in 2 per cent acetic acid.

For the adsorption behaviour of riboflavin on lactose, cf. Leviton; and on florisil, Rubin and DeRitter.

Ferrebee treated urine preparatory to the fluorometric estimation of riboflavin as follows: A quantity of 1–10 ml. (pH 5) was adsorbed on heat-activated floridin or supersorb (15 × 1 cm., washed with 2 per cent acetic acid and water). The riboflavin was eluted by washing with 20 ml. of a 20 per cent pyridine solution in 2 per cent acetic acid. This solution had to be cleared from contaminants by the addition of 2 drops of glacial acetic acid and 1½ drops of permanganate with finally, the addition of hydrogen peroxide.

VITAMIN B_6 (PYRIDOXINE). For the estimation in aqueous rice bran, Scudi precipitated 0·5 g. of concentrate in 5 ml. of water at pH 8 with saturated, clear lead acetate solution. The precipitate was removed and washed, and the filtrate was adjusted to pH 3 with HCl and diluted to 20 ml. Half of this solution was adsorbed on a mixture of 0·1 g. of super filtrol + 9 g. of decalso (diameter, 1 cm.). The column was washed with three 5-ml. portions of water containing 1/8 vol. of citrate buffer pH 3, and the vitamin was eluted with three 5-ml. portions of *N*/4-NaOH.

For the elimination with Amberlite IR-4 of such substances the subsequent adsorption of which on super filtrol is undesirable, see Brown, Bina and Thomas.

A fluorescent *metabolite of pyridoxine* (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine) was isolated by Huff and Perlzweig. After the ingestion of 1·25 g. of pyridoxine, a 24-hour sample of urine, adjusted to pH 4·5 with acetic acid, was adsorbed on decalso (42 × 2·5 cm., 60–80 mesh, washed with acetic acid, 25 per cent KCl solution and water). The effectiveness of the adsorption was checked by examination in ultraviolet light. After having "saturated" the zeolite with the metabolite, the latter was removed with 25 per cent KCl solution, evaporated and separated from KCl by extraction with hot alcohol. After evaporation, the material was taken up in 10 ml. of hot water and the solution cooled. The precipitate was centrifuged off, extracted with dilute alkali and the compound was precipitated with HCl. After recrystallisation from pyridine a yield of 10 mg. was obtained.

VITAMINS B_{10} AND B_{11}. Briggs, Luckey, Elvehjem and Hart gave a general scheme of purification in which two intermediate steps consisted of the development of chromatograms on super filtrol with water at pH 3.

PANTOTHENIC ACID (VITAMIN B_x). **Purification of Crude Synthetic *d,l*-Pantothenic Acid** (Kuhn and Th. Wieland 2). A 3-g. sample was adjusted with NaOH to pH 8·5, diluted with water to 0·5 *l.* and poured on to a 40-cm. alumina column (600 g.), which had been pretreated with 2*N*-HCl and washed until free from acid. After washing the column with 1·5 *l.* of water and discarding the top 5-cm. section, the moist 35-cm. section was transferred to a new tube and washed with baryta solution until the filtrate began to turn phenolphthalein paper slightly red. This filtrate was freed from chloride ions with silver sulphate, from silver ions with H_2S, and from sulphate ions with baryta. A vacuum evaporation yielded syrupy pantothenic acid of increased purity.

The same authors also achieved a partial accumulation of the pantothenic acid contained in tuna liver extracts. Adsorption on the " acid " alumina from water (pH 8·5) resulted in the formation of a bluish-white fluorescent zone near the bottom, above which other layers appeared. A partial chromatographic purification of pantothenic acid concentrates from liver was carried out by Kringstad and Jansen (cf. Lunde, Kringstad and Jansen).

VITAMIN C

Vitamin C does not seem to have been the object of any extensive chromatographic study. A. Kuhn and Gerhard investigated the behaviour of aqueous vitamin C solutions when shaken with active carbons. Partial adsorption took place together with partial dehydrogenation. Frankonite, bentonite or floridin did not adsorb ascorbic acid under conditions permitting of only slight oxidation. Fuller's earth displayed an opposite behaviour.

Vitamin C in Lemon Juice (Lew, Wolfrom and Goepp 2). Fresh juice, obtained by grinding the flesh with acid-washed

sand, was centrifuged and 1 ml. of the juice was mixed with 7 ml. of abs. alcohol. The mixture was centrifuged again and the solution was developed with 2 ml. of 95 per cent alcohol on a 6 × 0·9 cm. florex XXX column. The ascorbic acid could then be located by brushing with a solution of 5 mg. of 2 : 6-dichlorophenol-indophenol in 20 ml. of water. (Above the vitamin C zone a congo red streak showed the presence of acids.)

Vitamin D

Some minor modifications of the isolation procedure previously described were given by Brockmann and Busse (2). For spectrophotometric estimation of the vitamin in liver oils, Marcussen (1, 2) purified his saponified products by filtering the hexane solutions through a hydraffin column. The latter was washed with the same solvent until traces of vitamin A appeared in the filtrate (Carr-Price reaction).

In order to estimate vitamin D by the Carr-Price reaction, interfering substances like vitamin A, carotenoids and sterols can be removed as follows (De Witt and Sullivan). On the top of a 6 × 2 cm. column of magnesia (Baker's reagent + celite 1 : 1) a 1-cm. layer of anhydrous sodium sulphate is packed tightly. The chromatogram is developed with light petroleum and is observed in ultraviolet light of low intensity. The following zones appear, out of which III, IV and V are washed into the filtrate and collected separately.

I. Pale blue fluorescent narrow zone.
I. Broad zone, greenish-yellow fl. : vitamin A.
III. Narrow zone, grayish fl. : traces of A and D.
IV. Two narrow zones, bluish fl. : vitamin D.
V. Narrow zone, gray-blue fl. : inactive sterols.

In case of incomplete resolution, the process should be repeated. The vitamin D is estimated using a modified $SbCl_3$-reagent (in ethylene chloride).

An analytical method which was published recently by P. B. Müller (4) is based on earlier contributions of the same author concerning the application of different, calorimetrically standardized aluminas (p. 30) which form several sections of the column. For many details of this thorough study the original

paper should be consulted. Principle : The light petroleum solution is sent through a five-section column containing aluminas (Merck) of the following increasing activities (from top to bottom) as expressed in calories (Q) : 4, 11, 50, 56·5, and 83·5. In the second section (11 cal.) vitamin A alcohol and the vitamins D_2 and D_3 are retained ; the fourth section contains vitamin A ester.

While the vitamin A alcohol content of this section is determined by the Carr-Price test, a further purification of the vitamins D can be carried out by saponification and repeated adsorption on a three-section column with the aluminas characterized by 4, 11, and 83·5 cal. Saponifications were carried out using 5 *N*-ethanolic KOH in the presence of about 10 per cent of water. To develop vitamin D chromatograms, light petroleum + 10 per cent absolute alcohol was used. The vitamin was determined by the Carr-Price test.

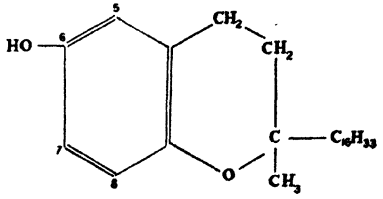
Calciferyl-3 : 5-dinitrobenzoate : Dasler and Bauer.

According to Werder (2), *dihydro-tachysterol* propionate or acetate can be purified by adsorption from ligroin on alumina, and elution with ether + methanol (1 : 1).

A possibly new member of the vitamin D class, termed *chromosterol* was isolated by van der Vliet by chromatographing crude animal sterol mixtures on alumina.

Vitamin E

Relative adsorption affinities of the tocopherols. M. Kofler (4) found that the adsorption affinities on alumina decrease in the following series : δ-tocopherol (8-methyl-tocol) ; γ-tocopherol (7 : 8-dimethyl-tocol) ; β-tocopherol (5 : 8-dimethyl-tocol) ; 5 : 7-dimethyl-tocol; and α-tocopherol (5: 7: 8-trimethyl-tocol).



It seems that the 6-hydroxyl group plays here a part in the anchoring of the molecule, but its effect is markedly weakened by the presence of methyl groups, especially in the 5 and 7 positions.

Estimation of Vitamin E. Principle: Retain interfering substances on a strong adsorbent and determine vitamin E in the chromatographic filtrate.

In order to estimate the tocopherol content of wheat germ oil concentrates by means of an α, α'-bipyridyl-ferric chloride coloration, Emmerie and Engel (1, 2) adsorb 5 ml. of a benzene solution on Floridin XS ($3 \times 1{\cdot}2$ cm.) and wash it with benzene until 25 ml. of filtrate are obtained. The latter is then free from carotenoids or vitamin A that would interfere with the analysis. In one experiment 0·775 g. of γ-tocopherol was used and 0·765 g. was recovered. Acetyl-tocopherol behaves in a similar manner. The floridin must be pre-treated by heating with strong HCl on a water bath for an hour and then standing with three or four portions of fresh acid at room temperature. Finally, it is washed with water, alcohol and benzene and dried at 20°. For an estimation in 10 ml. of blood *serum*, the following additions are made: 5 ml. of 0·2*N*-KOH, 15 ml. of 37% formaldehyde solution (neutralized against phenolphthalein), 15 ml. of ethanol and 50 ml. of peroxide-free ether. After shaking, the ether is replaced twice with the addition of 10 ml. of alcohol each time. The combined extracts are washed successively with 25 ml. of 2% KOH solution, the same volume of 1% sulphuric acid, and water. The solution is then dried over sodium sulphate, after the addition of 10 ml. of benzene, evaporated in vacuo and adsorbed as above.

A modification of the Emmerie and Engel method was recently proposed by Wall and Kelley, who remove interfering substances from plant extracts, by using activated magnesia + super cel columns. From such an adsorbate carotene and tocopherol are washed into the filtrate by light petroleum + 10 per cent acetone while chlorophyll and xanthophylls remain in the column.

Mayer and Sobotka adapted the Emmerie-Engel method for the photoelectric determination of *dl*-α-tocopherol in 10 ml. (or less) of human serum, describing all the necessary operations in detail. Meunier and Vinet (1), in order to estimate tocopherols in total lipoid extracts of tissues or blood, evaporated the chloroform extract and poured the benzene solution of the residue on to a montmorillonite column, which retained vitamin A, carotenoids, chlorophyll, other pyrrole pigments (bilirubin), sterols and phosphatides ; vitamin E and neutral fats passed through. For similar purposes purified florisil (" super-sorb ") was used by Devlin and Mattill (cf. Hines and Mattill), and activated alumina by Tošić and Moore who eliminated $FeCl_3$-reducing substances that would have interfered with vitamin E-estimations in the unsaponifiable residue of vegetable oils.

Estimation in animal fats : Chipault et al.

Separation and Estimation of α-and γ-(or δ-) Tocopherol. According to M. Kofler (4), the adsorption differences are sufficient for a direct and separate estimation of α-tocopherol. For example, the light petroleum solution of the unsaponifiable residue of 6 g. of cottonseed oil was filtered through a 20 × 1 cm. column of pretreated alumina (see below). After a washing with 100 ml. of the solvent mentioned, α-tocopherol was washed into the filtrate with 40 ml. of benzene and then the γ-compound with 30 ml. of methanol.

Pretreatment of alumina for the adsorption of the tocopherols. To 4 kg. of the oxide were added a solution of 200 g. of stannous chloride in 2 l. of conc. HCl, and then 2 l. of water. The material was kept for three days with occasional stirring. It was then washed with tap water until a yellow reaction with methyl red was reached ; after washing with acetone it should be dried in air. One portion was activated by heating ; and by mixing the two brands, the adsorbent was adjusted to the correct activity. This is attained if from a 20 × 1 cm. tube α-tocopherol is washed quantitatively into the filtrate with 40 ml. of benzene while γ-tocopherol is retained.

Some data with reference to the adsorption of the tocopherols from light petroleum on zinc carbonate + celite were given by Weisler, Robeson and Baxter ; *a*-tocopherol passes through first.

Estimation of Tocopherol in Animal and Vegetable Tissues by the Phenazin Method (M. Kofler 1-3). This method is based on the fluorometric determination of a phenazine derivative prepared from tocopherol red (p. 239). A tissue extract (2 g. to 10 g. of material) was obtained by boiling with 2*N* alcoholic KOH and then transferring into light petroleum. In order to exclude large quantities of unsaponifiable material or fluorescent substances other than tocopherol, the solution was adsorbed on alumina and the tocopherol was eluted with benzene. The strength of the adsorbent should be so adjusted, by mixing activated and unactivated alumina, that the column will retain the tocopherol quantitatively from light petroleum, but yield it quantitatively to benzene. (If necessary, tocopherol-quinone can then be eluted with methanol.) The above directions should eliminate carotenoids, if present in the extract, since the benzene solution should be colourless.

After formation of tocopherol red and then of its *phenazine derivative* the solution of the latter can be chromatographed. It is best adsorbed from light petroleum on activated alumina and the column developed with light petroleum containing 20 per cent of benzene. A yellowish-green fluorescent zone that migrates down the column is eluted with benzene + light petroleum (1 : 1) and estimated. Standard solutions used in the fluorometer should be subjected to the same procedure.

A phenazine obtained from a red, oily oxidation product of α-tocopherol could be purified only partially on alumina by Smith, Irwin and Ungnade.

