PARTITION CHROMATOGRAPHY OF STEROIDS

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Received March 18, 1955

CONTENTS

I. Introduction 679
II. Derivatives of steroids 681
III. Solvent systems 682
  A. Stationary phase: aqueous solvents 682
    1. Water 682
    2. Bases 683
    3. Alcohols 684
    4. Acids 687
  B. Stationary phase: non-aqueous solvents 688
    1. Stationary phase more polar 688
    2. Stationary phase less polar 690
  C. "Monophasic" solvent systems 691
IV. Analytical methods 693
  A. Detection methods 693
  B. Quantitative methods 696
V. Applications 697
  A. Estrogens 697
  B. C_{19}-Steroids 697
  C. C_{21}-Steroids 697
  D. Sterols and bile acids 699
  E. Plant steroids 699
VI. References 700

I. Introduction

Steroids are a group of natural products related to perhydro-1,2-cyclopentenophenanthrene, including (89) such biologically important compounds as sterols, bile acids, sex hormones, adrenocortical hormones, heart poisons, steroidal saponins, and certain alkaloids.[1] In nature, steroids occur in relatively low concentration and almost invariably in association with other, structurally related steroids, a fact which makes their isolation, separation, identification, and quantitative estimation quite difficult. As a result, chromatographic methods, being more efficient and convenient than batch processes (96, p. 170), have attracted the interest of chemists working in the steroid field.

Differential migration methods, including chromatography, have been classified according to the driving or resistive forces effecting separation (408), although usually more than one force is operative. Steroids have been subjected to adsorption chromatography, chemichromatography, electrochromatography,

[1] For the sake of completeness the alkaloids with modified steroid skeleton have been included in this review.

and partition chromatography. Chemichromatography (90, 314), particularly ion-exchange chromatography (5; 14, p. 71; 28, p. 75), and electrochromatography (46, 241, 242, 259, 300, 367, 373, 436, 437, 438, 439) have not yet been adequately explored, but the latter techniques are obviously limited to ionized steroids or ionized derivatives of steroids. Adsorption chromatography, on the other hand, is quite suitable for lipide-soluble compounds and has, indeed, found wide application in the steroid field (237, pp. 175 and 262). However, secondary reactions caused by adsorbents (237, p. 276) and tailing, due to non-linearity of the adsorption isotherm (96, p. 221), have complicated the application of this method to the steroids, and more recently attention has been focused on the use of partition chromatography.

Without going into the details of partition chromatography, which are adequately presented in various textbooks (21, 45, 96, 105, 237), it may be recalled that the position of a compound in the chromatogram depends on a characteristic physical constant of the molecule, its partition coefficient, and that the linear partition isotherm is responsible for a symmetrical distribution of the zone in the direction of its movement. The principal advantages of partition chromatography over adsorption chromatography are therefore the use of R values for identification purposes and the completeness of separation for quantitative analysis. The sensitivity and convenience of paper partition chromatography are additional advantages, which, however, are shared by adsorption chromatography on impregnated paper (77, 78, 112, 227, 229, 317, 339, 383, 400). The limited load capacity of filter paper, on the other hand, is sometimes considered a disadvantage, but thicker paper, bands instead of spots, and partition columns may be used to overcome this difficulty.

Technical details of partition chromatography are also adequately covered elsewhere, but a few words about the choice of methods and common errors in technique may not be out of place. Column chromatography is to be preferred over paper chromatography in preparative work, especially when a small amount of one component is to be separated from a large excess of other components, and for accurate quantitative determinations, especially with methods in which extractives from filter paper interfere. Theoretically, ascending, descending, and radial development of paper chromatograms give equivalent results, but in practice, the possibility of separating closely situated bands by flowing descending development may offer advantages over the technically simpler ascending method. Further resolution is obtainable by two-dimensional or repeated unidimensional paper chromatography. Radial development often results in sharper definition of bands than either ascending or descending development and offers the possibility of treating one or several segments of the paper with color reagents. The importance of temperature control, of equilibrating stationary and mobile phases, and of saturating the atmosphere in the chromatographic chamber cannot be overemphasized. While it is unnecessary to impregnate the filter paper with the stationary phase when working with aqueous solvent systems, this practice may often improve resolution. Finally, and above all, the limitations of chromatographic methods as a criterion of homogeneity or identity should always be borne in mind (81).

The term polarity, as used in partition chromatography, also needs a word of explanation. Especially in a discussion on partition chromatography of steroids, which are hydrophobic compounds with many possibilities of isomerism, polarity has more practical than theoretical meaning. The order of movement of a series of compounds may differ from one solvent system to another, and a rule which holds for one chromatographic system may not hold for another. At this stage of our knowledge it may be wise to refrain from generalizing about the effect of structure on chromatographic behavior.

With the exception of steroids containing a hydrophilic side-chain and such derivatives of steroids as glycosides, glucuronides, peptides, etc., steroids as a class are quite insoluble in water. This frequently makes it difficult to apply the conventional partition chromatographic methods with water as the stationary phase. Another disturbing factor is their strong adsorption on the supposedly inert migration media. Various schemes have been devised to overcome these difficulties: Steroids have been chromatographed in the form of derivatives, in aqueous solvent systems containing water-soluble solvents such as alcohol or acetic acid to increase their solubility in the stationary phase, and in partition systems consisting of two partially miscible organic solvents, holding either the more polar or the less polar phase stationary. Many steroids give characteristic color reactions which can be utilized for their detection, but some reagents are so corrosive that they will destroy paper chromatograms. Other steroids are so unreactive that special spot tests must be used.

This review is an attempt to organize the entire literature on the methods of partition chromatography of steroids and their applications, available at the beginning of 1955.[2] A review on the partition chromatography of lipides, including steroids (7), and one on the adsorption and partition chromatography of steroids (82) have appeared earlier. In addition, the reader is referred to textbooks of chromatography containing chapters on the partition chromatography of steroids (21, 45; 105, p. 91; 237, pp. 171 and 262), discussions on individual aspects of the subject (14, 81, 153, 154, 352, 452), and review articles on chromatography (58, 110, 219, 220, 372, 406, 407).

II. Derivatives of Steroids

The preparation of derivatives prior to chromatography for the purpose of changing the solubility behavior or imparting a color to colorless compounds, or both, has long been standard practice in adsorption chromatography. Certain derivatives of steroids have also shown promising properties in partition chromatography.

In 1949 Zaffaroni, Burton, and Keutmann (455) chromatographed hydrazones of ketosteroids formed with Girard's Reagent T in 1-butanol/water.[3] The derivatives are not only more water-soluble than the ketosteroids, but also more easily

[2] Reports of meetings have been included, unless the same material was published elsewhere.

[3] The diagonal line used in "butanol/water" and in other instances is a shorthand expression for a developing solvent (butanol) saturated with a partially miscible solvent (water), which serves as the stationary phase.

detectable on filter paper by the use of various alkaloid reagents. Since the R_F values are mainly determined by the number of hydrazone groups in the molecule, the separation of compounds having the same number of hydrazone groups is not very good. Besides, steroids with two ketone groups frequently give rise to two spots, corresponding to the mono- and dihydrazones, respectively. In a modification of this method (343) hydrazones were formed with I^{131}-labeled Girard's Reagent P, which permitted detection of the zones in paper chromatograms with a Geiger counter. Essentially the same R_F values, but more sharply defined zones, were obtained when the filter paper was impregnated with Quilon prior to development with 1-butanol/water (342). Tanaka and Takeda (416) separated Girard T hydrazones of bile acid methyl esters according to the number of ketone groups in the molecule by paper chromatography in a butanol–2-propanol–water (6:2:1)[4] system. The use of 2-hydroxy-3-naphthoic acid hydrazones (90), 2,4-dinitrophenylhydrazones (463), and thiosemicarbazones (79) in partition chromatography of ketosteroids has also been reported.

