REVERSED PHASE PAPER CHROMATOGRAPHY AND DETECTION OF STEROIDS OF THE CHOLESTEROL CLASS*

ROBERT P. MARTIN**

Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City, Utah (U.S.A.)

INTRODUCTION

The chromatography system to be described was originally developed in order to assist in the study of metabolism related to cholesterol in adrenal cortex tissue. A system was desired in which large amounts of "fat" from the tissue extract would remain behind at the origin yet allow some resolution of the less polar steroids such as cholestenones and cholestenols, and in which solvents and impregnators would neither interfere with scanning for ultraviolet absorption[1] nor require heating or long periods of drying for removal from the paper. For one or more reasons, methods previously available were considered to be unsatisfactory[2-7]. Consequently, as earlier reported[8], the general method of MILLS AND WERNER[9] was adapted. KODICEK AND ASHBY[10] have published an abstract concerning a similar method for Vitamin D and other sterols.

The present method allows mutual separation of individual steroids from large amounts of fat; for example, the separation of cholesteryl acetate, 3β-cholestanol, and cholesterol from material like cholesteryl palmitate, which remains at the origin, is possible. The solvent front is visible on the paper in and out of the tank. Furthermore, the applied solvents are sufficiently well removed by a short drying period at room temperature.

Application of the color reagent phosphomolybdic acid[11] and of the very little used reagent phosphotungstic acid[12] has been extended, and the possible significance of the latter in structure elucidation has been investigated.

METHODS

Chromatography

In order to separate various resins, MILLS AND WERNER[9] used a high-boiling hydrocarbon called "odorless kerosene" as the impregnating phase and aqueous *iso*propanol as the developing phase. Attempts were made to use the same system for the resolution of ether-soluble materials obtained from adrenal glands, but they were not successful. However, 60% (v/v) aqueous *n*-propanol was found to be quite satisfactory as the moving phase.

The chromatography tank is prepared as described by BUSH[13] with 60% aq. *n*-propanol covering the bottom of the tank to a depth of about 5 cm. In the center is placed a beaker

* This work was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service. It is taken in part from the doctoral thesis submitted by the author to the Graduate School, University of Utah, 1955.

** Armour and Company Predoctoral Fellow in Biochemistry. Present address: Department of Chemistry, Colorado State University, Ft. Collins, Colorado (U.S.A.).

References p. 416.

filled with odorless kerosene (Standard Oil Co. product "Base Oil C", boiling range 180–325° C) and fitted with a rolled filter paper extending nearly to the top. With the lids taped in place, tanks so prepared have been used at least 8 months without renewal. To prepare the chromatograms, Whatman filter paper No. 2 is impregnated by dipping in 12% (v/v) kerosene in petroleum ether. After momentary draining, the paper is evenly blotted and then clamped on a rack to be slightly inclined for the application of the material. For a preparative chromatogram in which an initial separation of crude extract material is desired, the paper should be as wide as the tank allows. The origin line must be at least 9 or 10 cm below the line at which the paper is suspended in the tank. The extract material, concentrated to an oily residue in the tip of a conical tube, is dissolved in a minimal amount of solvent (*e.g.*, 0.2 ml ethyl acetate) and applied by micropipette in a thin line at the origin. In hot summer months, because of evaporation of kerosene from the paper, this operation must be carried out promptly. In extreme cases, the amount of impregnating kerosene may be increased.

When a rather large amount of material (*ca.* 25 mg) has been applied at the origin, the kerosene is displaced away from the origin line during the application of the solvent, and a 10–12 hour equilibration of the paper in the tank is required. This allows a redistribution of the kerosene and an even spreading of the applied material. When microgram quantities of material are applied, the original displacement is not great, and the equilibration time may be decreased to 5 or 6 hours. The chromatography tanks should be placed in a room of moderately constant temperature, although it has been noted that variations of about 5° C have not been harmful. Furthermore, equilibration and development take place about equally well at temperatures of 22° and 37°.

After the papers have equilibrated, the descending solvent, 60% aq. *n*-propanol saturated with kerosene, is run into the troughs through the holes in the top of the tank. Optimal resolution times depend on the results one requires. Generally, for relatively pure compounds a 10 to 12 hour period is sufficient; for crude extracts a period of 12 to 15 hours may be necessary. However, shorter periods may give the separation desired. One may follow the descent of the developing solvent by observing the front of the paper while the back is being illuminated. Just after removal of the paper from the tank, the solvent front is easily visible; furthermore, when the paper has dried, there is a residual line at the solvent front which may be seen in the Haines ultraviolet scanner. Thus, for individual compounds front ratios (R_F's) may be calculated. Fortunately, a short drying period of about 20 minutes at room temperature is sufficient for further operations such as viewing in the UV scanner, spraying with various color reagents, and elution for immediate application to other resolving systems or for plating and counting for radioactivity. This short drying period without elevation of temperature is not only convenient but desirable in principle, as will be illustrated later.