Accumulation and Isolation of Tocopherols. (*a*) Moss and Drummond described a fourteen-fold concentration by chromatographing 10% solutions of *wheat germ oil* in light petroleum on Merck's alumina. Since most impurities passed into the filtrate, much more material than usual, viz., 150 g. of oil, could be handled on a 45 × 4·5 cm. column. The latter was washed with only 300 to 500 ml. of the same solvent, until the filtrate became free of fats and colourless. The chromatogram showed three sets of coloured sections, the yellow one

of which (located below a deep orange and above a pinkish zone) contained the tocopherol.

(*b*) Baxter, Robeson, Taylor and Lehman distilled their refined *cottonseed oil* in a cyclic molecular still and carried out a partial resolution of the light petroleum solution on special filtrol and ducil. By the liquid chromatogram method the impurities, mainly glycerides and hydrocarbons, were eliminated, and concentrates containing 52 per cent of α- and 75 per cent of γ-tocopherol respectively were obtained. The separation of the α- and β- compounds was incomplete ; the $\beta : \alpha$ ratio was 60 : 40 in the concentrates.

After formation of the *succinate* of an α-tocopherol concentrate (52 per cent) with the anhydride in pyridine, the mixture was diluted with ether and the excess anhydride was frozen out. The mother liquor was washed with HCl and then with water. The dark oil obtained after drying and evaporation (150 g.) was dissolved in 650 ml. of light petroleum (b.p. 30–65°) and forced through a 18 × 10 cm. column of special filtrol and hyflo super cel (7 : 3) under nitrogen pressure. Impurities were washed down with light petroleum, and the strongly adsorbed α-tocopherol succinate was eluted with 3 *l.* of ether. The dry residue left on evaporation yielded, after crystallization from light petroleum, 80 per cent of the expected amount (m.p. 76–7°).

For some data on *soyabean oil*, cf. Weisler, Baxter and Ludwig.

(*c*) The α-tocopherol content of a concentrate prepared from *Mangona shark liver oil* by Robeson and Baxter was increased from 59 per cent to 78·5 per cent when 1·2 g. of the substance in 15 ml. of light petroleum either was developed on floridin (23 × 1·2 cm.) with 80 ml. of solvent. The bluish-green upper half of the column was discarded and the colourless lower half was eluted with ether containing 5 per cent of methanol. The oily residue left on evaporation was used for the preparation of *α-tocopherol palmitate :* 0·28 g. in 2 ml. of ethylene chloride and 1 ml. of pyridine was left with 0·9 ml. of palmitoyl chloride for 20 hours. The mixture was poured into ether, and the solution was then washed with HCl, 0·5*N*-KOH and water. The residue left on evaporation of the

ether solution contained some free palmitic acid which was, however, easily retained when the light petroleum solution (15 ml.) was washed through a doucil column (sodium aluminium silicate, American Doucil Co.; 23 × 1·2 cm.) with 75 ml. of the same solvent. This filtrate yielded, upon evaporation and recrystallization from ethanol, pure α-tocopherol palmitate.

Purification of Synthetic Products. For the purification of synthetic *dl*-α-tocopherol, Bergel, Copping, Jacob, Todd and Work gave the following method (cf. also Karrer and Ringier; Smith, Ungnade, Hoehn and Wawzonek). The condensation product from 1 g. of phytol and 0·7 g. of φ-cumoquinol in the presence of zinc chloride was chromatographed from light petroleum on alumina. The top and bottom zones were discarded, and the colourless broad middle portion was eluted with benzene + acetone + methanol (8 : 1 : 1). The eluate served for the preparation of the allophanate.

Smith and Ungnade claim that it is more advantageous to carry out the α-tocopherol synthesis in the absence of solvent and catalysts because the chromatographic treatment subsequently required, which may cause considerable losses, can thus be avoided. This suggestion probably goes too far. It is true that *dl*-α-tocopherol is much more liable to autoxidation in the form of an adsorbate than in the free state; however, this behaviour is not shown by some of its esters (Isler).

γ-Tocopherol allophanate suffered considerable decomposition on calcium carbonate (Emerson and Smith).

Similar synthetic methods can be employed in the preparation of higher or lower *homologues* of the tocopherols. For example, *dl*-5 : 7-dimethyl-8-ethyl-tocol was obtained by Karrer and Hoffmann as follows: 2 g. of 3 : 5-dimethyl-2-ethyl-hydroquinone in 20 ml. of dry ligroin (b.p. 80–100°), 6·4 g. of phytyl bromide and 1·1 g. of anhydrous $ZnCl_2$, were heated in N_2, in a water bath for two hours. The product was washed with water, alkali and water; after drying, the solvent was evaporated. The residue when dissolved in light petroleum yielded on Brockmann's alumina three zones: I. (top) nearly colourless (content, 1·3 g.); II. violet (0·5 g.); and III.

yellow (0·1 g.). Zones I–II were eluted with ether + methanol and used for the preparation of the allophanate.

Synthetic *iso*-α-tocopherol can be isolated by developing the crude mixture of the allophanates on Brockmann alumina with benzene + light petroleum. In such an experiment the second fifth of the column contained the main portion of the desired product (John, Günther and Rathmann).

7 : 8-Cyclo-trimethylene-tocol and 7 : 8-cyclo-tetramethylene-tocol were purified by Karrer and Kugler from light petroleum on alumina and eluted with methanol + ether from the middle sections of the column. The physiologically inactive 5 : 7-dimethyl-*thio*tocol was treated in a similar manner on alumina, where it occupied a top position (Karrer and Leiser).

Further literature concerning racemic tocopherol homologues and related compounds: 5 : 7-dimethyl-tocol (Karrer and Fritzsche 1,2); 5 : 8-dimethyl-tocol (Karrer, König, Ringier and Salomon); 7 : 8-dimethyl-tocol (Karrer, Fritzsche and Escher); 5 : 7-diethyl-tocol (Karrer and Schläpfer); 5 : 8-diethyl-7-methyl-tocol (Karrer and Hoffmann 2); "homo-α-tocopherol" (Karrer and Yap 1); "nor-α-tocopherol" (John and Herrmann); 2 : 5 : 7 : 8-tetramethyl-2-[2'-methopentyl]-6-hydroxy-chromane (Karrer and Yap 2); 5 : 7 : 8-trimethyl-2 : 2-pentamethylene-6-hydroxy-chromane and 2 : 5 : 7 : 8-tetramethyl-pentadecyl-6-hydroxy-chromane (Karrer and Kehrer); 2 : 5 : 7 : 8-tetramethyl-2- 4' : 8'-dimethyl-nonyl -6-hydroxy-chromane (Karrer and Jensen); 6-hydroxy-2 : 8-dimethyl-2-(4' : 8' : 12'-trimethyltridecyl)-chromane (Jacob, Sutcliffe and Todd; cf. also Jacob, Steiger, Todd and Work); trimethyl-cyclotocol (Karrer and Rentschler 2); naphthotocopherol (Fernholz, McPhillamy and Ansbacher). For an allylation product of γ-tocopherol, see Emerson and Smith.

α-Tocopherol Red (**α-Chromane Red**) was obtained by John and Emte (1, 2) by oxidizing α-tocopherol in alcohol with silver nitrate in a water bath for two hours. The ether extract of the diluted liquid was evaporated and the residue adsorbed from benzene on silica. The intensely red main zone can be either eluted with chloroform or washed into the filtrate with more benzene. However, even after a repeated adsorption, the product contained only 80 per cent of pigment.

α-Tocopherol red can be freed from the more strongly adsorbed *p*-quinoid contaminants by chromatographing on silicic acid. Karrer and Geiger stressed the complex nature of the zinc carbonate chromatogram.

Isomerization products of α-tocopherol red or chromane red " 109 " can be developed with benzene + light petroleum on silicic acid and eluted with alcohol + ether (John and Emte 1, 2).

Biotin (Vitamin H)

Isolation of Biotin from Milk (Melville, Hofmann, Hague and Du Vigneaud). The best starting material for the chromatographic treatment of biotin is its methyl ester. A crude concentrate was esterified with methanol + HCl and the solution of 55 g. of solids (containing 81 mg. of ester) in 400 ml. of chloroform was adsorbed on 250 g. of decalso (60—100 mesh). After washing with 3·6 *l.* of chloroform, the ester was removed from the column with 1·2 *l.* of acetone containing 5% of methanol. The resulting solution (solids, 10·7 g.; biotin ester, 66 mg.) was evaporated in vacuo and the solution of the residue in 500 ml. of acetone was chromatographed on 220 g. of alumina (alorco A, 120–150 mesh). After washing with 2·3 *l.* of acetone, 900 ml. of acetone containing 10% of methanol were introduced and the filtrate was collected in 50-ml. fractions. The main part of the biotin methyl ester was contained in the second to eighth fractions. From the fourth and fifth fractions the ester crystallized out upon the addition of a few drops of methanol to the residue, after evaporation of the solvent; and from the third and sixth, upon seeding. The semi-crystalline samples were further purified by washing with ethyl acetate, by high vacuum sublimation, and by recrystallization from hot methanol + excess ether. Yield, 25–40%, m.p. 166–7°.

β-Biotin from Liver. Kögl and ten Ham isolated β-biotin from a commercial concentrate by a similar method; however, the crude material, which contained biotin methyl ester, was hydrolyzed before its solution (125 g. in 100 ml. of water; acidified to congo red; biotin, 45 mg.) was adsorbed on norit (17 × 3 cm.). The column was washed with 1750 ml. of water and then with a mixture of 600 ml. of acetone + 395 ml. of water + 5 ml. of 25% NH_4OH. The main portion of the biotin accumulated in the fraction obtained by collecting between 1150 and 1340 ml. of the acetone + ammonia filtrates.

It was evaporated, esterified and adsorbed from chloroform on Brockmann's alumina. This column was washed with 1 *l.* of chloroform, and then with 400 ml. of acetone. The eluate was re-adsorbed on alumina from acetone and a liquid chromatogram again was prepared by washing with acetone + methanol (9 : 1). The first fraction contained 30 mg. of β-biotin methyl ester which was crystallizable.

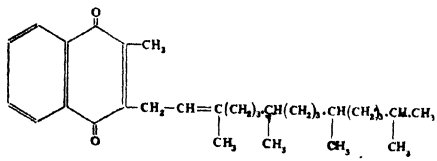
Biotin from Beef Liver Concentrates (Du Vigneaud, Hofmann, Melville and György). The concentrate was esterified with methanol and HCl and the crude product (18 g., 83 H- units/mg. of solid) was dissolved in 150 ml. of chloroform and adsorbed on 50 g. of alumina (Brockmann). The column was washed with 300 ml. of chloroform, the washings being discarded, then with 300 ml. of acetone, then with three 50-ml. portions of acetone + methanol (9 : 1). The latter four fractions from several preparations (2·55 g.), containing 1210–2140 units/mg., were rechromatographed from chloroform on 100 g. of alumina. The washings obtained with 200 ml. of acetone and then with 150 ml. of the acetone + methanol mixture contained low grade material, but the next seven 10-ml. portions of the mixture yielded fractions containing 3420–6670 units/mg. of solid. The chloroform solution of this material was extracted with fifteen 10-ml. portions of 3*N*-HCl and the residue left, upon evaporation of the acid, was esterified. After the removal of the methanol in vacuo, the residue was dissolved in water and neutralized with bicarbonate. The solution was extracted with cold ethyl acetate. This extract deposited 35 mg. of crystals, m.p. 166–7° (after sublimation).

Purification of Crude, Synthetic d,l-β-Biotin Methyl Ester (7·2 g. containing 274 mg. of biotin). The ester was adsorbed from benzene on 210 g. of alumina. After washing with 3·75 l. of benzene, then with 3 l. of chloroform and finally with 1 l. of acetone, which removed 65 mg. of *rac.* biotin from the column, the next 2·25 l. of acetone eluted 110 mg. of biotin, nearly the whole of which could be crystallized from ethyl acetate (Grüssner, Bourquin and Schnider).

Vitamin K

Vitamin K_1 was isolated from alfalfa by Doisy *et al.* and Dam, Karrer *et al.*, independently, by means of a chromatographic accumulation. The latter group of investigators also isolated vitamin K_2 from fish meal (see the formulæ below).

Isolation of Vitamin K_1 from Alfalfa (Binkley, MacCorquodale, Thayer and Doisy). These authors found that vitamin K_1 is inactivated by alumina, magnesium oxide or infusorial earth but is stable toward sucrose, norit, calcium carbonate (or sulphate), permutit, and decalso. The two latter adsorbents were selected for use. If a 2·5% solution of a crude extract in petroleum (b.p. 90–105°) is employed, 1 part of solid material requires 25 to 60 parts of adsorbent. The chromatograms are developed with petroleum and then with the same solvent containing first 10% and later 20% of benzene. The filtrates are collected in fractions, each of which is tested for activity. In general four or five adsorptions are needed to obtain the pure vitamin, viz., 1 to 2 on decalso, 1 to 2 on permutit and, finally, one on Darco. In the original paper details are given of the treatment of a 500-kg. sample of alfalfa; this was carried out in an industrial plant. The extracts were concentrated to about 400 l. for the first adsorption in which about 350 kg. of Decalso were used. That an efficient accumulation of vitamin K_1 is possible even on such scale is shown in Table 20. Work on a smaller scale follows the procedure described below for vitamin K_2.