Heftmann (178) coupled estrogens with diazotized *p*-nitrobenzeneazodimethoxyaniline (Fast Black Salt K) prior to chromatography in a toluene–petroleum ether–ethanol–water (20:10:3:7) system. All estrogens formed purple dyes, except equilenin (blue), which separated in paper chromatograms in the order[5] estriol < estradiol < estrone. A similar solvent system (179) separated epimeric estradiols (estradiol-17α > estradiol-17β) and equine estrogens (equilin > equilenin). Steroids containing a Δ^4-3-keto group coupled to give yellow dyes.

III. SOLVENT SYSTEMS

A. STATIONARY PHASE: AQUEOUS SOLVENTS

1. Water

As previously stated, only the more water-soluble steroids or hydrophilic derivatives can be successfully chromatographed in the classical partition systems. The more hydrophobic steroids move with the solvent front or undesirable streaking occurs, owing to adsorption on the migration media. Rather bad tailing due to adsorption was observed when adrenal hormones were chromatographed on paper with xylene/water (418) or benzene/water (383), and Edgar (121) reported similar experiences with progesterone in a petroleum ether/water system. However, Lata and Vestling (235) were able to isolate cholesterol and its esters from tissue preparations with 1-butanol/water, and Serchi (379) isolated 16-ketoestrone from urine by radial chromatography on thick filter paper with this solvent. Water-saturated 2-ethylhexanol has been used for separating 11-dehydro-17-hydroxycorticosterone from 11-deoxycorticosterone (74). 1-Butanol–benzene/water, 1-butanol–chloroform/water, and isoamyl alcohol–chloroform/water systems have been employed in the separation of saponins (428). Auterhoff (8) used phenol/water for paper chromatography of steroidal

[4] This designation is to signify that the solvents were mixed in the proportions given, and that, after equilibration, the phase richer in organic solvents was used as the mobile phase and the phase richer in water as the stationary phase. All proportions in this paper are v/v.

[5] Throughout this review the order given is that of rate of movement, elution, or R_F.

alkaloids, but found this solvent unsuitable for use on cellulose columns (10). In butyl acetate/water steroid amines without oxygen function in the molecule were well separated, particularly C-3 isomers (317).

For paper chromatography of the more hydrophilic digitalis glycosides many water-saturated solvents may be used: isoamyl alcohol–isobutyl alcohol (205), 1-butanol and amyl alcohol (150), ethyl acetate (350), ethyl acetate–phenol, ethyl acetate–eucalyptole, etc. (264), ethyl acetate–chloroform (432, 433, 434), ethyl acetate–benzene (350), ethyl acetate–chloroform–benzene (386), and benzene–chloroform (296, 386). Hassall and Martin (164) proposed a number of solvent systems suitable for the separation of cardiac glycosides on either sheets or piles of filter paper, some of which have already been mentioned. The significant observation was made that single compounds give rise to two spots when chloroform/water or ethyl acetate/aqueous sodium benzoate are used as solvents, a phenomenon which is in all likelihood due to irreversible adsorption on the paper (163). Schenker, Hunger, and Reichstein (359) found 1-butanol/water (*cf.* 279) and 1-butanol–toluene (1:1)/water (*cf.* 215) useful for paper chromatography of very polar cardiac glycosides and aglycones. Impregnation of the paper with the stationary phase and incorporation of 1-amino-2-methylanthraquinone in the mobile phase for marking the solvent front was recommended.

Stoll and Kreis (403) have isolated eight new cardiac-active glycosides from white squill, largely by use of chromatography on columns of cotton linters or diatomaceous earth with ethyl acetate/water, or, for more polar compounds, ethyl acetate–1-butanol (9:1)/water. Similar experiments with silica gel columns (402) showed that resolution was increased as the water content of the silica gel column was decreased (apparently owing to added adsorption effects) and that the addition of methanol to the eluant (ethyl acetate or chloroform) was useful in the case of very polar glycosides. Cardiac glycosides which are very difficult to separate by other methods have been handled effectively by Hegedüs, Tamm, and Reichstein (184) on large columns of Hyflo Supercel impregnated with water. The polarity of the mobile phase was increased in steps by using in succession petroleum ether–benzene mixtures, benzene, benzene–chloroform mixtures, chloroform, and finally chloroform–1-butanol mixtures. Heftmann and Johnson (182) were able to separate quantitatively all six of the then known active hormones of the adrenal gland on columns of silicic acid carrying water as the stationary phase. The polarity of the mobile phase was progressively increased by continually adding dichloromethane to petroleum ether. Gradient elution eliminated tailing and overcame the large differences in polarity between these steroids. The order of emergence from the column was: 11-deoxycorticosterone, dehydrocorticosterone, 17-hydroxydeoxycorticosterone, corticosterone, 11-dehydro-17-hydroxycorticosterone, and 17-hydroxycorticosterone.

2. Bases

Estrogens, being phenolic steroids, dissolve in alkali, and effective partition systems for estrogens may be devised in which the stationary phase is an aqueous

base. Another approach toward increasing the water-solubility, which was suggested by Boscott (54) and is also applicable to other classes of steroids, is the use of hydrotropic agents in the stationary phase. Boscott impregnated filter paper with a saturated solution of sodium *p*-toluenesulfonate and used toluene equilibrated with the latter as the mobile phase.

For partition chromatography of estrogens Heusghem (190, 191) saturated filter paper with ammonia vapors and then developed the chromatogram with the organic solvent phase of a chloroform–benzene–1 *N* ammonium hydroxide (1:9:1) mixture. Boute (55) obtained similar results with a stationary phase of 1 *N* sodium hydroxide and a mobile phase of either benzene, toluene, or xylene.

Partition chromatography of estrogens on columns of Celite holding 0.4 *N* sodium hydroxide was described by Haenni, Carol, and Banes (151, 152). Various factors affecting separation were investigated and benzene was recommended as an eluant for separating either estradiols and dihydroequilins from dihydroequilenins on a short column or estradiols from dihydroequilins on a long column. Braunsberg, Stern, and Swyer (60, 399) developed a similar procedure for the separation of estrone, estradiol, and estriol. Using a stationary phase of 3.1 *N* sodium hydroxide on a Celite column, estrone and then estradiol was eluted with benzene–petroleum ether (4:1). Estriol, which is much more hydrophilic, was later eluted with chloroform–1-butanol (17:3). To elute estriol, Bitman and Sykes (40) changed the stationary phase instead of the mobile phase by passing carbon dioxide through the column after elution of estradiol. Estriol was then readily eluted from the sodium bicarbonate–Celite column with benzene.

Aqueous alkali is also a suitable stationary phase for steroid acids. Zaffaroni and Burton (453) used 1-butanol/10 per cent ammonium hydroxide for separating etio acids derived from the oxidative degradation of adrenocortical hormones. Procházka (316) later employed isoamyl alcohol equilibrated with 2 per cent ammonium hydroxide or an ammonium hydroxide–ammonium carbonate buffer for the paper chromatography of other steroid acids. The R_F values in these systems were chiefly determined by the number of carboxyl groups in the acids, while the hydroxyl groups had a lesser effect. In a collidine/water system and ammonia atmosphere (392) conjugated bile acids, especially taurine conjugates, moved faster than the free acids and the mobilities were in the order monohydroxy acids > dihydroxy acids > trihydroxy acids. Tschesche and Seehofer (424) found a 1-butanol–pyridine–water (3:1:3) system applicable to polar cardiac glycosides.

3. Alcohols

One of the most successful methods of overcoming the poor solubility of steroids in the stationary water phase is the incorporation of a water-soluble alcohol in the solvent mixture. The relative proportion of alcohol in the mixture may be increased or decreased at will to increase or decrease R_F values. Higher alcohol concentrations are preferable for chromatographing the less polar steroids. Such solvent systems are relatively sensitive to variations in temperature, and adsorption effects (tailing) are not uncommon.

The separation of various sterols by radial partition chromatography at 0°C. with phenol–methanol–water (27:60:113) and with a "monophasic" system of petroleum ether–benzene–methanol (1:1:8) was described in 1950 (260). At the same time Svendsen and Jensen (409) demonstrated the separation of digitalis glycosides by various chloroform–methanol–water partition systems. If chromatographed at 22°C., gitoxin interfered with the quantitative determination of gitoxigenin, but at 17°C. the two substances were separated (207). The difficulty was traced to inadequate saturation of the atmosphere in the chromatographic chamber (208). Jaminet (204, 205) modified the solvent system by addition of benzene (*cf.* 385) and Vastagh and Tuzson (433) by substitution of dioxane for methanol. Sasakawa (350) used ethyl acetate–methanol–water and amyl acetate–dioxane–water mixtures. Silberman and Thorp (385) improved the separation of digilanids by the use of ethyl acetate–benzene–ethanol–water mixtures. The order was digilanid A > B > C. The exact position of each glycoside was dependent on the relative quantity of the glycoside component in its direct vicinity. Thus, in high concentration digilanid C displaced digilanid B farther to the front. This phenomenon is of practical value as well as theoretical interest and illustrates the effect of processes other than partition.

In 1952 Bush (78) described a technique for separating the six then known active hormones of the adrenal cortex. The organic phases of various mixtures of petroleum ether, of toluene or benzene, or of petroleum ether and toluene or benzene with aqueous methanol were used as developing solvents at 34°C. At lower temperatures and with low methanol concentrations streaking occurred.[6] Steroids of lesser polarity (testosterone, progesterone, Δ^4-androstene-3,17-dione, adrenosterone, 11-deoxycorticosterone, and acetates of adrenocortical hormones) were fractionated in a petroleum ether–methanol–water (5:4:1) system, while very polar steroids (11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone) separated best in a more polar solvent mixture of toluene–ethyl acetate–methanol–water (9:1:5:5). The order was 11-deoxycorticosterone > dehydrocorticosterone > 17-hydroxydeoxycorticosterone > corticosterone > 11-dehydro-17-hydroxycorticosterone > 17-hydroxycorticosterone in all solvent systems. Steroids differing from one another in minor structural details (geometric, position, and double-bond isomers) may be separated in these solvent systems. The effect of small structural differences on chromatographic behavior was discussed by Bush (81, p. 325). In later publications Bush described the modifications of the original solvent systems xylene–methanol–water (85) and benzene–chloroform–methanol–water (81). A benzene/50 per cent or 60 per cent aqueous methanol system was used for chromatographing cardiac aglycones (87).

Bloch, Zimmermann, and Cohen (43) have applied the solvent systems described by Bush (78) to steroids related to pregnanediol, Haslewood (159) to bile acids and their esters, and Migeon and Plager (271) to various 17-keto-

[6] Shull (382) has recently found that in this method the use of an elevated temperature and lengthy preincubation of the filter paper can be dispensed with if the filter paper is first impregnated with water (by immersion in acetone-water (7:3) and evaporation of the acetone).

steroids. Mitchell (273, 274) chromatographed estriol in the benzene–methanol–water system of Bush, but used a "monophasic" system of petroleum ether–methanol to separate estrone from estradiol. For the paper chromatographic separation of steroidal sapogenins Heftmann and Hayden (181) used modifications of the petroleum ether–toluene–ethanol–water mixtures previously described for use with estrogen derivatives (178). Boscott (53) also chromatographed uncombined estriol in petroleum ether–toluene–ethanol–water.

Using cyclohexane/20 per cent methanol, McDonough (257) chromatographed the neutral 17-ketosteroids in urine. Paper chromatography of adrenocortical hormones with 1-butanol–methanol–water and 1-butanol–benzene–methanol–water systems (318), and with petroleum ether–toluene–1-butanol–ethanol–water (301) has also been described. Pechet (302) has recently shown that this method is suitable for the separation of very polar corticosteroids, notably aldosterone, and that their order on the chromatogram may be varied by suitable choice of developing solvents. This is of practical interest as additional proof of identity.

The separation of progesterone, Δ^4-androstene-3,17-dione, 11-deoxycorticosterone, and testosterone[7] by partition chromatography on Hyflo Supercel columns with a stationary phase of 40–80 per cent aqueous methanol and a mobile phase of *n*-hexane was described by Butt, Morris, Morris, and Williams (88) in 1951. Subsequently, Morris and Williams (276) published a method for the determination of minute amounts of individual adrenocortical hormones by chromatography on two partition columns of Hyflo Supercel. The first column, which contained 25 per cent aqueous ethanol as the stationary phase and toluene as the mobile phase, gave a fore-fraction of dehydrocorticosterone, corticosterone, and 17-hydroxydeoxycorticosterone (11-deoxycorticosterone was not tested) and then two fractions, containing, in order, 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone. The fore-fraction was separated by rechromatographing on a second column with 1,2-ethanediol as the stationary phase and petroleum ether–toluene (1:4) as the mobile phase. Carol (93) separated progesterone from testosterone on a Celite column, using 80 per cent ethanol as the stationary and isoöctane as the mobile phase, and Banes (23) used the same method, but 50 per cent ethanol, for separating dianhydrogitoxigenin from digoxin. For the isolation of 16-epiestriol from pregnancy urine Marrian and Bauld (255) used 70 per cent methanol on a Celite column and an eluant of 1,2-dichloroethane. Products of the oxidation of cholesterol by air were separated (277) on Celite partition columns with cyclohexane/95 per cent methanol, and lithocholic acid from stearic acid with *n*-heptane/95 per cent methanol (278). The solvent systems investigated by Bush (78) are also applicable to partition chromatography on Supercel (157) or cellulose powder (389, 390). In the work on the isolation of aldosterone (390) a modified system was found valuable. The proportions of benzene–petroleum ether–methanol–water were changed in the course of chromatographic development from 1:1:1:1 to 6:4:5:5 and finally to 2:0:1:1.

[7] In the order of elution.

Boscott (52, 53) devised partition systems of toluene and absolute ethanol or methanol by separating the mixture into two layers with addition of lithium chloride or calcium chloride. A similar situation may exist in the "partition type" silica gel columns of Katzenellenbogen, Dobriner, and Kritchevsky (216), if they are indeed partition columns. Absolute ethanol was used as the stationary phase and dichloromethane containing ethanol as the mobile phase for the fractionation of the acetates of adrenal steroids. In later work (140) that group used 40 per cent ethanol as the stationary phase and dichloromethane–petroleum ether (1:1) as the mobile phase. Rosselet and Lieberman (337) also used ethanol, adsorbed on Florisil, as the stationary phase and dichloromethane–petroleum ether (1:1) as the mobile phase for column partition of corticoid acetates, but gradually increased the ethanol content of the eluant from 0 to 2 per cent.

4. Acids

The function of water-soluble organic acids in partition systems for steroids is, similar to that of water-soluble alcohols, to increase the solubility in the stationary phase. Organic or mineral acids may be used for the basic steroids, which form salts. In the case of the acidic steroids, the acids may help to reduce tailing by repressing their ionization.