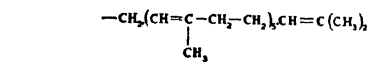


TABLE 20

FRACTIONATION OF 10 KG. OF SOLIDS (0·4 UNITS/MG.) CONTAINED IN A CRUDE ALFALFA EXTRACT ON DECALSO (BINKLEY *et al.*)

Fraction	Solvent	Solid content (g)	Vitamin K_1 units/mg.
1	petroleum	1034	0·3
2	,,	456	2
3	,,	506	4
4	,,	200	7·5
5	petroleum with 10 per cent benzene	72	9

Isolation of Vitamin K_1 ("α-Phylloquinone") from Alfalfa (Karrer, Geiger, Legler, Rüegger and Salomon (cf. Dam, Geiger, Glavind, Karrer, Karrer, Rotschild and Salomon). Each 30-kg. portion of dry alfalfa powder was extracted with 40 *l.* of boiling light petroleum (b.p. 40–60°). From the extract (40 *l.*, after concentration), chlorophyll was eliminated by mixing 10-*l.* portions with finely powdered zinc carbonate (20 × the solid content of each solution). If after two hours of mechanical stirring and sedimentation the solution is still green (chlorophyll), a little more of the carbonate should be employed; an excess should be avoided. The reddish orange solution is drained off and the carbonate is extracted with 5 *l.* of fresh light petroleum. The adsorbent is washed with light petroleum and the combined extracts are adjusted to a volume of 500 ml. After standing at -10° for two days, a colourless precipitate can be filtered off and the solution evaporated to dryness in vacuo.

The residue is then submitted to molecular distillation. The fraction with b.p. <150° is liquefied by gentle heating and poured into acetone; the white precipitate that separates is removed. Further fractions are filtered off after cooling and concentration. The filtrate is evaporated to dryness, the residue is dissolved in 18 parts of light petroleum and chromatographed on magnesium sulphate (activated by strong heating for four hours). Each column (35 × 8 cm.) was moistened with 350 ml. of light petroleum, and 240 ml. of the above solution was then adsorbed. The column was washed with 800 ml. of light petroleum. Finally, the total vitamin content was washed

out with 1,500 ml. of light petroleum + 10 per cent benzene. The dry residue from the latter solution was chromatographed on 27 zinc carbonate columns (40 × 3 cm. ; treated with 50 ml. of light petroleum). Each tube adsorbs 1·5 g. of substance from 50 ml. of light petroleum and is developed with 150 ml. of this solvent and then with 100 ml. of light petroleum containing 10 per cent benzene.

In each of the five zones (6, 4, 18, 3, and 8 cm. high), the value of $E^{1\%}_{1\,cm}$ was measured at 248 mμ and the 4-cm. and 18-cm. zones, with values of 15 and 55, were combined from all 27 tubes (none of the other zones showed a maximum at 248 mμ). The chromatographic procedure using zinc carbonate must be repeated 7 or 8 times under spectroscopic control. The zones with similar values are then combined. From 200 g. of molecular distillate, 0·5 g. of pure vitamin K_1 was obtained by means of several hundred individual chromatograms. Yield, 15–20 per cent.

Vitamin K concentrates chromatographed from light petroleum on calcium sulphate (activated by slight heating), do not give a colour reaction with sodium ethylate in alcohol (Fernholz, Ansbacher and Moore).

Isolation of Vitamin K_2 from Putrefied Fish Meal (McKee, Binkley, Thayer, MacCorquodale and Doisy). The light petroleum extract (2 *l.*) of 25 kg. of material (125 g. of solids ; 5·7 units/mg.) was developed on a 106 × 10 cm. decalso column with 12 *l.* of high boiling petroleum. After the pigments had separated into definite zones, the vitamin was washed through with six 3-l. portions of petroleum containing 20 per cent of benzene. Nearly the whole vitamin content was found in the two first fractions of the filtrate (4·2 g. of solids ; 167 units/mg.), which did not contain appreciable amounts of other substances. The bulk of the adsorbed impurities remained on the column. After evaporation, the product (about 3 g.) was dissolved in 300 ml. of petroleum and chromatographed on a permutit column, 70 × 5 cm. Some impurities were washed into the filtrate with 700 ml. of petroleum and by further washing with 3 l. of petroleum containing 10 per cent benzene, together with a little vitamin. Finally, 3 l

of petroleum containing 15 per cent benzene and 1·5 l. containing 20 per cent were applied. About 90 per cent of the vitamin was found in the second 500-ml. portion of the 15 per cent benzene solution and in the first 1,000-ml. portion of the 20 per cent benzene fraction (500 units/mg.). After evaporation of the solvent, the oily residue from these combined fractions solidified upon cooling and yielded, after six recrystallizations from ethanol + acetone, two from acetone and two from low boiling petroleum, plates of pure vitamin K_2, m.p. 52–53·5°.

SYNTHETIC COMPOUNDS related to vitamin K can be effectively purified from light petroleum solution on zinc carbonate; e.g. crude 2-phytyl-naphthaquinone-1 : 4 (Karrer, Geiger, Rüegger and Schwab); 2-methyl-3-octadecyl- and 2-methyl-3-dihydrophytyl-naphthaquinone-1 : 4 (Karrer, Epprecht and Konig).

VITAMIN P

This vitamin, detected by Szent-Györgyi, was first chromatographed by Robeznieks. Later Mager was successful in isolating the pure eriodictyol component: An aqueous solution of 25 mg. of "citrin" was washed on a 15 × 3 cm. column of alumina (Brockmann) with 4–5 l. of water. The following zones were formed: dark brown (top); yellowish brown; light brown; and light yellow. The eriodictyol rhamnoside was eluted from the light brown zone by repeated shaking with 200 ml. of 1 per cent sulphuric acid. Most of this acid was eliminated with baryta and the slightly acid filtrate was evaporated in vacuo. The residue was treated with absolute alcohol, filtered, evaporated, extracted with ethyl acetate and crystallized; m.p. 184–6°.

FURTHER VITAMINS

Folic acid. Quantitative data concerning the adsorption on charcoal were given by Frieden, Mitchell and Williams. In a long series of operations which did not, however, yield crystalline folic acid, chromatography was used at certain stages by Mitchell, Snell and Williams. A substantial concentration was achieved. For example, a purified sample (88·3 mg.; potency, 46,000 units) in 1 ml. of 40 per cent methanol was adsorbed on Brockmann's alumina (10 × 1·5 cm.;

treated with the same solvent). The column was washed with 40 per cent methanol containing 1 drop of conc. ammonia per 100 ml. Nine 4-to 6-ml. fractions were collected. The sixth contained 5·8 mg. of solids with a potency of 105,000 units.

The operations which led to the isolation of crystalline liver *Lactobacillus casei* factor from liver extracts and of the *Lactobacillus casei* factor from an aerobic fermentation liquor of a *Coryne bacterium* have been recently described (Stokstad, Hutchings and SubbaRow ; Stokstad, Hutchings, Mowat, Boothe, Waller, Angier, Semb and SubbaRow ; Hutchings *et al*).

Rhizopterin, a new pterin-like growth factor "S.L.R. factor") which promotes the growth of *Streptococcus lactis R* (*S. faecalis R*) was purified 200,000-fold by Rickes, Chaiet and Keresztesy. After previous adsorption on norit and then fuller's earth, precipitation at pH 7, and other operations, the solution was filtered at pH 4 through alumina. From this column impurities were removed with 95 per cent ethanol and the active material was recovered by elution with aqueous methanol + ammonia followed by further chromatographic purifications. For details the original paper should be consulted.

Unidentified vitamins produced by a strain of *Mycobacterium tuberculosis*, and promoting growth and feathering in chicks, were partially purified by adsorption on norit and chromatography on super filtrol at pH 5 (Mills, Briggs, Luckey and Elvehjem).

CHAPTER XIX

ANTIBIOTICS

Penicillin

For obvious reasons, little has been reported on this subject during recent years in spite of evident intensive activity. Penicillin can be adsorbed from protein-free solutions, at pH 2–7, by activated charcoal or fuller's earth. The elution from charcoal may be effected by means of ethanol, methanol, acetone or pyridine (Clayton, Hems, Robinson, Andrews and Hunwicke). A preliminary statement concerning the purification of penicillin was also made by Catch, Cook and Heilbron (p.26). Amyl acetate or ether solutions were adsorbed on a water-retentive support such as silica gel or hyflo supercel, which was intimately mixed with the hydroxide or carbonate of an alkali or alkaline earth metal. The base is best precipitated on the carrier before use, e.g., a 2·5% precipitate of an alkaline earth carbonate is appropriate. Apparently, the constituents of the crude penicillin are separated primarily according to their relative acid strengths in such a system.

Abraham and Chain passed the ether solution of partially purified penicillin, which had been pre-treated with animal charcoal, through Brockmann's alumina (40 × 3·6 cm.). The top zone (1 cm.) was dark brown and contained little penicillin, the bulk of which was present in the next, pale yellow zone, 12–14 cm. broad ; the other coloured zones were practically free from the compound. Zone 2 was eluted by four prolonged shakings with M/15-phosphate buffer, pH 7. The eluate was extracted at pH 2 with three 0·3-vol. portions of peroxide-free ether at 4°. The penicillin was transferred back to water by adjusting to pH 5·8–6·0 with *N*/30-baryta, re-transferred into ether and re-chromatographed. Whereas the initial solution contained 15–25 units/mg., this was increased by the second chromatogram to 100–150. After a reduction process with aluminium amalgam the corresponding

figure was found to be 300, and, upon further chromatography from amyl acetate solution, 450–500 units/mg.

Penicillin G from Aspergillus parasiticus (Arnstein and A. H. Cook). The crude sodium salt was treated essentially as indicated by Catch, Cook and Heilbron (p. 247). An ethereal extract (70 ml., 140,000 Oxford units) was adsorbed on a column 25 cm. long (65 g. of silica gel loaded with water and potassium phosphate buffer, pH 6·5) and developed with 1,400 ml. of ether at 0°. The fractions (1·5 to 2 cm. zones) were extracted with neutral buffer (50 ml.). The ninth to twelfth fractions showed the following potencies in Oxford units : 1,200 ; 5,000 ; 50,000 ; and 31,250. After rechromatography at pH 6·7, fractions containing 37,500, 75,000 and 37,500 units were obtained.

Filter paper chromotograms. In order to detect the presence of various penicillins, Goodall and Levi soaked filter paper in 30 per cent potassium phosphate buffer pH 6–7. The 33 × 1·8 cm. paper strips were dried in air but kept in a damp atmosphere before use. A 1-µl. spot of the sample (Na salts, in the buffer) was placed on the strip and a chromatogram was developed with wet ether. The position of the invisible zones can be determined by pressing the strip on flat, uniform sheets of agar pre-inoculated with *Bac. subtilis*. The respective penicillin spots diffuse out into the medium and form, on incubation, elliptical inhibition areas. Quantitative estimates can be made by comparison. Using this technique, Winsten and Spark added in turn to the same broth, samples of pure, identified penicillins and chromatographed as above. They observed which of the inhibition zones were accentuated by such an addition. (Photographs in the original papers.)

Streptomycin

A comprehensive survey of this field was recently given by Peck.

After Fried and Wintersteiner made the first, rather brief communication referring in part to reineckates, detailed directions for the chromatographic purification of streptomycin were worked out by different investigators.

Kuehl, Peck, Walti and Folkers as well as Kuehl, Peck, Hoffhine, Graber and Folkers isolated streptomycin from *Streptomyces griseus* by the following steps: adsorption on charcoal and elution with methanolic formic acid, conversion to the picrates and hydrochlorides, and chromatography of the latter on a mixture of Darco G–60 and filter paper or acid-washed alumina in the same manner as given below for streptothricin.

Another group of investigators, viz., Carter, Clark, Dickman, Loo, Skell and Strong found that streptomycin can be removed from slightly acid aqueous methanol (70–80%) solutions, brought to pH 6·3 with LiOH, by alumina (Merck or Harshaw) which had been pretreated with sulphuric acid. The column is washed with 80 per cent methanol. In such adsorbents the crude streptomycin hydrochloride is converted into the sulphate. The antibiotic activity in the successive filtrate fractions reach a peak and then decreases again as shown by the Sakaguchi reaction for guanidino groups. In the best fractions 600–900 units/mg. were found, in contrast to the initial value of 150–300. A 490-ml. packed column (diameter, 3·2 cm.) is needed for handling 8–10 g. of concentrate; and 100-ml. filtrate fractions are taken. After the Sakaguchi reaction began to decrease, the washing was continued with water. The recovery was 80 per cent of the initial activity.

Vander Brook, Wick, De Vries, Harris and Cartland used for a similar purpose alumina columns which had been pretreated with HCl. For some data on the isolation of streptomycin B see Fried and Titus.

Mercaptolysis products of the calcium chloride compound of streptomycin trihydrochloride, viz. two anomeric forms of ethyl tetraacetylthiostrepto-biosaminide diethyl thioacetal can be isolated by developing on magnesol + celite (5 : 1) with benzene + alcohol (100 : 1) and are located by streaking the column with alkaline permanganate. The yellow bottom zone was eluted with acetone and the product crystallized from ethanol-water (Hooper, Klemm, Polglase and Wolfrom).

Streptolin, in many respects a streptomycin-like antibiotic produced by *Streptomyces*, was purified by Rivett and Peterson by passing the crude solution at pH 7·9 through

filter-cel (Johns-Manville Co.), in a press. After washing with water, streptolin was eluted with 7·5 per cent pyridine hydrochloride at pH 1·5. It was further purified by adsorption at pH 2·3 on Darco G–60, washing, and elution with 0·03 *N*-HCl in 50 per cent ethanol.

Streptothricin

A short communication on this subject was made by Vander Brook, Wick, De Vries, Harris and Cartland. A detailed investigation was published recently by Peck, Walti, Graber, Flynn, Hoffhine, Allfrey and Folkers on the isolation of this antibiotic from *Streptomyces lavendulae*. In the course of other operations, the hydrochloride concentrates were chromatographed on alumina or Darco as follows :

Purification on alumina. The columns were filled with 10–30 g. of acid-washed adsorbent per g. of concentrate, together with methanol, and the solvent was allowed to drain until the top surface was almost reached. A 10–15 per cent methanol solution of the concentrate was then introduced. The column was washed with methanol (by gravity or slight pressure). When precipitates were formed by the addition of acetone or ether to samples of the filtrate, the collection of fractions began.

Purification on Darco G–60. The column consisted of a dry Darco + paper pulp mixture. The methanol was introduced under pressure and the above technique was followed.

This antibiotic which is also basic can, according to Kocholaty and Junowicz-Kocholaty, be purified on a Decalso column from which the material is quantitatively released by conc. NaCl solution. Under certain conditions an exchange on Amberlite IR–100 may also be used.

Clavacin

was isolated from *Aspergillus clavatus* by Katzman, Hays, Cain, van Wyk, Reithel, Thayer, Doisy, Gaby, Carroll, Muir, Jones and Wade. The chilled and filtered culture medium (pH 5·5–6) was adsorbed on norit A (20–50 mesh ; washed

with dilute HCl, water, and dried), 3 to 10 million units being adsorbed per kg. The appearance of antibacterial activity in the filtrate was always preceded by the appearance of colour. The adsorbate was dried on filter paper and extracted with chloroform in a Soxhlet apparatus. High potency material crystallized out upon evaporation. In other instances, the norit was extracted with acetone. This extract was evaporated in vacuo, dissolved in the minimum amount of warm ethanol and precipitated with excess acetone at dry ice temperature; the precipitate was inactive. After repeated extraction and precipitation, the solution was concentrated to a small volume, when impurities were precipitated as an inactive oil. Finally, the solution was evaporated to dryness. Then the residue was dissolved in warm anhydrous chloroform (10–50 ml. per 0·5–0·7 g.) and chromatographed on permutit (25 × 1 cm. to 50 × 2·5 cm.). The clavacin was obtained by washing the column with chloroform containing, if necessary, 0·25–0·5% of ethanol; it crystallized on concentration; m.p. 109–10°.

Further Antibiotics

Antibiotics from Pseudomonas aeruginosa (Hays, Wells, Katzman, Cain, Jacobs, Thayer, Doisy, Gaby, Roberts, Muir, Carrol, Jones and Wade). The 95% alcoholic extract was diluted to 80% and exhaustively extracted with light petroleum. The alcoholic solution was evaporated in vacuo and its residue was extracted with ether. By repeated shakings with 5% bicarbonate solution, then with 5% carbonate solution, and finally with *N*-NaOH, several main fractions and a residue ("neutral fraction") were obtained. Crystalline substances were isolated from the carbonate soluble fraction ("Pyo II"), as well as from the neutral fraction ("Pyos I, III, and IV").

Carbonate soluble fraction. The benzene solution was chromatographed on a 10 × 1 cm. column of dried oxalic acid. Most of the solid material and the brown colour were not retained. After washing with benzene the column was dissolved in saturated bicarbonate solution + ether, and the

ether was extracted 6 times with bicarbonate solution. The active material was then removed by six extractions with bicarbonate solution and transferred into ether after acidification with HCl (pH 4–5). After washing with water, the ether extract was evaporated and the residue crystallized from benzene. Decolourization with charcoal in alcohol and recrystallization of " Pyo II " from acetone, methanol, and ethanol yielded yellowish scales of m.p. 149°.

Neutral fraction. The chloroform solution of this brown oil was adsorbed on several permutit columns, which had previously been washed with chloroform. The liberation of active material was accelerated by the addition of 0·5% absolute alcohol to the chloroform. For the fractionation of 10·5 g. of material a 20 × 5 cm. column was used ; about 40 fractions (mostly 200 ml.) were required. A spectroscopic examination of the successive fractions showed three characteristic curves, corresponding, in order of elution, to Pyo IV, Pyo III, and Pyo I. Pyocyanine remains on the permutit. Pyo IV is contaminated with a reddish oil from which it can be freed by rechromatography as above. Pyo III was obtained by repeated crystallization of Pyo I containing 25% of Pyo III (estimated spectroscopically). Pyo I was present in the 33rd fraction of the main chromatogram (see above), and was crystallized from that fraction. These crystals were separated into the components, Pyo Ib and Ic, by procedures that cannot be described in detail. The following antibiotics were obtained in analytically pure state :

Pyo I b, m.p. 146·2–147° (colourless) ;
Pyo I c, m.p. 138·8–139·2° (colourless) ;
Pyo II, m.p. 149–149·5° (light yellow) ;
Pyo III, m.p. 152·8–153·5° (colourless) ;
Pyo IV, m.p. 131–2° (colourless).

Chlorellin, contained in the green alga, *Chlorella*, was obtained by Spoehr, Smith, Strain, Milner and Hardin. To each 15-l. portion of cell-free culture fluid, 30 ml. of acetic acid were added and the liquid was passed through a florisin column (40 × 3·7 cm. ; 200 g.) over a period of 4 hours. Chlorellin could then be eluted with ethanol.

Actinomycin. An orange-red, nitrogen containing antibacterial pigment, *actinomycin A* (occurring in the soil organism, *Actinomyces antibioticus*) was extracted by Waksman and Tishler with ether. After the elimination of colourless compounds with light petroleum, the benzene solution of the residue was developed on Brockmann's alumina with acetone + benzene (15 : 85); the main zone was eluted with a mixture (30 : 70) of the same two solvents, evaporated, and crystallized from ethyl acetate.

Iodinin, the pigment of *Chromobacterium iodinum*, a di-N-oxide of a dihydroxyphenazine, can be purified by chromotographing its benzene solution on alumina (McIlwain).

Notatin. (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw and Raistrick). This antibacterial substance is a glucose-dehydrogenase obtained from *Penicillium notatum* and *P. reticulosum*. After other purification steps, it was dissolved in a phosphate-citric acid buffer pH 3 and chromatographed on alumina, previously boiled with strong HCl; it gave a buff-coloured zone. Notatin was eluted by phosphate buffer pH 5. During these operations the activity has increased by 40%, and a recovery of 20–35% was obtained.

Brief mention was made by Mull, Townley and Scholz of the chromatographic isolation on alumina of an unnamed antibiotic that occurs in *Penicillium obscurum*. As starting material the mother liquors from the preparation of gliotoxin were used.

Crepin, a growth inhibitor of *Staphylococcus aureus* was isolated by Heatly from the petals of *Crepis taraxacifolia*, by adsorption from acid solution on charcoal and elution with 80% aqueous acetone.

Sulphactin was isolated from a soil *Actinomyces* and adsorbed on florisil. The column was first washed with chloroform; later filtrate fractions, obtained with chloroform + alcohol 9 : 1 contained the crystallizable compound (Junowicz-Kocholaty, Kocholaty and Kelner).

Gramicidine : pp. 47, 163; cf. also Synge and Tiselius.

ENZYMES

Enzyme chromatography, which is a special case of the almost unstudied chromatographic treatment of proteins, has mainly been useful so far in two respects : as a step in purifying crude solutions of a certain enzyme, and for the separation of different enzymes which occur in the same extract. Furthermore, the resolution of some components of enzyme systems has been carried out in a few cases, like the splitting of the "old" yellow enzyme into the protein and chromogen or the separation of the coenzyme from a glycerophosphatase (p. 255).

For the literature till 1940, cf. also Grundmann (2).

Chromatography as a Step in Purifying Crude Solutions

α-Amylase (*ex* saliva). The exchanger, Amberlite IR–4B was used in a step of the purification and crystallization process by K. H. Meyer, Fischer, Bernfeld and Staub.

Succinodehydrase. In heart muscle extracts Euler, Hellström and Günther succeeded in increasing the ratio, succinodehydrase/diaphorase tenfold by chromatography on calcium carbonate (elution with disodium phosphate).

Horse Liver Catalase. Agner further improved his partially purified extracts (0·2 mg./ml.) by adjusting them with KH_2PO_4 to pH 5·5 and chromatographing on tricalcium phosphate. The enzyme was adsorbed in a distinct zone at the top and could be eluted with phosphate buffer pH 8. According to Sumner, Dounce and Frampton, catalase can be crystallized either before or after the application of Agner's chromatographic method ; however, the latter procedure is preferable.

Photographs of chromatograms prepared by using beef liver catalase and further information can be found in the well-known monograph by Sumner and Somers.

"Old" yellow enzyme (degree of purity 40 to 60 per cent) was further purified by Weygand and Birkofer who drew a solution in $N/10$-$N/20$ phosphate buffer of pH 5–7 through frankonite SB (20 × 6 cm.) which had been moistened with water. They also carried out a chromatographic splitting of the yellow enzyme into flavine-phosphoric acid and protein.

For this purpose, a microtube (diameter, 0·5 cm.) was filled with 50 mg. of finely powdered granosil upon which 100 mg. of the frankonite was placed. First, water was sucked through and then, without using the pump, within 90 min., about 50 mg. of yellow enzyme, dissolved in 10 ml. of $N/50$-HCl. From the colourless filtrate HCl was eliminated by dialysis against water, whereupon the protein was able to couple with lactoflavine-phosphoric acid.

Resolution of β-Glycerophosphatase into an Apoenzyme and Coenzyme. von Euler and Fenó drew the solution (ex intestinal mucosa) at pH 4 to 7 through an alumina column and collected the filtrate in fractions. The first portions of the flow contained a thermostable coenzyme of low molecular weight, which was able to activate the apoenzyme component. The latter can be eluted with 1 per cent ammonia solution. In some other experiments a neutral, precipitated suspension of the starting material was poured on to the column.

Rennin. A partial accumulation of this enzyme was carried out by Schöberl and Rambacher. The commercial product (20 g.) was shaken with 0·5 *l.* of water for 2 hours, filtered, dialyzed at 0° against water for 3 days, centrifuged, diluted to 1 *l.*, and adjusted with lactic acid to pH 4. The ice-cold solution was sucked through two 8-cm. alumina (Brockmann) columns within 10 hours and developed with $M/15$ phosphate buffer (pH 5·6). The column was divided into 7 parts and each was eluted with 100 ml. of phosphate buffer pH 7. The 5th and 6th sections contained brownish zones. The relative clogging times from top to bottom were: >240, 38, *16*, 21, 125 and 173 min. It seems that this purification method could be improved further.

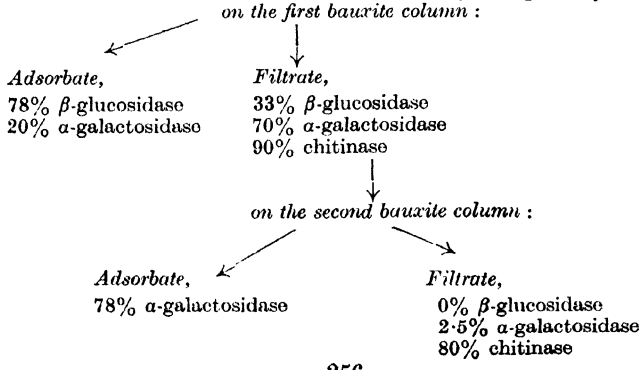
Separation of Different Enzymes Contained in the Same Extract

In the investigations reported below empirical conditions were found for the separation of enzyme mixtures which occur in crude or only slightly purified extracts of some vegetable and animal tissues. Furthermore, a chromatographic treatment was useful for a clearer definition than before of the

specificity limits in the action of some enzymes. If the relative activity of an enzyme solution on two different substrates is markedly altered by chromatography, then the presence of two different enzymes must be admitted because it would be very improbable to suppose that such effects were caused generally by the elimination of some activators or inhibitors.

The essential difference between current chromatography and this type of enzyme separations is that in the latter case both the starting material and the fractions obtained consist in overwhelming degree of foreign substances; the enzymes are only present in minute quantities. The reproducibility of such experiments depends to an unusual degree on the origin and quality of solutions and adsorbents as well as the height of the column. In the following experiments each operation was a simple filtration through an adequate adsorbent (author and Tóth, in part with Bálint, Vajda, Fürth and Bársony).

Separation of β-d-Glucosidase, α-d-Galactosidase and Chitinase of Emulsin. Under suitable conditions the greatest part of the β-glucosidase contained in Emulsin Merck can be retained on bauxite (from Hungary) while more than two-thirds of the β-galactosidase and nearly all of the chitinase reach the filtrate. The galactosidase (but not the chitinase) is then retained on a fresh column. All adsorbed enzymes can be eluted by means of very weak ammonia. The scheme of such resolutions can be given as follows:



The respective substrates for β-glucosidase, α-galactosidase and chitinase were salicin, raffinose and chitodextrin.