In 1949 Munier and Macheboeuf (280) described general methods for the paper partition chromatography of alkaloids which are applicable to the steroid alkaloids. 1-Butanol–acetic acid–water mixtures gave good results with various alkaloids, including conessine and veratrine. This solvent system has recently been applied to the alkaloids of Kurchee bark by Tschesche and Petersen (423). Nash and Brooker (281) used a variety of acetic acid-containing solvent mixtures for the separation of *Veratrum* alkaloids, particularly a 1,2-dichloroethane–acetic acid–water (99:2:99) mixture, and Kuhn and Löw (233) used ethyl acetate–acetic acid–water (3:1:3) for fractionating *Solanum* alkaloids. Filter paper impregnated with potassium chloride and a stationary phase of dilute hydrochloric acid were used by both Auterhoff (9), who chromatographed *Veratrum* alkaloids with 1-butanol, and Procházka, Lábler, and Kotásek (317), who used 2-butanol for various steroid amines. The separation of cevadine from veratridine on silica columns with acidic buffers as stationary phase was reported by both Auterhoff and Strausberg (10), who used petroleum ether–chloroform (1:3) as the mobile phase, and Svoboda and Parks (410), who used chloroform as the eluant.

For the separation of bile acids Sjövall (393) proposed a partition system of isopropyl ether–heptane (7:3)/70 per cent acetic acid. Impregnation of the filter paper with the stationary phase was necessary to eliminate tailing, but R_F values varied depending on the amount of acetic acid in the paper (*cf.* 395, 161). Deoxycholic acid moved appreciably faster than hyodeoxycholic acid, but chenodeoxycholic acid did not separate from deoxycholic acid. The latter pair was subsequently separated with an isopropyl ether–heptane (1:4) developer which was allowed to overflow the paper (395). Heptane alone gave separation, but the load capacity of the system was much smaller. For bile acids conjugated

with glycine, Sjövall (395) used isopropyl ether–heptane (4:1)/70 per cent acetic acid, but taurine conjugates were chromatographed with a mobile phase of 1-butanol equilibrated with 3 per cent acetic acid, but not with the stationary phase of 70 per cent acetic acid. In both cases separation was incomplete. For column chromatography Sjövall (394) used 60 per cent acetic acid, held stationary on Supercel, and an eluant of isopropyl ether–heptane (4:1). Mosbach, Zomzely, and Kendall (278) separated bile acids by fractional elution from a Celite column containing 70 per cent acetic acid. Lithocholic acid was eluted with petroleum ether, deoxycholic acid and then α-hyodeoxycholic acid with isopropyl ether–petroleum ether (2:3), and finally cholic acid followed by (contrary to expectation) dehydrocholic acid with isopropyl ether–petroleum ether (3:2). For the paper chromatography of bile acids toluene–acetic acid–water mixtures have also been found useful (39).

Other uses of acetic acid in partition chromatography include "countercurrent" partition chromatography (118) with unspecified results, and a separation of cardiac glycosides (266). Sannié and Lapin (349) have separated sixteen steroidal sapogenins by paper chromatography with petroleum–chloroform–acetic acid (100:4:1 or 25:10:1). It is not clear to what extent partition was involved in the separation, since only the petroleum was saturated with water. Certain generalizations on the effect of configuration on chromatographic mobility were made which may be valuable for predicting the structure of unknown sapogenins. For paper chromatography of the saponins 1-butanol– or isoamyl alcohol–acetic acid–water (4:1:5) mixtures have been proposed (429).

B. STATIONARY PHASE: NON-AQUEOUS SOLVENTS

1. Stationary phase more polar

One of the most useful techniques for separating steroids of relatively low polarity was devised by Zaffaroni, Burton, and Keutmann (456, 75) in 1950. Descending chromatograms were developed with benzene on formamide-impregnated filter paper or with toluene on paper impregnated with 1,2-propanediol. To prevent lateral diffusion of the spots the paper was cut into lanes. By partition between the more polar stationary organic solvent and the partially miscible, less polar, moving solvent mixtures of corticosteroids were resolved in accordance with the partition coefficients of their component steroids. Zaffaroni (452) and Savard (352) have discussed the relation between the structure and the chromatographic behavior of steroids in solvent systems of this type. This relation has been described in mathematical form (147), leading to an erroneous prediction for the structure of aldosterone, and Bush (82, p. 234) has pointed out several instances in which it differs from that in aqueous solvent systems. Bush has also compared the relative advantages and disadvantages of the two types of solvent systems (81, p. 329). From a practical point of view, solvent systems with non-aqueous stationary phase are less convenient to use, require more time, and give less information than aqueous systems, but they accommodate larger quantities of more impure mixtures and are better suited to the separation of less polar compounds. Their resolving power is excellent, if a suit-

able mobile phase is allowed to overflow the paper for a prolonged period of time or to ascend the paper repeatedly (200). The order of movement is the same in both solvent systems (11-deoxycorticosterone > dehydrocorticosterone > corticosterone > 17-hydroxydeoxycorticosterone; pregnane-3,21-diol-11,20-dione > Δ^4-pregnene-17β,20,21-triol-3-one > Δ^4-pregnene-17α,20,21-triol-3-one; 11-dehydro-17-hydroxycorticosterone > pregnane-3,12,21-triol-20-one > 17-hydroxycorticosterone), but the rate of movement is faster in benzene/formamide and the separation is less satisfactory. Diffusion of the spots increases with the amount of substance chromatographed and with the distance traveled. Esters give more diffuse spots than the corresponding alcohols. Since the solvent is allowed to run off the paper, R_F values cannot be determined. The rate of movement is dependent on a number of factors, including the amount of stationary phase on the paper (37). More even running rates and greater convenience in handling chromatograms have resulted from dipping the paper into formamide or 1,2-propanediol diluted with volatile solvents (19, 282, 453) and allowing the diluent to evaporate, but if the dilution is too great streaking may occur (208).

The solvent systems benzene/formamide and toluene/1,2-propanediol have also been applied to paper chromatography of cardiac glycosides (361, 183, 208).[8] For this application the paper does not have to be cut into lanes, but spots near the edge of the sheet will tend to skew outward (183, 210). The same solvents have also found use in the separation of estrogens (462, 336, 206, 55, 138). As Siblíková-Zbudovská and Hais (384) have pointed out, the results of Rosenkrantz (336) must be in error and the correct order for the estrogens should be estrone > estradiol-17β and estradiol-17α > estradiol-17β. Progesterone (138) and compounds related to pregnanediol (43) as well as C_{19}-17-ketosteroids (351, 138) have been chromatographed in the above-mentioned systems. Columns of powdered cellulose (19) or silica gel (256) with a stationary phase of 1,2-propanediol and a mobile phase of toluene have been used in the isolation of corticosteroids.

Other mobile phases have been used to chromatograph more polar or less polar steroids (452, p. 64). Schindler and Reichstein (361) have added chloroform to the benzene to increase the mobility of cardiac glycosides in benzene/formamide, and Zbudovská and Hais (462) have applied this modification to the chromatography of estrogens. Substitution of chloroform for benzene–chloroform mixtures may reverse the order of mobilities (208). For the chromatography of adrenal steroids on filter paper impregnated with 1,2-propanediol, 1,2-dichloroethane–toluene (3:7) has also been used (98, 154). On formamide-impregnated columns adrenal steroids have been chromatographed with benzene–isoöctane (22) or with cyclohexane–benzene or cyclohexane (216) as the mobile phase. Digitoxin preparations were analyzed on formamide columns with benzene–chloroform as the eluant (25, 24). Cyclohexane/formamide has also been used in the paper chromatography of corticoids (318), and cyclohexane–benzene (1:1)/formamide was used by Kochakian and Stidworthy (222) for the paper

[8] The references should preferably be read in the sequences given.

chromatography of 17-ketosteroids. Richardson (*cf.* 56) introduced the ligroin/1,2-propanediol system which Savard (351, 352) applied to 17-ketosteroids of low polarity. This system has also been used in partition columns for the separation of isomeric 7-hydroxycholesterols (277). Axelrod (12, 14) quantitatively separated estrogens by the use of *o*-dichlorobenzene, dichloromethane, or cyclohexene, saturated with formamide. Solvent systems of decalin/formamide, cyclohexene/formamide, and methylcyclohexane/1,2-propanediol have resolved complicated mixtures of C_{21}- and C_{19}-steroids (13, 6). In the course of this work Axelrod made some interesting observations with regard to the order of movement of steroids in the above solvent systems. Other solvent systems in this category are xylene–1,2-propanediol, which was used for estrogens by Boute (55), and xylene/formamide or xylene/formamide–ammonia (202), xylene–acetonitrile/formamide (268), and carbon tetrachloride/formamide (439, 373), which have been used to separate corticosteroids.