A bauxite-sand mixture 3 : 1 (18 g.) was lightly stamped into a tube (10 × 2 cm.) to a height of exactly 5 cm. At first under slight suction, an acetate buffer (10 ml., pH 4·7) was introduced of which 2–3 ml. reached the filtrate within half an hour (pH 6·8). Then a solution of 1 g. emulsin (Weidenhagen β-glucosidase number, 0·22) + 5 ml. of the buffer mentioned was filtered through paper and diluted to 25 ml. While the enzyme content of a parallel solution was tested, 10 ml. was poured on to the column which let 9 to 10 ml. of an enzyme-free solution through (pH 6·5) within 45 min. Further 15 ml. of the solution were then introduced : under full suction, the whole solution penetrated the column within 5 hours. The volume of the filtrate amounted then to 14 ml. ("A").

The column was extruded, roughly pulverized and shaken in a centrifuge tube with 25 ml. of N/10-ammonia for 15 min. After the addition of 25 ml. of water it was centrifuged and washed with 2 × 50 ml. of water in the centrifuge (10 min. each time). The combined solution (150 ml.) was centrifuged again (for half an hour) in order to eliminate turbidity. It was adjusted to pH 4·5 with a few drops of strong acetic acid and used for enzyme estimations.

Two filtrates "A" were combined and 13 ml. was chromatographed on 10 g. of fresh bauxite (height, 3·5 cm.) which had been moistened with 5 ml. of the acetate buffer. First, 5 ml. was introduced (its filtrate was discarded), then 8 ml. more of the solution. The filtration required an hour. The filtrate was buffered and its enzymes were tested. The column was eluted as above (13 ml. of N/10-ammonia + 1 vol. of water : washing with 2 × 25 ml. water etc.).

Separation of Emulsin Salicinase and Cellobiase. For this partial separation, aluminum oxide (Brockmann) is superior to bauxite. The solution was prepared by dissolving 4 g. of almond emulsin (Merck) in 20 ml. of N/10-acetate buffer (pH 4·6) and 80 ml. of water (centrifugation). The alumina

(9 g.) was stamped into a tube (diameter, 2 cm.) and under the action of the pump 7 ml. of $N/10$-acetate buffer (pH 4·6) was drawn through. The height of the column was 3·8 cm. Thereupon 25 ml. of the emulsin solution was introduced and passed through the column in $\frac{3}{4}$ hours (no suction). The first 7 ml. were discarded and the rest was employed for the estimations. About $\frac{2}{3}$ of the initial cellobiase potency but only $\frac{1}{4}$ of the salicinase potency was recovered.

Separation of Amygdalase, Gentiobiase, and Salicinase present in the House Fungus Merulius lacrimans. In a mortar 12 g. of the fungus, 6 g. of sand and 100 ml. of water were worked up to a homogeneous mass. After centrifugation, the residue was triturated with 30 ml. of $N/10$-acetate buffer (pH 4·6), centrifuged again, and, after the combination of the two fractions extracted for a third time. A 25-ml. portion of the solution was drawn through 8 g. of Brockmann's alumina (2 × 3·5 cm., pre-treated with 7 ml. of the acetate buffer) within 90 min. The first 7 ml. of the filtrate were discarded, and about 16 ml. used for the analysis. Found, 6 per cent of the initial amygdalase, 47 per cent of the gentiobiase and 28 per cent of the salicinase activity.

Enzymes Acting on Chitin or its Breakdown Products. The " chitinase " of the emulsin (p. 256) can be fractionated into two components, viz. chitinase which acts on chitodextrin but not on chitobiose-N-diacetate, and chitobiase of the opposite specificity. Chitinase shows a smaller adsorption affinity on bauxite than the biase. For example, 2 g. of emulsin (Merck) was triturated with 10 ml. of $N/10$-acetate buffer (pH 4·7) and 40 ml. of water, and after standing at 0° for 30 min., centrifuged. Of this solution 30 ml. were shaken with 2·5 g. of bauxite for 15 min. and centrifuged again ; no enzyme was eliminated. A mixture of 23 g. of bauxite and 13 g. of sand was filled into a tube (diameter, 2 cm.) to a height of exactly 9 cm. Under suction 7 ml. of the buffer and then 25 ml. of the solution were introduced and the first 10 ml. of the filtrate discarded. The next 13–14 ml. were used for the estimations (substrates : chitodextrin and chitobiose). The

filtrate contained 83 per cent of the initial chitinase but only 25 per cent of the chitobiase (author and Tóth 1).

The " chitinase " can be fractionated in a similar manner when hepatopancreas extracts of the edible snail (*Helix pomatia*) are used. A chromatographic filtration through an 8-9 cm. bauxite column is adequate. Many individual cases showed, however, that sometimes the chitinase, but in other cases the chitobiase was preferentially adsorbed. The technique employed corresponds about to that used for emulsin.

In one experiment the effect of the hepatopancreas extract on the following substrates was tested, before and after filtration through an 8-cm. bauxite layer (pH 4·8) : chitodextrin, chitobiose, β- and α-phenyl-N-acetyl-glucosaminid and salicin. About half of the initial activity on chitodextrin and β-phenyl-N-acetyl-glucosaminid was found in the filtrate but none of the other enzymes (author, Tóth and Vajda).

A separation of glucosaminase from β-glucosidase of *Helix pomatia* extracts was also carried out by Neuberger and Rivers. Although some experiments failed, two were successful. For example, 18 g. of a mixture of roughly ground (Hungarian) bauxite and fine sand (43 : 1) was made into a 5 cm. high column in a 10 × 2 cm. tube. Then 10 ml. of a $N/50$-acetate buffer (pH 4·7) was sucked in. As soon as its level reached the top of the column, 25 ml. of the enzyme solution was added. It is advisable to reject the first 10 ml. of the filtrate and use the rest for the estimations. The activity towards β-phenyl-N-acetyl-glucosaminid was tested at pH 4·0 and 38°. When (after action for 14 hours) the splitting power of the crude solution was : glucosidase, 100 per cent and glucosaminidase, 90 per cent, then the corresponding figures for the filtered solution were : 20 per cent and 65 per cent.

Chromatographic Experiments with Tannase (Tóth and Bársony). These investigators demonstrated that the hydrolysis of various gallic acid esters was not effected by a single enzyme but that *Aspergillus niger* extracts contain a gallic acid-esterase and a depsidase.

40 ml. of extract (pH 7·6) was run, without suction, through a flat disc of Brockmann's alumina (0·9 × 2 cm. ; 2 g.). Strong suction was applied after half an hour. The filtrate showed 0–4% of the initial esterase activity but 81–93% of the initial tannase activity (substrates : phenylacetate and methyl gallate).

Using a similar technique it was shown that the relative activities of the initial solution and the chromatographic filtrate on some other substrates were essentially altered by the adsorption experiment. The following figures indicate the amount of enzyme present in the filtrate, expressed as a percentage of the initial value, with reference to the respective substrates : methyl gallate, 50–87 ; glucogallin, 67–92 ; chinese tannin, 31–53 ; and *m*-digallic acid, 11–36%.

An artificial mixture of emulsin solution and *Aspergillus* extract was resolved on bauxite. Under favourable conditions the following fractions of the initial activities were present in the filtrate : action on methyl gallate, 90% ; on salicin, 6–9% ; and on glucogallin, 49%.

Phenolases (Enselme, Creyssel and Rapatel). Pretreated extracts obtained from potato peels (or from the mushroom, *Psalliota campestris*) can be further purified on aluminium hydroxide. Some sections of the column were eluted with disodium phosphate at pH 7·8 and then showed increased *o*-diphenolase but much decreased monophenolase activity.

Prothrombin was prepared by Munro and Munro by adsorption on aluminium hydroxide at pH 8, washing this adsorbate repeatedly by suspending it in oxalated saline, and elution with 0·2 *M* phosphate buffer at pH 8.

MISCELLANEOUS BIOLOGICAL SUBSTANCES

(*a*) The stimulating substance of **Mimosa pudica** which can be accumulated to a certain degree by precipitation methods with lead acetate and mercuric acetate, was further purified by Hesse (3). An aqueous solution of 36·7 g. substance in 100 ml. of air-free water was adsorbed on 1 kg. of alumina (Merck, puriss.) and washed with 1 l. of air-free water. Nearly the whole active material was located between a dark brown top section and a yellow zone, and could be eluted with 2 *N*-acetic acid. This product was about 10 times purer than the starting material (yield, 80–90 per cent).

(*b*) **Allergens.** Spies, Coulson, Bernton and Stevens prepared a concentrate of allergenic constituents of the *cottonseed embryo* and found that it formed a picrate with similar activity. Filtered solutions of 20 g. of this substance in 5 l. of 50 per cent warm ethanol were allowed to flow through Brockmann's alumina (18·8 × 4·4 cm.). Development with the same solvent resulted in the formation of two yellow zones. Elution was done by repeated stirring with *N*/20–NaOH. The content of the two zones could not be differentiated by clinical or immunological tests. The chromatographic filtrate was inactive. A partial accumulation of the allergen in aqueous *pollen* extracts obtained from *Calamagrostis epigeios* was carried out by P. Westphal, using alumina. Among a number of zones an intensely greenish fluorescing one proved to be strongly active.

(*c*) **Sex Hormones in Achlya** (Raper and Haagen-Smit). Hormone "A" which is contained in the female plants of *Achlya ambisexualis*, a fungus, can be adsorbed on alumina from water, ether, chloroform or CCl_4. It is eluted by acetone which leaves inert material in the column.

(d) Haller, Acree and Potts were able to fractionate on alumina the **attracting principle** in benzene extracts prepared from the abdominal tips of virgin female gypsy *moths* (*Porthetria dispar*). In an experiment, the first and second

percolates caught 114 and 26 male moths respectively while the third had no attractive power.

(*e*) **Hypertensin** which is able to raise the blood pressure under certain conditions, can be adsorbed from methanol on alumina and eluted with aqueous methanol (Edman).

(*f*) **Chorionic Gonadotropin** (Katzman, Godfrid, Cain and Doisy). Chilled and filtered pregnancy urine (brought to pH 3·5 with glacial acetic acid) was adsorbed on permutit The length of the column is important. A 38 × 10 cm. column (= 2 kg.) can take 700 litres (= 16 million rat units) at the rate of 10 l. per hour. The adsorbate is washed with water until the filtrate becomes neutral and colourless. Washings with 76 per cent ethanol, then with the same solvent containing 10 per cent ammonium acetate remove coloured impurities. The hormone is then washed down with 38 per cent ethanol containing 10 per cent ammonium acetate. The active material can be precipitated by increasing the alcohol concentration.

(*g*) **Oxidized Insulin,** obtained by a treatment with performic acid, was submitted to frontal analysis by Tiselius and Sanger.

(*h*) **Thyroglobulin** has been resolved into several fractions, containing various amounts of thyroxin, by Rivière, Gautron and Thély. A 5 per cent solution was adsorbed on aluminium hydroxide, freshly precipitated on a kieselguhr support. The empirically divided sections were eluted at pH 2 : the respective fractions were purified in the form of their picrates and liberated with ammonia.

(*i*) The chromatographic purification of *tuberculine* was unsuccessful because of difficulties in the elution (Gözsy and Vásárhelyi).

CHAPTER XXII

APPLICATIONS TO TECHNOLOGY

Foods

Boekenoogen (3) published a short survey on the adsorption methods used in the technology of fats. The bleaching of fats by adsorbents is discussed by Yamamoto, and the elimination of vegetable oil pigments on fuller's earth by Kulkarni and Jatkar. For the estimation of vitamin A in foods, cf. pp. 221–3.

Detection of added Artificial Dyes in Foodstuffs. (Thaler and Schulte 1). Salmon: Added tar dye is not adsorbed on alumina from light petroleum, while the native red pigment shows a sharp chromatogram. The opposite occurs in pyridine solution. For such tests 3 g. of material were treated with 30 ml. of solvent at 30° for 2 hours.

Egg powder or doughs containing it: From extracts obtained with 70% acetone only tar dyes are adsorbed in sharp zones on alumina while the carotenoid pigments of the yolk can be easily washed through the column.

Canned tomatoes: Sometimes unripe tomatoes are canned and a water soluble red dye is added. It can be detected by using 70% acetone as above.

Benzene extracts of oils, vegetables, fish, etc., were tested by Balavoine on alumina, on which the fluorescence of added dyes was also observed. Synthetic dyes in fruit juices remain near the top of an alumina column (Mischon); cf. also Koslow; capillary analysis: Taub and Ortega y Canet. Bixin is easily detected in butter (MgO, light petroleum; van Duuren).

Cane Juice and Molasses. From acetylated juice α-and β-glucoes pentaacetate, β-fructose tetraacetate and sucrose octaacetate were isolated by developing with benzene + 1 per cent abs. alcohol on magnesol + celite 5 : 1, and brushing with alkaline permanganate (Binkley and Wolfrom 1). Some inositol can be obtained from sugar cane juice or blackstrap molasses by similar procedures (Binkley, Blair and Wolfrom). From beet molasses or cane blackstrap molasses pure sucrose

was prepared by adsorption on florex XXX + 20 per cent celite by developing with 95 per cent alcohol (Binkley and Wolfrom 3).

Black Tea (Valentin 3). If 10 ml. of extract (2 g. of tea, 100 ml. of boiling water) is adsorbed on alumina (Merck; diam., 0·5 cm.), several coloured zones appear, followed by a bottom caffeine zone (violet fluorescence in ultraviolet light). The latter can be quantitatively washed into the filtrate with 40 ml. of water from which, after transfer into chloroform and evaporation, the base crystallizes. In large scale experiments *l*-malic acid was also found. When the column was washed with acetone, vanillin crystals were obtained from the filtrate.