Other organic solvents may be used as the stationary phase, provided they are not miscible with the mobile phase. For the paper chromatography of weakly polar compounds Neher and Wettstein (283) impregnated the filter paper with 2-phenoxyethanol (phenyl cellosolve) and separated sterols and ethers or esters of saturated steroids by continued or repeated descending chromatography with either heptane, hexane, ligroin, or cyclohexane as the mobile phase. Savard (351) applied this method to ketosteroids, using either heptane or ligroin as the mobile phase. Axelrod (13) found a methylcyclohexane/1,3-butanediol system suitable for the separation of C_{19}- and C_{21}-steroids, and Patton and Trunnell (299) recently reported the separation of estrogens in a benzene–hexane–pyridine–water (20:25:4:1)/2,3-butanediol solvent system.

For column chromatography of adrenal cortex hormones Haines (153, 154) impregnated silica gel with 1,2-ethanediol and developed with cyclohexane–dichloromethane mixtures. A 4:1 mixture eluted 11-deoxycorticosterone, a 2:1 mixture eluted corticosterone, a 1:4 mixture eluted 11-dehydro-17-hydroxycorticosterone, and pure dichloromethane eluted 17-hydroxycorticosterone. The stepwise elution was carried out by an automatic device. Morris and Williams (276) separated dehydrocorticosterone, corticosterone, and 17-hydroxydeoxycorticosterone[7] on Supercel columns containing 1,2-ethanediol with a petroleum ether–toluene (1:4) mixture. In the separation of ketosteroids according to Jones and Stitch (211) the stationary phase is nitromethane on a silicic acid column and the mobile phase is 3 per cent chloroform in petroleum ether. With columns of sufficient length such closely related steroids as androstan-3β-ol-17-one and Δ^5-androsten-3β-ol-17-one can be separated.

2. Stationary phase less polar

In order to use the less polar solvent as the stationary phase in a partition chromatogram the migration medium must be hydrophobic and it may or may not be impregnated with the stationary phase. The order of movement is the reverse of that expected in ordinary partition chromatography, i.e., the more hydrophobic compounds move more slowly than the more hydrophilic ones, and

the process is called reversed-phase partition chromatography. Reversed-phase systems are very valuable for fractionation of the least polar steroids.

Kritchevsky and Tiselius (232) in 1951 made filter paper hydrophobic with silicone and separated C_{19}-ketosteroids with a water–ethanol–chloroform (3:5:5) mixture. The method was later extended to estrogens (231), digitalis glycosides (149), and adrenal steroids (439). Silicone-treated paper and a solvent mixture of water–methanol–benzene–petroleum ether (2:7:3:6) have been employed in the identification of estrogen esters by Markwardt (254). Davis, McMahon, and Kalnitsky (107) have used Quilon-impregnated paper for separating D vitamins from other sterols. Many of the solvent combinations tried were not partition systems and in these cases the mechanism of separation was probably chiefly adsorption. Kodicek and Ashby (225) have reported the separation of sterols on filter paper impregnated with liquid paraffin, which served as the stationary phase, using dilute alcohols as mobile solvents.

Reversed-phase partition chromatography on columns has also been investigated. It is a valuable tool for the preliminary concentration of steroids from natural sources (5, 276). Estrogens may be fractionated on columns of vulcanized rubber powder containing benzene (289). Estriol is eluted with 20 per cent aqueous methanol, next estradiol with 40 per cent methanol, and then estrone with 60 per cent methanol. In their work on the metabolism of bile acids Bergström, Sjövall, and Norman have made extensive use of columns of Supercel, made hydrophobic by exposure to dimethyldichlorosilane (34). With a stationary phase of chloroform–heptane (9:1) and a mobile phase of 60 per cent aqueous methanol the order of elution was cholic acid > deoxycholic acid > 3-hydroxy-12-ketocholanic acid > lithocholic acid (394). One hydroxyl group had roughly the same effect on polarity as two keto groups, so that 3,7,12-triketocholanic acid was not separated from 3-hydroxy-12-ketocholanic acid. By increasing either the methanol concentration or the heptane content of the stationary phase the movement was accelerated. Taurocholic, taurocholanic, glycocholic, and cholic acids were eluted in the order given by the use of isoöctanol–chloroform (1:1) as stationary and methanol–water (1:1) as mobile phase (32, 286). This solvent system also separated tauroconjugates > glycoconjugates > free bile acids (287). A stationary phase of 1-butanol and a mobile phase of water were required to fractionate the polar tauroconjugates taurocholic acid > taurodeoxycholic acid > taurolithocholic acid (286).

C. "MONOPHASIC" SOLVENT SYSTEMS

While chromatography on filter paper impregnated with adsorbents is obviously a form of adsorption chromatography and therefore falls outside of the scope of this review, there appears to be some evidence (105, p. 12) that partition is, at least to some extent, involved in the chromatographic development on untreated filter paper with water-soluble solvents or solvent mixtures not previously saturated with aqueous solvents. As long as the mechanism of separation in these so-called "monophasic" solvent systems is not clearly understood, it is appropriate to include such separations in this review. Generally, "mono-

phasic" solvent systems are not ideal for steroid chromatography. Spots are elongated or show tails and variable R_F values.

Boscott (51) has used aqueous alkali, aqueous pyridine, and aqueous potassium chloride solution (52) in attempts to chromatograph urinary estrogens on paper. In spite of a failure of earlier investigators (288) to separate estrogens with 10 per cent aqueous 1-propanol, Heusghem (190, 191) took up the problem again and was able to achieve separation of estrone, estradiol, and estriol with 33 per cent aqueous methanol, 0.05 N sodium hydroxide, or 15 per cent aqueous pyridine, but stated that the results were unsatisfactory, owing to tailing and variable R_F values. Under these conditions the more polar steroids moved more rapidly than the less polar ones, and the order was estriol > estradiol > estrone. A simple separation of progesterone from oily vehicles by paper chromatography with 80 per cent aqueous ethanol has also been carried out (158). The paper chromatography of bile acids with 1-propanol–ammonia–water (45:1:4) and with 1-propanol–ethanolamine–water (18:1:1) was described by Kritchevsky and Kirk (230). These "monophasic" systems fractionated bile acids in the order deoxycholic acid > dehydrocholic acid > cholic acid and norcholic acid, i.e., the less polar acids moved faster than the more polar acids.

Cardiac glycosides have been chromatographed using water, saturated with 1-butanol (164), with *n*-amyl alcohol or isoamyl alcohol (150), with 1-butanol–pyridine mixtures (264), and with 2-propanol–eucalyptole, 2-propanol–eucalyptole–acetic acid or even more complicated mixtures (266), but Wegner (440) has recently shown that small amounts of cardiac genins are separable with water alone. For what was called "genuine partition chromatography on paper" Tschesche, Grimmer, and Seehofer (422, 424) impregnated filter paper with the organic solvent phase of 1-pentanol–water, isobutyl alcohol–water, or even more complicated 1-octanol–1-pentanol–water–formamide mixtures and then separated cardiac genins and glycosides with the aqueous phase. It was noted, however, that the sequence of the steroids was not reversed when the aqueous phase was used for impregnation and the organic solvent phase for development. Procházka used what he called "reversed-phase systems" of petroleum–petroleum ether (2:3), saturated with either 57 per cent aqueous ethanol or 80 per cent aqueous acetic acid, for the separation of esters of steroid acids (316) and the same mixture, but saturated with 72 per cent aqueous ethanol or 72 per cent ethanol in 2 per cent ammonium hydroxide, for the separation of steroid amines (317). It is difficult to see why the petroleum–petroleum ether mixture should have remained stationary on untreated filter paper, even though it was impregnated with and kept in an atmosphere of this mixture.

A similar situation exists in the chromatography of corticosteroids. Hofmann and Staudinger first used water, saturated with 1-butanol, for fractionation on filter paper (195) or cellulose columns (197). Aqueous ethanol (5–10 per cent) could also be used in the latter case, and Meyerheim and Hübener (268) have shown that 25 per cent aqueous methanol is suitable for the fractionation of adrenal steroids by paper chromatography. Schmidt, Staudinger, and Bauer (371) later improved the separation by the use of water saturated with 1-heptanol

and impregnation of the filter paper, first with water and then with 1-heptanol. This solvent system, which was called a "reversed system," apparently because the more polar steroids moved faster than the less polar ones, did not separate 11-dehydro-17-hydroxycorticosterone from 17-hydroxycorticosterone but readily separated both from aldosterone (370). Finally, Schmidt and Staudinger (368) found that water alone is a suitable developer for cellulose columns.

Sakal and Merrill (347) proposed a "monophasic" solvent system of xylene–methanol (3:1) for the paper chromatography of cortical hormones. The spots on the chromatogram showed little tendency toward lateral diffusion (*cf.* 456), but, owing to adsorption on the paper, they were elongated to such an extent that separation became difficult, especially when the paper had been dried prior to use. The zones were shorter when the paper was not predried, but then the R_F values were closer to one another. Various mixtures of absolute methanol, ethyl acetate, and chloroform have been used in two-dimensional chromatography of cardiac glycosides by Jaminet (203, 205). Carbon tetrachloride (containing 1–2 per cent acetic acid) or hexane (containing 3.5–4 per cent methanol) was found suitable for the paper chromatography of bile acids (316); the former solvent mixture was also suitable for steroid amines (317).

IV. Analytical Methods

Any of the known qualitative or quantitative methods may be applied to steroids eluted from partition columns or paper chromatograms, but in connection with this review only procedures applicable to paper chromatography are of interest. It is well to note, however, that established methods may fail if separation or elution is incomplete, and that extractives from the migration media, especially filter paper, may interfere. The interference is particularly serious in the case of spectroscopic procedures, and various methods had to be devised to remove interfering substances from filter paper prior to use. Axelrod (13), for example, washed Whatman No. 1 filter paper by allowing 2 *N* acetic acid, distilled water, and distilled methanol to overflow the paper for 3 days each. Romanoff, Wolf, Constandse, and Pincus (334) recommended Whatman No. 540 as cleaner filter paper, but extracted it in a Soxhlet extractor with water–methanol (3:1) for a week. To elute steroids from filter paper, it may be steeped in absolute methanol, 95 per cent ethanol, or other suitable solvents. Various elution devices have also been described (376, 14, 153, 461).[8] The importance of using the eluate of a comparable piece of filter paper for a spectroscopic blank determination cannot be overemphasized (452, 14).

A. DETECTION METHODS

Steroids which absorb ultraviolet light or fluoresce after treatment with a suitable reagent may be located on paper chromatograms by inspection or contact photography. Technical details have been described (77, 153, 202, 385), but elaborate equipment is not required. Especially in quantitative work, the destructive effect of ultraviolet light on steroids (148, p. 212; 358; 371, p. 131) should be considered and exposure should be kept at a minimum (390, p. 1164).

A number of papers have appeared in which radioactive steroids have been detected by the use of Geiger counters (31, 173, 312, 313, 343) or radioautography (188, 392). Since these techniques do not differ from the general methods used in paper chromatography, they will not be discussed further.

Chemical reagents are applied to the dried chromatogram by spraying or dipping and the paper is then usually heated. Inasmuch as steroids are generally rather unreactive, relatively many non-selective reagents acting in an obscure manner are in use. These are nevertheless sometimes quite specific, giving different colors or fluorescence with different steroids. Some reagents are applied without regard to their corrosive effect on the paper. Glass-fiber paper, which can withstand strong acids, has been used for adsorption chromatography of steroids (112), but not for partition chromatography (376, p. 83).

Concentrated sulfuric acid (322) and fuming sulfuric acid (12) give characteristic colors or fluorescence with many steroids (462, 13, 6, 281, 425, 316)[8] but the filter paper soon disintegrates. The Liebermann–Burchard reagent (sulfuric acid–acetic anhydride) (298, 235, 215, 316, 119, 429) is less destructive but gives a fluorescence similar to that of the more convenient 15 per cent phosphoric acid reagent proposed by Neher and Wettstein (282). The latter reagent gives a variety of colors in ultraviolet light with minute amounts of all types of steroids (298, 190, 230, 393, 150, 316, 39). It can be made more sensitive by exposure of the finished chromatogram to iodine vapors (316). Trichloroacetic acid in chloroform was first used for cardiac glycosides by Svendsen and Jensen (409). It produces an intense fluorescence and permits differentiation between the digitalis glycosides of the A and B series. The reagent was also found to be applicable to the detection of steroidal sapogenins (181). Jensen (208) later found that the fluorescence of the A substances was due to decomposition of the reagent. In ethanol solution trichloroacetic acid gives no color with the A series, but chloramine can be added to the reagent to produce a fluorescence with such compounds. Hydrochloric acid fumes (264) give a color reaction with cardiac glycosides, but the paper becomes brittle. Nitrous fumes (46) stain estrogens yellow and the color can be intensified by exposure to ammonia vapors (55), but the reaction is not specific. Millon's reagent (nitrous acid–mercuric nitrate) and Kober's reagent (sodium *p*-phenolsulfonate in phosphoric acid) give colored and fluorescent spots with estrogens (12).

The Carr–Price reaction with antimony trichloride in chloroform (282), nitrobenzene (400), or acetic acid (188) has been applied to all types of steroids (236, 336, 91, 349, 190, 462, 43, 316, 317).[8] It is not so sensitive as the phosphoric acid reaction mentioned previously, but produces colored and fluorescent spots for additional identification of steroids. Addition of zinc chloride seems to intensify the color (138). The reaction has been modified by either exposing the paper to chlorine (383) or spraying it with chlorine in chloroform (316) before spraying with antimony trichloride in acetic anhydride. On addition of chlorine to antimony trichloride in chloroform the pentachloride is formed, which is also a non-selective reagent for steroids (260, 228, 12, 13, 316, 317). Antimony pentachloride gives additional color tests but is more difficult to handle. Zinc chloride

(288) produces colored or fluorescent spots with many steroids (43). A modified reagent contains zinc chloride and benzoyl chloride (283, 12). Ferric chloride in methanol (12) and ferric ferricyanide (299) have been used for estrogens.