Green Tea. The polyphenol fraction of green tea was investigated by Bradfield, Penney and Wright. From an infusion chloroform removed caffeine, chlorophyll degradation products, etc. After the elimination of this solvent, the residue was extracted with ethyl acetate and the evaporation residue of the latter (orange powder, 2 g.) was repeatedly rubbed with wet ether. Finally, the solution was partition chromatographed on silica gel (45 × 3 cm.). One of the filtrate fractions (700 to 1,000 ml.) yielded crystalline *d*-gallocatechin (probably), and the next one, *l*-gallocatechin.

Wine. The residue left on vacuum evaporation of red wine samples, when extracted with hot acetone and adsorbed on activated magnesia gives, in addition to a blue anthocyanin zone, yellow zones (probably flavones) and in some cases also a green zone (alkaline form of flavone or chlorophyll ?). White wines yield yellow zones only. The chromatographic filtrates contain quercetin (Gentilini).

Pharmacy, Drugs and Galenicals

Individual Compounds. Barbiturates are readily adsorbed from urine on suitable charcoals (Paget and Tilly). A benzene solution of free mepacrine base can be purified on alumina (Welsh). Quinosol in alcohol can be recognized on alumina by its yellowish-green fluorescence which, however,

is shown also by other hydroxyquinolines (Hoffmann). Concerning a spotting method for stilbamidine and mepacrine (atebrine) cf. Henry and Grindley.

A method of estimating prontosil solubile in total blood or plasma (made non-clotting with "liquoid" Roche and haemolyzed with HCl) can be based on the following principle (Stelzer). A talc column retains the prontosil and a part of the blood pigments while the greater portion of the latter, as well as the serum colloids and salts, pass into the filtrate. Prontosil and some pigments are washed into the filtrate with $N/10$-NaOH. This brownish filtrate is adjusted to $N/40$-HCl and filtered through a frankonite column which does not retain the prontosil.

Drugs, Galenicals etc. For the estimation of *alkaloid* salts by titration of the free base in chromatographic filtrates, it is essential that the adsorbent should firmly bind the acid component but let the alkaloid pass. Alumina samples (5 g.) can be tested by shaking them with 20 ml. of a 1% procaine hydrochloride solution and titration of the filtrate (bromophenol blue). For the estimation of alkaloids in *Tinctura* or *Extractum Nucis Vomicæ* (Christensen 1), the mixture of 25 g. of tincture and 15 g. of absolute alcohol is passed through 10 g. of alumina (diameter, 1 cm.). After washing with 15 ml. of alcohol and dilution with water, the titration of the base is carried out against bromophenol blue. Half a gram of extract is sufficient for an analogous estimation (Christensen 1, 2; Reimers, Gottlieb and Christensen). Cf. also Ulrix.

Similar methods were used by Björling (1, 2), and, for homeopathic tinctures of *Hydrastis*, *Berberis*, *Colombo*, *Chelidonium*, etc., by Neugebauer and Brunner. A number of alkaloids were also estimated by Reimers and Gottlieb (cf. Reimers) by sucking the 90% alcohol solution of their hydrochlorides through alumina and titration of the free base after the addition of 1 vol. of water. The method was unsuccessful with the following alkaloids: narcotine, papaverine (cannot be titrated in 50% alcohol); amphetamine (the base is adsorbed too strongly); apomorphine (the whole salt passes

through) etc. On the other hand, the method is applicable to percaine, eukodal or pantocaine.

As was recently pointed out by Björling (3), if an alkaloid salt with a polyvalent acid is chromatographed on Brockmann's alumina and washed into the filtrate with alcohol, then much less than 100% of the alkaloid base can be recovered. This was the case, for example when procaine sulphate, tartrate, citrate or oxalate was used, while the base liberated from the hydrochloride by the alumina passed through quantitatively as shown by titration. This anomaly can be prevented by the addition of 2*M*-HCl (equivalent to the total base) before chromatography or by the use as eluent of alcoholic solutions of LiCl, $LiNO_3$, NaI, NaBr, KI, NH_4Br or $MgCl_2$ instead of pure alcohol. The method was applied to codeine phosphate, atropine sulphate, dicodid bitartrate, ephedrine sulphate, benzedrine sulphate, and oxedrin tartrate.

Ergot. A. Kofler determined the ergotamine and the (non-effective) ergotaminine content in Gynergen tablets (Ergotamin Stoll) by washing with chloroform on alumina. Ergotaminine appeared in the filtrate first. In order to estimate the ergometrine and ergotoxine alkaloids, Fischer-Jörgensen and Tönnesen developed 5 ml. of the fluid extract on alumina Merck (11 × 2 cm.) with 50 ml. of ether. From 15 ml. of the chromatographic filtrate the ergometrine group was extracted by shaking six times with phosphate buffer pH 6·8; and the ergotoxine alkaloids by shaking a 15-ml. sample three times with 1% tartaric acid solution. In both fractions the alkaloids were estimated photometrically, after the addition of *p*-dimethylamino-benzaldehyde.

Colchicum. The alkaloid content was estimated by Mühlemann and Tobler, after having adsorbed 50 g. of the tincture on a 30 × 1·5 cm. alumina column which was then eluted with alcohol.

Frangula. For the isolation of emodin contained in fluid extracts see Fischer-Jörgensen.

Digitalis extracts can, after preliminary operations, be purified from chloroform on alumina without the loss of digitalin (Lecoq 1).

Extractum filicis, prepared according to the Swiss Pharmacopœia, shows great variations in the chromatogram obtained from ether on alumina (Fichler).

Colour reproductions of the chromatograms of most of the tinctures of the Pharm. Danica have been given by Christensen and Jensen.

Chromatography in forensic medicine : Frache.

Insecticides

Natural Products. Norton and Hansberry (cf. also Norton) divided the ether extract of Mexican Yam Bean (*Pachyrrhizus erosus*) seeds into a non-toxic oil and a toxic resin. A 6% benzene solution of the latter, when developed with benzene on alumina, gave three groups of strongly retained zones and three filtrates. The latter were collected (A) from the appearance of a blue fluorescence in the filtrate until the appearance of a positive Meyer colour test ; (B) to the appearance of a yellow colour and a blue-green fluorescence ; and (C) to the disappearance of the Goodhue test. Upon concentration and fractional crystallization of (C), first, a non-toxic compound, then rotenone, $C_{23}H_{22}O_6$, and, finally, a strongly toxic rotenoid, $C_{20}H_{16}O_6$, were isolated ; for further details, the original paper should be consulted. In all, six well-characterized compounds were obtained, but some heterogeneous fractions also showed toxicity.

Rotenone was isolated from the seeds of the African berebera tree (*Millettia ferruginea*), and the residual mother liquors were worked up by Clark. Upon the addition of alkali, a crystalline product was obtained that could not be resolved by further crystallizations. Chromatography on alumina from chloroform and then from benzene was tedious, as the adsorption had to be repeated many times, using the liquid chromatogram method. Finally, however, tephrosin, m.p. 198°, was obtained. If an artificial mixture of this compound and deguelin in chloroform is passed through alumina,

pure deguelin appears first in the filtrate. A neutral *Derris elliptica* resin, from which the main bulk of rotenone had been removed, was adsorbed from benzene on alumina (moistened with 1% phenol in benzene in order to bind the free alkali), and the column was developed with benzene + acetone (1 : 1). The first filtrate contained rotenone, etc., the second elliptone (Harper). Cf. also Meijer and Koolhaas.

DDT (*p,p'*-dichloro-diphenyl-trichloroethane), $(Cl.C_6H_4)_2CH{\cdot}CCl_3$. "Technical" DDT was resolved by Haller, Bartlett, Drake, Newman, Cristol, Eaker, Hayes, Kilmer, Magerlein, Mueller, Schneider and Wheatley who obtained an "Oil D" by methods other than chromatographic adsorption. Ten grams of this oil dissolved in 25 ml. of CCl_4 was poured on to a 20 × 3 cm. activated alumina column which was developed with 100 ml. portions of carbon tetrachloride (twice ; D-1 and D-2), benzene, (D-3), benzene + 5% ethanol (D-4), and absolute ethanol (D-5). Upon recrystallization and refractionation these main portions yielded : (D-1) = *p,p'*-DDT and *o,p'*-DDT ; (D-3) = *p,p'*-DDD (1 : 1-dichloro-2 : 2-bis-[*p*-chlorophenyl]-ethane) ; (D-4) = $C_{14}H_9O_3Cl_5S$; (D-5) = $(Cl{\cdot}C_6H_4)_2SO_4$ and $C_{14}H_9O_3Cl_5S$ and $Cl{\cdot}C_6H_4{\cdot}CHOH{\cdot}CCl_3$.

The isolation of the *o,o'*—DDT isomer from commercial DDT has been described by Cristol, Soloway and Haller. The procedure also involves a number of operations other than chromatography. The yield was 0·1%.

Smokeless Powder

Schroeder (2) has described the following routine method for the determination of diphenylamine (the stabilizer) and diethyl phthalate (the plasticizer) in an American smokeless powder. The subdivided powder was extracted in a Soxhlet with methylene chloride for several hours to remove diphenylamine, diethyl phthalate, and nitroglycerin from the insoluble nitrocellulose. Appropriate portions of the extract were taken and diluted with light petroleum so that for the determination of diphenylamine (see below) approximately 1 mg. of diphenylamine was contained in 10–15 ml. of methylene chloride +

light petroleum (1 : 4) and, for the determination of diethyl phthalate, 15 mg. of this compound was present in 15-20 ml. of a 1 : 1 solvent mixture.

Determination of diphenylamine. The sample was chromatographed on a 15 × 2·5 cm. 4 : 1 silicic acid + Celite column which had been pre-washed with 55 ml. of anhydrous ether and 110 ml. of light petroleum. The zone of diphenylamine was developed to the middle of the column with about 50 ml. of 1 : 4 benzene + light petroleum and then with 75 ml. of light petroleum, whilst diethyl phthalate and nitroglycerin remained strongly adsorbed. A 1% solution of sodium nitrite, in conc. sulphuric acid was used to locate the diphenylamine by streaking from the top and bottom of the extruded column until the *limits* of the zone were detected (blue colour). This streaking was done by permitting the reagent to flow from a dropper down a glass rod. (In order to determine that the separation from nitroglycerin was satisfactory, its zone was located using a 1% diphenylamine solution in conc. sulphuric acid.) The diphenylamine zone was eluted with ether and estimated spectrophotometrically.

Determination of diethyl phthalate. This compound was isolated by chromatographing on a 15 × 1·9 cm. column of 2 : 1 silicic acid + Celite which had been pre-washed with 30 ml. of anhydrous ether and then with 60 ml. of light petroleum. Nitroglycerin and diphenylamine were washed into the filtrate with 50 ml. of 1 : 1 benzene + light petroleum and 50 ml. of light petroleum. The upper half of the column which contained the diethyl phthalate (for which no streak reagent was available) was eluted with 100 ml. of anhydrous ether; this solvent was evaporated, and the residue was taken up in alcohol for spectrophotometric estimation.

The maximum spread of duplicate determinations of either compound in separate samples of the same powder was only 3 per cent of the amount present.

Tannins

The adsorption of natural and synthetic tannins on columns composed of hide powder was studied by Pollak and Patzenhauer who described some fluorescence chromatograms.

Earth Oils, Asphalts, Bitumens, Lignites

One of the important analytical methods of the petroleum industry is the estimation of aromatics and some olefins in mixtures with paraffins and naphthenes. This is, according to Mair (1), conveniently carried out using silica gel and employing the liquid chromatogram principle. The diagnosis of each fraction is made on the basis of its refractive index. The sequence is: aromatics (top, last eluted); monoolefins; paraffins + naphthenes. The refractive index shows a sudden rise when the aromatic material begins to appear in the filtrate.

The usual chromatographic device consists of two tubes sealed together, the upper, measuring 50 × 2·2 cm., and the lower, 75 × 1 cm. This apparatus permits the separation of about 20 ml. of aromatic hydrocarbons from a mixture. It is equipped with a jacket and cooled to 0°. Ethyl alcohol seems to be the best developer.

On a larger scale, the hydrocarbons contained in the gasoline and kerosene fractions of petroleum can be separated into two portions, viz., aromatic hydrocarbons, and paraffin plus naphthene hydrocarbons (Mair 2; further literature in the original paper). The mixture is adsorbed on a silica gel column and then paraffin and naphthene hydrocarbons can be eluted with methanol. For example, when 15 kg. of silica gel (28–200 mesh) were used in a 300 × 10 cm. tube, 0·5 to 1 l. of aromatics could be separated, the refractive indices being used as a guide. The method can be conveniently modified as follows. The hydrocarbon mixture is introduced into the column and when the level has just reached the surface, ethanol or isopropyl alcohol is added in order to achieve fractional desorption. The paraffins + naphthenes emerge first in the filtrate, followed by the aromatic fraction. On a small scale this method was also used for the separation of mono-olefins from paraffins and aromatics; however, at room temperature considerable polymerization of the olefins may take place; this can be diminished, but not eliminated, by working in nitrogen and at low temperature.

In a recent communication by Hirschler and Amon the preparation of high-purity hydrocarbons of various types is described in detail, using either commercially available or synthetic mixtures. For the desorption from silica methanol or ethanol, and for the desorption from active carbon benzene was used. The diagnosis of the filtrate fractions was made on the basis of the refractive index.

For the detection of dimethyl yellow in *vaseline* Karlovitz recommends that the benzene solution be shaken with bolus alba ; the suspension turns red if the dye is present.

In the analysis of oil soluble *petroleum sulphonates* the mineral oil content can be estimated by filtration through a clay column (Brooks, Peters and Lykken).

For a technical analysis of *asphalts* etc., a resolution of the extract into typical fractions may be of use. Grader recommends that 1 g. of material in 50 ml. of CCl_4 be adsorbed on alumina (activated with calcium bicarbonate ; $70 \times 2{\cdot}5$ cm.) and that the column be washed in turn with 800 ml. of the same solvent (easiest soluble fraction) ; then with 1500 ml. of benzene (earth oil resins) ; and with 1500 ml. of chloroform (soft asphalts). The hard asphalt remains in the tube and is estimated by difference. In a similar manner Mukherjee and Indra differentiated various types of crude oils, refined oils and artificial mixtures. Their fractions were also characterized by fluorescence.

By chromatography of light petroleum extracts of *lignite* on calcium hydroxide, needles of a highly reducing potassium salt, and a crystalline terpene were isolated (author and Frehden ; author and Stewart).

Sulphur can be partially removed from crude lignite oil by adsorption on silica gel (Hofmeier, Wisselinck and Müller).

Partial Resolution of Coal Hydrogenation Products (Neuworth). From the hydrogenation distillate (60 g.) the material was accumulated on a short column of activated alumina + super cel (1 : 1), eluted with benzene, evaporated and adsorbed from light petroleum on a $50 \times 4{\cdot}8$ cm. column. When

developed with 1 l. of the same solvent, the following four zones appeared in ultraviolet light: Zone IV (top), orange-brown fluorescence, contained two-thirds of the N and nine-tenths of the O of the distillate; this zone included the most highly condensed aromatic compounds. Zone III, yellowish green fluorescence, qualitatively similar to IV. Zone II (essentially hydrocarbons), blue fluorescence, contained mainly aromatic hydrocarbons with three or more condensed rings. Zone I (hydrocarbons), colourless in ultraviolet light, was a mixture of paraffins, naphthenes and aromatics with various molecular weights.

CHAPTER XXIII

INORGANIC CHROMATOGRAPHY

Recent work in this field has dealt mainly with the separation of cations. A short review of the microanalytical applications was given by Beaucourt and Masters. For studies on moving boundary systems formed by strong electrolytes, especially salts, cf. Longsworth as well as Dole. An electrochromatographic separation method was outlined by Lecoq (3).

Separation of Cations

In spite of many efforts, no generally applicable analytical method is available for the resolution of cation mixtures and the quantitative estimation of the individual metals. However, a chromatographic filtration, in order to eliminate interfering heavy metal ions, seems to have gained practical importance. For example, traces of iron contained in commercial caustic soda solutions can be eliminated by adsorption on strontium salts (Caldwell and Boyd, cf. Pinterović). In luminescence chemistry the chromatographic purification of materials like the " Lenard phosphor " (sulphide mixtures of zinc, magnesium and alkaline earths) was found to be particularly effective (Tiede and Schikore). By filtration through alumina, iron as well as the phosphorogenic metals, copper and silver, are eliminated ; the two latter are eventually replaced by definite quantities of copper and silver. All of these heavy metals are more strongly adsorbed on alumina than is zinc.

An extremely pure aluminium hydroxide, suitable for the chromatography of heavy metals was prepared from the ethylate by Schmäh. For noble metals on carbon, cf. Dubrisay.

A special technique was outlined by Karschulin and Svarc.

Traces of copper can be accumulated on cotton wool which has been saturated with sodium stearate in alcohol and dried (Seekles, Havinga and Bijkerk). For gold, cf. Stark.

Separation of Cations on Alumina according to Schwab.

This matter has been thoroughly discussed by Jacobs and Tompkins (1–3), who investigated the rate of advance and width of the zones and related problems. They pointed out that commercial alumina is an amphoteric ionic adsorbent which is able to exchange both cations and anions. Generally,

anions and cations are not adsorbed in equivalent amounts, the latter being preferentially retained. A sodium-cation exchange must be held responsible for this phenomenon, due to the presence of sodium aluminate in the adsorbent.

With reference to the chromatographic sequence of metals on alumina, as earlier given by Schwab and Jockers (somewhat extended by Venturello and Agliardi), it must be stressed that this sequence is subject to alterations if the pH is modified (cf. below the inversion of the arsenic-antimony sequence as observed by Schwab and Ghosh 1). Further inversions may be caused by changing the anion (Jacobs and Tompkins 2).

If a single cation is present, the limits of microanalytical detection are remarkably low (Schwab and Ghosh 2 ; Table 21). For example, when 0·01 ml. of a solution containing 0·5 μg. of ferric ion was developed with one drop of ferrocyanide + one drop of HCl on a 1–2 mm. column, a definite colour resulted. However, in practical metal analysis the Schwab method cannot be used for a complicated mixture of cations. Schwab and Ghosh (1), therefore, compromised by combining the classic group-precipitation method with chromatography. The latter is then used within the current analytical groups only. Even so the results are of restricted applicability ; they are relatively best in the $(NH_4)_2S$ group of metals.

TABLE 21

LIMIT OF DETECTION OF SOME CATIONS ON THE ALUMINA COLUMN (SCHWAB AND GHOSH)

Cation	Reagent	Limit of detection (μg.)
Fe^{+++}	$K_4[Fe(CN)_6]$	0·01
Cu^{++}	,,	0·02
Cu^{++}	H_2S	0·4
Co^{++}	α-nitroso-β-naphthol	0·06
Co^{++}	$(NH_4)_2S$	0·2
Ni^{++}	,,	0·3
Ni^{++}	rubeanic acid + NH_3	0·04
Tl^{+}	KI	0·12
UO_2^{++}	$K_4[Fe(CN)_6]$	0·45
Ag^{+}	$(NH_4)_2S$	1·0
Ag^{+}	p-dimethylamino-benzylidene rhodanin	0·02
Pb^{++}	$(NH_4)_2S$ or K_2CrO_4	0·54
Cd^{++}	$(NH_4)_2S$	0·54

H_2S group. The earlier procedure has been modified by acidifying the solution with tartaric acid instead of HCl. Thus, the reported sequence on alumina, As^{+++} (top) and Sb^{+++}, has been inverted. Furthermore, most of the Bi^{+++} is now adsorbed together with Sb^{+++}; Hg^{+++} and Sn^{++++} could not be handled at all and small amounts of Cd^{++} could not be separated from Zn^{++}. An example of a good resolution is provided by Sb^{+++}, As^{+++} and Cd^{++} in tartaric acid solution. When the column was washed with an aqueous solution of tartaric acid, the following zones were formed: orange (top): Sb^{+++}; yellow: As^{+++}; interzone; yellow: Cd^{++}. The colour of the two upper zones disappears on elution with ammonium sulphide.

$(NH_4)_2S$ group. A mixture of Fe^{+++}, Cr^{+++}, UO_2^{++}, Zn^{++}, Co^{++}, Ni^{++} and Mn^{++}, in the form of chlorides, nitrates or sulphates was washed with water on the column and treated with ammonia. Finally, air was sucked through the column in order to oxidize Mn to MnO_2. The top to bottom sequence was:

brown:	Fe^{+++}
green:	Cr^{+++}
yellow:	UO_2^{++}
white:	Zn^{++} (cannot be differentiated from Al^{+++})
pink to reddish-yellow:	Co^{++}
bluish green:	Ni^{++}
dark brown:	MnO_2

HCl-group. (Hg^{+} cannot be handled.) A mixture of Pb^{++}, Ag^{+} and Tl^{+} (nitrates), washed with water and treated with potassium chromate, gave:

(top)	yellow:	Pb^{++}
	red:	Ag^{+} (with some Tl^{+})
	yellow:	Tl^{+}

Only a beginning has been made so far in *quantitative* cation estimations by chromatography. Schwab and Dattler (2) found the breadth of zones of copper sulphate and of cobalt sulphate to be proportional to the quantities of the cations within wide concentration limits.

Disturbing complications may occur, however, when different anions are present on alumina. For example, an

equimolecular mixture of copper nitrate and cobaltous sulphate gave *two* cobalt zones of different colour below the copper zone. Furthermore, excess copper nitrate caused the appearance of two copper zones and a single cobalt zone. No sulphate ion was present in the washings. Evidently, a fraction of the anions was also retained. Schwab and Dattler suggested that aluminate double salts might be formed. However, a simpler interpretation of the presence of the extra zones is that the adsorption of cupric ions is greater from a sulphate solution than from an equivalent nitrate solution. Hence, in some instances a narrower but more intensely coloured zone will also appear (Jacobs and Tompkins 2, 3). The latter authors also pointed to the loss of sharpness of the boundaries during development and the strong "tailing", as a possible cause of the inaccuracies of the estimation, especially, since empty interzones are not formed between the individual cation adsorbates.

Some experiments with *rare earths* on Brockmann's alumina were carried out by Erämetsä as well as Erämetsä, Sahama and Kanula. They observed that the adsorbability of Y is stronger than that of the lanthanides proper. Within the latter series the addition of citric acid caused inversion of some of the sequences. According to Croatto (1, 2), Ce^{+++} is retained more strongly than La^{+++} by alumina.

Separation of Cations on Complex-Forming Adsorbents (Erlenmeyer and Dahn; Erlenmeyer and Schoenauer; Erlenmeyer and Schmidlin).

On 8-*hydroxyquinoline* (mixed with 1–2 parts of starch or kieselguhr; 8 × 0·3 cm.), when an aqueous solution of some acetates is developed with water, the following sequence appears (this was established by working mostly with pairs of ions):

(top) grayish black: VO_3^-
yellow: WO_4^{--}
green: Cu^{++}
yellow: Bi^{+++}
green: Ni^{++}
reddish: Co^{++}
yellow (green fluorescence): Zn^{++}
black: Fe^{+++}
reddish-orange: UO_2^{++}

This sequence is dependent on the pH ; e.g., in acetic acid solution Fe stands above Zn. The separation of Co and Ni becomes possible only if the column is developed with acetic acid.

The same principle was combined by Hopf with the filter paper technique in the form of spot tests as follows.

The paper is loaded with colloidal alumina by precipitation of sodium aluminate with acetic acid and washing. Then the reagent is soaked in and the paper is dried. For example, when a 5 per cent 8-hydroxyquinoline solution in 10 per cent acetic acid has been used, spotting with a ferromolybdenum solution will show a yellow central area (Mo) and a black outer zone (Fe). The former is precipitated and adsorbed as a complex ; it displaces the iron whose ion migrates and forms a visible complex only on drying.

Goto and Kakita recommend soaking filter paper with a suspension of aluminium hydroxide, drying, dipping in the solution (containing the cation) and developing with oxine which forms fluorescent complexes with Al, Zn, Cd, Mg, Ca, Zr and Be.

On a *violuric acid* (+ starch) column the following cases of distinct separation were observed upon developing the acetates with water (Erlenmeyer *et al.*).

(top) violet : K^+	(top) violet-red : Ba^{++} or Sr^{++}
red : Ca^{++}	red : Ca^{++}
brick-red : Mg^{++}	
red-violet : Na^+	
(top) green : Cu^{++}	(top) green : Cu^{++}
brown : Pb^{++}	yellowish : Hg^{++}

Some other pairs could not be satisfactorily resolved.
Separations on violuric acid-starch paper : Hopf.

A method of separating *potassium* and *sodium* ions can be based on complex formation with *violuric acid* or 5-*oxo*-4-*oximino*-3-*phenyl-isoxazoline*. For example, a $N/4$-mixture of the two acetates is easily resolved ; a yellow top zone of K appears, followed by a reddish Na-zone of which only the latter can be washed rapidly with water into the filtrate. Both alkali metals are retained if the top half of the column is composed of the isoxazoline derivative and the bottom half of

violuric acid (both halves mixed with 1-2 parts of starch). In a 5 × 0·25 cm. tube $N/50$ solutions can be handled.

Some attempt at quantitative estimation of K and Na has been made by measuring the maximum breadth of their zones after prolonged development with water. While a rough estimate seems to be possible in this manner with a few micrograms of substance in a tube of 0·14 cm. inner diameter, micro-determinations are not practical since it takes 1–2 days until the coloured zones attain their maximum breadth.

The complexes mentioned have also been studied by G. Robinson.

Further complexes. Dithizonates of some heavy metals can be adsorbed from chloroform on alumina forming characteristic coloured zones (Erametsä 3).

For the removal of traces of cerium Vickery recommends the addition of *p*-phenetidine (saturated, in water) which forms a violet complex, and subsequent adsorption on decolourizing charcoal. This adsorbate can then be eluted with chloroform.

Micro-chromatographic detection of cadmium was recommended by Korenman and Krainova by adding excess KCN and filtering through silica gel on which only the Ag and Cd complexes are retained of those which can be decomposed by ammonium sulphide.

Brush Method on Alumina. An ideal procedure for the qualitative analysis of metal ions would be, after adsorption, the use of specific colour reagents, by means of which each cation is detected by drawing a number of parallel streaks on the same column. (A clear separation into zones is not always required for this purpose). Only a small advance has so far been made in this direction ; this permits the simultaneous detection of a limited number of certain metals in the same solution (author and Frehden 2).