Exposure of the chromatogram to iodine vapors (280, 228) produces brown spots with many organic compounds, including steroids. The paper may also be suspended over a bromine solution and then sprayed with potassium iodide and starch solutions (260). Similar results may be obtained with solutions of iodine in petroleum ether (77, 339, 316), alcohol (39), or hydriodic acid (13). The reaction with chlorine, potassium iodide, and starch appears to be more specific (393). Sudan Black (118) and Rhodamine B (393) are generally applicable stains. Potassium permanganate in alkali (75, 8) is reduced by many organic compounds. Phosphomolybdic acid is converted to molybdenum blue by many compounds, including steroids (280, 228, 393, 222, 159, 254). Molybdic acid gives a rose color with estrogens (46) and various colors with the various Kurchee alkaloids (423). Phosphotungstic or phosphomolybdic acid gives a purple color with cholesterol (118). The Folin–Ciocalteu reagent (phosphotungstomolybdate) (273, 190) is more sensitive toward estrogens than toward other steroids. Silicotungstic acid (228) gives a variety of colors with many steroids.

Many aromatic aldehydes give a characteristic but fleeting color with steroids on paper chromatograms. Cinnamaldehyde, hydrocinnamaldehyde, salicylaldehyde, and *p*-nitrosalicylaldehyde have been used for sapogenins (349), *p*-nitrobenzaldehyde and *p*-nitrocinnamaldehyde for steroid acids (316), and *p*-dimethylaminobenzaldehyde and vanillin for sapogenins (349), steroid acids (316), and cardiac glycosides (137). Anisaldehyde (282) gives colored and fluorescent spots with a variety of steroids, including those already mentioned (349, 298, 149, 137, 316). Furfural has been used for cardiac glycosides (215) but apparently blackens the paper (349).

The Raymond reagent or the Zimmermann reagent (*m*-dinitrobenzene and alkali) is widely used for the detection of cardiac-active glycosides (203, 361, 208) and various ketosteroids (158, 232, 222, 393). With cardiac glycosides it gives a fleeting purple color (361) and with ketosteroids a blue, tan, or violet color, depending on whether the keto group is in the 3-, 16-, or 17-position (222). The product may be sprayed with 2,4-dinitrophenylhydrazine (222) to bring out the yellow or orange color of the dinitrophenylhydrazone. The Legal reagent (sodium nitroprusside) (283), the Baljet reagent (sodium picrate) (228, 208), *p*-nitroaniline followed by periodic acid (137), and 3,5-dinitrobenzoic acid (87) have also been used for the detection of cardiac glycosides.

Diazonium salts (e.g., the Pauly reagent), sprayed on the chromatograms, couple with estrogens (52, 190, 378, 462). Tollens' reagent (alkaline silver nitrate) is a suitable spray for such reducing steroids as corticosteroids (456) or cardiac glycosides (183). The α-ketol group of the former also reduces Nelson's arsenomolybdate reagent (374, 375), and triphenyltetrazolium chloride (75) and its chemical relatives (291, 129). Axelrod (11) used the destruction of the dihydroxyacetone side-chain of corticoids by alkali to differentiate between such com-

pounds and corticoids with a 20-keto-21-hydroxy side-chain by means of triphenyltetrazolium chloride.

In contrast to the iodine solutions mentioned previously, iodine in potassium iodide (456) is a surprisingly specific reagent (376). It gives a blue color with only 11-dehydro-17-hydroxycorticosterone (75), 17α-hydroxyprogesterone, allopregnan-3β-ol-20-one, isoandrosterone, dehydroisoandrosterone (138), cholic acid (27, 393), and some etiocholenic acids (27). The methanolic sodium hydroxide reagent introduced by Bush (78) is very sensitive and appears to be specific for Δ^4-3-ketosteroids (81, p. 326). The sensitivity of the fluorescence reaction can be increased by using aqueous instead of alcoholic sodium hydroxide containing a trace of triphenyltetrazolium chloride to quench the background fluorescence of the paper (82, p. 233). Osmium tetroxide vapors (253) are apparently also a specific reagent for Δ^4-3-ketosteroids, but the reagent is not so safe to use.

Blood is hemolyzed by sapogenins and can be used as a spraying reagent (181). The reagent appears to be specific but lacks sensitivity. Steroidal alkaloids and Girard derivatives of ketosteroids (455) may be revealed by various alkaloid reagents (280): potassium iodobismuthate (the Kraut–Dragendorff reagent) (455, 280), potassium iodoplatinate (455, 280), potassium iodomercuriate (the Mayer reagent) (423), and indicators (8) have been used. Procházka (316) recommended fluorescein as a specific reagent for keto acids.

B. QUANTITATIVE METHODS

A roughly quantitative estimate of the amount of substance in a paper chromatogram can be obtained by comparing the size and color of a stained spot with a simultaneously stained spot containing a known quantity of material. This method has been applied to steroids (346, 453, 76, 41, 146),[8] but its accuracy is very low. A refinement of this technique consists in comparing a dilution series (213, 462, 150, 301) or photographs of ultraviolet-absorbing (78) or fluorescing spots (385). Tennent, Whitla, and Florey (418) designed an adapter for the direct ultraviolet photometry of paper strips, and Oertel used a densitometer to determine accurately the 17-ketosteroid (290) or corticoid (291) concentration in stained paper strips. Radiochemical determination of ketosteroids (343), bile acids (31), corticosteroids (192, 313), and other steroids (173) on paper chromatograms has been reported.

As indicated above, any sensitive quantitative method can be applied to the determination of steroids in eluates from either paper chromatograms or columns. These methods are outside the scope of a review on partition chromatography, but the chromatographic methods which have been adapted to quantitative work will be mentioned.

Paper chromatograms of cortical hormones have been evaluated quantitatively after elution of the section stained with triphenyltetrazolium chloride (194, 195, 103), arsenomolybdate (375, 376), or 2,4-dinitrophenylhydrazine (111), or by ultraviolet spectrometry (153, 154, 202, 452). Methods for estimating 17-ketosteroids (340, 271, 450, 455), estrogens (12, 55, 191, 274), progesterone

(121, 158, 461), bile acids (128), cardiac glycosides (150, 210, 422, 433), and sterols (226, 235) after elution from paper chromatograms are available.

Eluates from partition columns can be measured with greater precision. Progesterone (88, 417, 93), 17-ketosteroids (211), estrogens (40, 60, 151), adrenocortical hormones (22, 182, 197, 276), bile acids (278, 286, 394), and cardiac glycosides (23, 24, 25) have been determined by various methods after column chromatography.

V. Applications

In the short time since Zaffaroni's first work on the partition chromatography of steroids appeared in 1949 this method has become one of the most important tools in steroid research. Space does not permit a detailed discussion of the results obtained by its application, but the following sections, arranged by classes of steroids, will serve as an illustration and as a guide to the literature.[8] For orientation in steroid metabolism the reader is referred to the monograph of Dorfman and Ungar (116). Preliminary concentration and purification of steroids are essential for the successful application of partition chromatography. Practical aspects, such as techniques for preparing samples and choice of chromatographic methods, depend on the application and will be found in the pertinent literature.

A. Estrogens

The preliminary purification of urinary estrogens by ion-exchange resins and other methods has been described by Bauld (28) and by Axelrod (14). The identification of urinary estrogens by paper chromatography has been reported by various investigators (462, 378, 190, 53, 381, 143, 12, 47, 48). Estriol (50), 16-ketoestrone (379), and 16-epiestriol (255) have been isolated, new equine estrogens have been identified (26), and an unknown estrogen has been partly characterized (141, 142). Partition chromatography has been employed in the identification of estrogens in tissues (145, 380, 274) and commercial estrogen preparations (92, 59, 344) and in the study of enzymatic reactions (324, 325).

B. C_{19}-Steroids

Adsorption chromatography (*cf.* 99, 292) has been in use for some time for the identification and quantitative estimation of the relatively large amounts of 17-ketosteroids in the urine, but partition chromatography is rapidly gaining favor for qualitative (341, 353, 401, 292, 257, 108, 120, 217) and quantitative (41, 340, 211, 99, 323) analysis. It is a valuable tool for the study of the biosynthesis (56, 15, 117, 354) and tissue metabolism (238, 329, 450, 451, 449, 223) of androgens, and for the study of enzymatic reactions (414, 415, 114). Ketosteroids have been identified by paper chromatography in blood (442, 271), in adrenal perfusates (42), and partially identified in sperm (113).

C. C_{21}-Steroids

Partition chromatography is of greatest importance in the study of corticosteroids. Some aspects of this work have already been reviewed (311). Methods

for the extraction of corticosteroids from blood and tissues may be found in the individual reports which follow and in the papers of Lombardo, Mann, Viscelli, and Hudson (239) and Axelrod and Zaffaroni (17, 452).

Several papers on the composition of adrenal cortex extracts have appeared (453, 19, 195, 78, 383, 375, 22, 463, 439, 115). Partition chromatography has been of special value in the isolation of aldosterone from adrenal extracts (412, 147, 148, 221, 256, 387, 389, 390, 157). Some attempts were also made to identify corticosteroids in the thymus (318) and the placenta (252) by partition chromatography. The nature of the adrenal secretion was studied by analysis of the adrenal venous blood of various species (80, 388), particularly of dogs (322, 454, 134, 132, 131, 321, 135, 133), sheep (85), rats (391), rabbits (214), and human subjects (331). The corticosteroids in human peripheral blood have also been studied by partition chromatography (284, 357, 276, 86, 49, 186).

A great deal of information about biogenesis and metabolic conversion of cortical hormones and related C_{21}-steroids has been obtained by application of partition chromatography to adrenal perfusates (457, 332, 176, 267, 251, 405, 335, 431, 319); some of this work has been reviewed (177, 174). Adrenal tissue slices (155, 170, 171, 57, 172, 193), homogenates (258, 377, 356, 435, 213, 196, 200, 458, 153, 165, 270, 168, 250, 345, 368, 369), or fractions (411, 166, 167, 249, 312, 313, 63, 64) were incubated with steroids and the metabolic products were examined by partition chromatography. Similarly, metabolic transformations of steroids in the liver have been studied by perfusion (175, 95, 94, 16, 272) and incubation with liver slices (124), homogenates (136, 4, 200, 201, 198, 199), and fractions (3, 419).

Microbiological transformations of steroids are of considerable importance. Much of this work has been carried out by Peterson (306) and his associates, who used partition chromatography routinely in production control (308, 212, 304, 97, 98, 309, 261, 126, 307, 262, 310, 127, 156, 263, 269, 459, 285).[8]

The analysis of urine extracts for adrenocortical hormones and related C_{21}-steroids by partition chromatography is made difficult by the presence of interfering pigments. Methods for hydrolysis, extraction, and purification may be found in individual papers and in a recent publication by Lombardo, Viscelli, Mittelman, and Hudson (240). On the other hand, Cope and Hurlock (101) found that the results of conventional corticoid assays are more reliable if the urine extract has been previously purified by partition chromatography. Normal human urine has been analyzed by paper chromatography before and after hydrolysis by various methods (74, 456, 76, 346, 83, 84, 146, 109, 443, 446, 103, 18, 334, 333, 65, 186). The urine of mice (305) and guinea pigs (66) has been so investigated, and Δ^4-pregnene-6β,11β,17α,21-tetrol-3,20-dione and 17-hydroxycorticosterone were isolated from the latter (67). Partition chromatography has also played a part in the isolation from human urine of Δ^4-pregnene-6β,11β,17α,21-tetrol-3,20-dione (68), pregnane-3α,17α,20α-triol (104), and other C_{21}-steroids (139, 140). Several investigators, notably Luetscher, have contributed to the isolation of aldosterone from urine by means of partition chromatography (246, 245, 243, 244, 247, 248, 100, 303). Pregnancy urine (268, 444,

38) and urine from patients with leukemia (301) and adrenocortical carcinoma (421) have also been investigated. From the latter pregnane-3α,17α,21-triol-20-one was isolated. The analysis of urine after the administration of certain drugs or hormones by partition chromatography has been used to study the *in vivo* metabolism of 11-dehydro-17-hydroxycorticosterone (72, 69, 326, 445), 17-hydroxycorticosterone (70, 186, 326), corticosterone (125), cholesterol (441), Δ^4-pregnen-17α-ol-3,11,20-trione (71), pregnane-11β,17α,21-triol-3,20-dione and pregnane-3α,11β,17α,21-tetrol-20-one (355), and pregnane-17α,21-diol-3,20-dione and pregnane-17α,21-diol-3,11,20-trione (430), and the effect of corticotropin (73, 420) and salicylates (396).

Other published chromatographic studies on corticoids include the analysis of ascitic (102) and synovial (448, 447) fluid, and the examination of synthetic (397, 413, 2, 218) and enzymatic (192) reaction products.

Not only commercial progesterone preparations (158) but also the minute quantities of progesterone in blood (88, 122, 123, 460, 461) have been determined by partition chromatography. This technique was also employed in the isolation of progesterone from pregnancy plasma (348) and in *in vitro* studies of progesterone metabolism (417, 169). The pregnanediol fraction of urine was found to consist of at least two fractions (44).

D. STEROLS AND BILE ACIDS

Paper chromatography has been used in the determination of adrenal cholesterol (235), in the identification of sterols in human aorta (187), and metabolic studies on cholesterol (392), epicholesterol (188), and vitamin D (224, 226). The products of the oxidation of cholesterol by air have been separated by column partition chromatography (277). Several investigators have taken advantage of chromatographic techniques to study the composition of bile from various species (161), including the pig (160), dog (278), and coypu (159), and the *in vivo* (29, 32, 36, 33, 35, 287) and *in vitro* (30, 31, 144) metabolism of bile acids.

E. PLANT STEROIDS

Most of the work on identification of the cardiac glycosides and aglycones in *Strophanthus* plants by partition chromatography originated at the Reichstein school (361, 362, 1, 130, 189, 20, 363, 184, 338, 364, 365, 327, 320, 328, 366, 315, 129).[8] The sensitivity and comparative simplicity of chromatographic methods have resulted in great savings during the British (87) and American (180) surveys of this genus. *Digitalis* glycosides have been analyzed qualitatively (264, 265, 234, 293, 297, 294, 295, 208, 209, 150, 61, 62, 440, 350, 404) and quantitatively (210, 385, 386, 422, 106, 25, 23, 24). Other genera examined for cardiac glycosides by partition chromatography were *Scilla* (403, 119), *Bowiea* (215, 425), *Urechites* (162), *Nerium* (330), *Acokanthera* (279), *Cheiranthus* (275), *Periploca* (360), and *Convallaria* (424).

The steroidal sapogenins of agaves and yuccas grown in France were surveyed by paper chromatography (185), and new saponins were discovered in *Smilax*

(298), *Narthecium* (398), and Japanese *Dioscoreaceae* (426, 428, 429, 427). Steroidal alkaloids identified by partition chromatography included those from *Veratrum* (8, 281), *Holarrhena* (423), and *Solanum* (233).

The author wishes to take this opportunity to thank Dr. Erich Mosettig for his encouragement and helpful criticism, to thank the authors who have supplied him with reprints of their work, and to invite his readers to send in further criticism and future publications in this field.

VI. References

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