Alumina columns (purissimum, Merck ; 12 × 2 cm.) were used. The chromatograms were developed with water, and, after having washed the wet column with light petroleum in order to facilitate extrusion, the various reagents were applied along the main axis by means of brushes. In the

following examples, 2 ml. of a 5% solution of the nitrate of each metal was used. Care must be taken to avoid the formation of basic salts on the alumina by adding acid. Examples :

(*a*) Ferric, cupric, colbaltous and nickel nitrates, developed with 100 ml. of water. Potassium ferrocyanide brush : Fe, blue ; Cu, brown ; saturated ammonium rhodanate (in acetone) brush : Co, greenish-blue ; dimethyl glyoxime brush ; Ni, red.

(*b*) Uranyl, silver and cadmium ; 10 ml. of water. Potassium chromate (1% in dilute acetic acid) brush : Ag, dark red (in uviol light, black) ; hydrogen sulphide (in dilute acetic acid) brush : Cd, yellow (intense orange fluorescence). Without brush : greenish fluorescent zone in uviol light : UO_2.

(*c*) Mercury, lead and silver ; 25 ml. of water. Potassium chromate (1% in dilute acetic acid) brush : Hg, dark orange (top zone) ; Pb, light yellow ; Ag, dark red (bottom).

Separation of Cations on Paper Impregnated with an Adsorbent. This method was recommended by Flood (1–4 ; Flood and Smedsass 1–3 ; Flood and Risberg). The adsorbent is aluminium hydroxide which contains sodium aluminate, precipitated on thick blotting paper strips.

The paper is first impregnated with sodium aluminate solution (prepared by the addition of aluminum nitrate solution to conc. NaOH solution until a precipitate begins to appear). It is then dried and dipped into saturated sodium bicarbonate solution which converts most of the aluminate into hydroxide. After washing with distilled water for several days, the paper is suspended for a few days. It is kept in closed bottles and used in the form of 0·5–1 cm. broad strips. First, 1 ml. of water is sucked in, followed by the solution (0·1 to 0·01 ml. ; >0·001 molar) ; finally, the chromatogram is developed with water, which should rise 10–15 cm. high. It is advantageous to clamp the strip between two glass plates. The detection of the cations can be carried out by the brush method.

Flood has also discussed the resolution of metal cation mixtures and determined a number of exchange equilibrium constants. On this basis the shape of the chromatograms could be predicted, including those obtained with complexes (unpublished).

Separation of Cations on Exchange Adsorbents. This field is rapidly expanding as demonstrated by Applezweig (3) in his recent survey. Some pertinent theoretical discussions were given by Bray. For data concerning the fixation of Ca, Ba, Zn and Pb on zeolites, see Dolique and Macabet, and for the partial fractionation of rare earths on the same adsorbent, cf. Russell and Pearce.

In most of the recent work *synthetic exchange resins* were used rather than zeolites. Several investigations were made of the factors governing the rate of adsorption of cations on such resins. Boyd, Schubert and Adamson formulated equations concerning the heterogeneous base-exchange reactions both according to the Langmuir adsorption mechanism and the mass action law. They found that the adsorption affinities are determined chiefly by the radii of the hydrated ions in the solution and by their charge. On Amberlite IR–1 the sequence of exchange adsorption affinity is: $La^{+++} > Y^{+++} \gg Ba^{++} \gg Cs^{+} > Rb^{+} > K^{+} > Na^{+} > H^{+} > Li^{+}$. Furthermore, it was found experimentally by Boyd, Adamson and Myers, Jr. that two rate processes are responsible for the adsorption velocity. In case the total electrolyte concentration is 0·1 *M* or greater, the diffusion in and through the particle is decisive. However, in case of 0·003 *M* or less, the adsorption rate is limited by diffusion through a liquid film at the surface of the particles.

Examples of the use of organic exchangers. The alkali metal content of a vanadate solution can be estimated according to Samuelson (1) by filtration through a resin which has been saturated with ammonium ions. After washing, the alkali metals were eluted with HCl. The exchange of silver ions by H-ions on a sulphonated organic adsorbent was tested by Sillén and Ekedahl and compared with a theoretical treatment given by Sillén. Traces of Cu in milk products can be accumulated (after treatment with perchloric acid) on Amberlite IR–100 (Cranston and Thompson). The exchange of cations applied as acetates or oxalates was investigated by Djurfelt, Hansen and Samuelson.

Separation of Rare Earths on Exchange Resins. An astonishing development took place in this field in connection with the Manhattan Project, some results of which were published recently. Only a very brief outline can be given here of this fundamental work which has demonstrated the clear superiority of the ionic exchange method over the classical fractionation procedures. Detailed equilibrium studies of the exchange process between rare earth complexes and resins were carried out by Tompkins and Mayer.

Amberlite. The successful separation of rare earths was made possible by the observation that complexes formed with some organic acids can be differentiated by fractional elution from the cation exchanger. It was also shown that the sequence of elution of rare earth ions from Amberlite IR–1 is the reverse of the atomic number if buffered citrate solutions are used (Marinsky, Glendenin and Coryell). The same authors succeeded in the chemical identification of radio-isotopes of neodymium and of element 61.

According to Tompkins, Khym and Cohn a mixture of fission-produced radio isotopes including some rare earths can be resolved by using Amberlite IR–1 columns. Zr and Cb are removed quantitatively by means of a 0·5% solution of oxalic acid while one of the tri- or divalent elements reaches the filtrate. Excellent results were obtained applying ammonium citrate-citric acid solutions at controlled pH. At pH 3 the trivalent fission species (and also Zr and Cb) are removed from the exchanger which takes place for the remaining di- and monovalent species at pH 5. By a closer variation of pH, Y and Ce can be fractionally obtained. By methods, in principle similar to those mentioned, the separation of the individual rare earths was also successful.

Following a similar principle, the conditions for the quantitative separation of cerium and yttrium, which were obtained in spectroscopically pure state, were worked out by Spedding, Voigt, Gladrow and Sleight; and for the separation of neodymium and praseodymium by Spedding, Voigt, Gladrow, Sleight, Powell, Wright, Butler and Figard. The

tracer technique was also of substantial help. The procedures for the large scale (pilot plant scale) separation of the rare earths, using Amberlite IR–100 and 0·5% ammonium acid citric acid solutions, have been described by Spedding, Fulmer, Butler, Gladrow, Gobush, Porter, Powell and Wright.

Purification of zirconium (Ayres). When crude zirconium nitrate (from ores) is dissolved in water, the resulting system contains a colloidal hydrated oxide of this metal which is not subject to ionic exchange, while contaminant metals such as iron, beryllium, rare earths and titanium are present as ions. If Amberlite IR–100 is used in its H^+ form, all the positive ions mentioned are exchanged and the effluent contains, after a single passage, 97%, and after a second passage 99·4% pure zirconium in an acid medium.

Dowex. For the separation of several earths of the cerium group, viz., La, Ce, Pr and Nd, it was found by Harris and Tompkins that using the ammonium acid citrate method, Dowex 50 is far superior to the Amberlites. The disadvantage of Dowex, viz., the slowness of achieving equilibrium can be compensated by reduced rates of flow. Under these conditions quantitative separations were obtained in a single adsorption-elution cycle.

Ketelle and Boyd showed, using Dowex 50 columns, that at 100° the separation of all rare earths is accelerated. For example, milligram amounts of lutecium, ytterbium, thulium, erbium, holmium amd yttrium required only 30 hours for a complete separation. For a 97 cm. long and 0·26 cm.^{2} bed (adsorbent, 270–325 mesh) the optimum pH is 3·2 for the separation of yttrium earths and 3·4 for cerium earths if a 4·75 per cent citric acid solution is used for the fractional elution.

Separation of Anions

Using an " acid " alumina column (pre-treated with acid) Kubli has considerably extended the series of anions given earlier by Schwab and Dattler. The following sequence, with

decreasing adsorption affinities, has been established. (For limits of detection and some quantitative separations cf. the original paper.)

$OH^- > PO_4^{---} > C_2O_4^{--} > F^- > SO_3^{--}$ and $[Fe(CN)_6]^{----}$ and $CrO_4^{--} > S_2O_3^{--} > SO_4^{--} > [Fe(CN)_6]^{---}$ and $Cr_2O_7^{--} > NO_2^-$ and $CNS^- > I^- > Br^- > Cl^- > NO_3^- > MnO_4^- > ClO_4^- > CH_3 \cdot COO^- > S^{--}$.

The adsorbent was prepared by treating basic alumina "Neuhausen" with perchloric acid (1 : 1), and filtration and washing two hours later. This adsorbent should be dried at 120° for an hour. The micro-experiments were carried out in glass or acetylcellulose tubes (drinking straws, 70 × 2–3 mm.; column height, 30–60 mm.) with a cotton plug and using 100–200 mg. of alumina. The adsorbent was washed with 0·1–0·2 ml. of thrice distilled water. The reagents are either introduced as usual or the acetylcellulose tube is cut open and suitable spot tests are carried out.

Separations on Zeo-Karb: Frizzell.

Separations on strips of alumina-coated filter paper were carried out by Flood (4) who treated the strips first with a strong acid like $HClO_4$. The sequence of the anions was the same as that found earlier with alumina columns.

The detection of *boric acid* in the presence of some strong oxidizers can be carried out by immersing the end of a turmeric (curcuma) paper strip in the solution. Only boric acid reaches a considerable height, where it induces the well-known colour change, while hypochlorite, permanganate or chromate undergoes reduction in the lower sections of the paper (Flood and Risberg).

The removal of inorganic cations by means of columns of organic *exchange resins* was recommended by Samuelson (1–3). One of the practical applications of this method is the quantitative elimination of all metal cations from a solution by filtration through hydrogen-permutit (pre-treated with acid). Such an elimination may be useful for the investigations of the anions in the filtrate; for example, metals which would

hinder the estimations of phosphate can be retained in the exchanger within a few minutes.

Estimation of sulphate in technical aluminium sulphates. The solution is filtered through an exchange resin which has been saturated with H^+. The Al-ions of the solution are quantitatively exchanged with H^+, whereupon the sulphuric acid thus formed is titrated in the filtrate.

The exchange equilibria on the polyamine resin, Amberlite IR–4B, were recently studied by Kunin and Myers; they were found dependent on the valence and structure of the anion and, furthermore, on the ionization constant of the corresponding acid. The sequence is: hydroxide > sulphate chromate > citrate > tartrate > nitrate > arsenate > phosphate > molybdate > acetate and iodide and bromide > chloride > fluoride.

Fractionation of Isotopes on Ion Exchangers

This important, because simple, method was introduced by T. I. Taylor and Urey (1,2) who also gave a general discussion of the selective exchange of metal ions on zeolites (see also p. 20). The artificial zeolites used were complex aluminium silicates with much exchangeable sodium. For the exchange on such materials the following sequence was observed:

$$Mg^{++} < Li^+ < Ca^{++} < Na^+ < Ba^{++} < NH_4^+ < K^+ < Rb^+ < Cs^+ < H^+$$

The experiments were made with lithium, potassium or ammonium, the isotopes of which behave differently enough on the zeolite to permit a partial fractionation. After the adsorption a "development" may follow by washing the column with a cation which is very strongly held. Under the influence of this treatment, differences in the isotopic ratio can be detected between the leading and tailing fractions of the chromatographic filtrate. The analyses are conveniently carried out in the mass spectrometer.

The following results show that sodium zeolites preferentially exchange the lighter isotope of lithium, but the heavier isotope of potassium or of nitrogen (when present in the ammonium ion).

Lithium. ($^6Li : {}^7Li = 11{\cdot}6$). The possibility of isotopic fractionation can be demonstrated by shaking a LiCl solution several times with permutit ; a ratio of 12·7 was found in the last filtrate.

The most satisfactory chromatographic experiment was carried out with sodium-decalso (Permutit Co.) in a hard rubber pipe about 35 m. (100 ft.) × 3 cm. which took 13 kg. of the exchanger. The zeolite was filled in 3·5-m. units as a slurry (dist. water). When 15 g. of LiCl in 150 ml. of water had been washed down with 3 per cent NaCl, the leading fraction showed a ratio of 14·1, and the tailing one a ratio of 8·8. The corresponding figures in another column (about $\frac{1}{3}$ the size of the former), with the use of a weaker developer (0·5 per cent NaCl), were 12·6 and 8·9. The first appearance of Li in the filtrate required the use of 70 g. of NaCl and was checked spectroscopically.

Potassium ($^{39}K : {}^{41}K = 14{\cdot}1$). It is more difficult to obtain a separation of potassium than of lithium isotopes on the sodium zeolite column (in some instances the results were negative), especially for the leading fractions of the filtrate. However, the following experiment demonstrates that a change in the ratio is possible. A solution of 100 g. of KCl was washed down on a sodium-zeolite column (about 10 m. × 3 cm. ; 4 kg.) with 5 per cent NaCl. The ratios in the leading and tailing portions were 14·3 and 12·9 respectively. Another experiment in which $BaCl_2$ was used as a displacer, gave similar ratios of 14·6 and 13·5.

Nitrogen ($^{14}N^{14}N : {}^{14}N^{15}N = 124$). An ammonium chloride solution was washed down with water on a sodium-permutit column (11 m. × 2 cm.). After the liberation of nitrogen from the filtrate, the ratio was found to have increased to 137.

For related problems and techniques cf. also : H. S. Taylor ; Thode and Walkling ; Groth and Harteck.

General survey on isotope separations : Urey (1, 2).

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INDEX OF NAMES

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