Color reagents

For application of color reagents to the preparative type of chromatogram a strip 4 mm wide is cut lengthwise from the center of the paper. Spraying with an 8% alcoholic solution of phosphomolybdic acid[11] has been useful in the delineation of cholesterol and cholesteryl ester areas resulting from chromatography of extracts of adrenal tissue and testis tissue. Uniform color development with a minimum of background color has been achieved by placing the sprayed strip in a chamber of *ca.* 37° C for about 10 minutes.

A reagent apparently previously used only for cholesterol, phosphotungstic acid[12], has been found applicable to a wider range of steroids. The paper strip is sprayed with a 15% alcoholic solution (stable for storage), then warmed in a chamber at 37° or heated more strongly as over a hot plate. In actual practice when dealing with chromatograms of unknown substances, one should cut out two strips, spray, then warm one gently and heat the other more strongly in order to obtain the most information. The phosphotungstic acid reagent can be quite sensitive, and it is much more specific than either phosphomolybdic acid (see below) or silicotungstic acid. The latter was reported to give brown with pregnenolone, testosterone propionate, and 5,6-dehydro-*epi*androsterone but pink with testosterone, stigmasterol and cholesterol[11].

Elution

After the various areas have been located as described above, they may be cut out for elution. In several experiments it was satisfactory and convenient to elute the large areas in Soxhlet apparatus, ethanol being used for the most mobile compounds in the prefrontal region, ethyl acetate for the center or cholesterol region, and ethyl acetate for the origin or cholesterol ester region. (It was found that ligroin even after a 3-hour period of refluxing did not elute all the material from tissue extracts which remains at the origin.) In many cases, where the prefrontal material has chromatographed into a rather well defined thin area, this part may be cut out and eluted by a descending flow of cold ethanol, as described by BUSH[13].

If one is interested in prefrontal material only, another method of elution may be used which takes advantage of the fact that the usual chromatography order is reversed in this system.

References p. 416.

The chromatogram is cut to be about 30 cm long with a very sharp bevel leading to a point in the center of the lower edge. A cylinder with funnel is placed in the tank directly beneath the pointed end of the paper, and it is better if the tip of the paper touches the inside of the funnel to drain off the drops of developing solvent as fast as they collect. In this way, *a chromatographic separation and elution may be accomplished at the same time.* Thus, in one operation compounds having a mobility equal to or greater than 7-ketocholesterol may be eluted free from cholesterol and other material of equal or less mobility.

RESULTS AND DISCUSSION

Individual compounds

A list of R_F values is given in Table I. These values were obtained by spotting about 50–60 μg of the authentic compound at the origin of a strip, chromatographing as previously described, then locating the compounds by spraying with phosphomolybdic acid solution and by viewing in the UV scanner. Commercial so-called "chemically pure cholesterol" gave two phosphomolybdic acid-positive spots, one at the prefront (R_F 0.87) and one at R_F 0.55. When the material of the lower R_F was eluted and chromatographed again, only one spot appeared, this being at R_F 0.55. Also, when the commercial cholesterol had been purified via the dibromide derivative[14], only one spot appeared and this at R_F 0.55. The faster running impurities are presumed to be cholesterol congeners with more oxygen functions, and the system can be applied to both separation and detection of such companions. It can be seen from Table I that this system can be used to separate cholesterol from its dihydro derivatives[15], from 7α- and 7β-hydroxylcholesterol[16], and from 3β, 5α, 6β-trihydroxycholestane and 3β, 5α-dihydroxy-6-ketocholestane. This is advantageous since both the latter two compounds also form insoluble digitonides, and the 6-keto compound, at least, is known to coprecipitate with cholesterol[17]. It is true, however, that in a 12–15 hour developing period this system will not separate cholesterol from another

TABLE I

R_F VALUES FOR REVERSED PHASE SYSTEM ON PAPER

The stationary phase was odorless kerosene, the descending phase 60% aq. *n*-propanol. Each compound was applied in the amount of 50–60 μg, chromatographed, and located by viewing in the Haines ultraviolet scanner and by spraying with 8% alcoholic phosphomolybdic acid solution, then warming.

Compound	R_F	*Compound*	R_F
Cholesteryl palmitate*	0.00	7-Ketocholesterol*	0.87
Cholesteryl acetate*	0.14	4-Cholesten-6β-ol-3-one*	0.87
		4-Cholesten-3β-ol-6-one	0.87
4-Cholesten-3-one	0.32		
Cholestan-3α-ol	0.40	Testosterone*	0.87
Cholestan-3β-ol*	0.46	Testosterone acetate	0.87
		17α-Methyltestosterone	0.87
Cholesterol	0.55	4-Androstene-3,17-dione*	0.87
7-Cholesten-3β-ol	0.55		
7,9-Cholestadien-3β-ol	0.55	Progesterone*	0.87
		17α-Hydroxyprogesterone*	0.87
4-Cholestene-3,6-dione*	0.65		
3β-Methoxy-20-keto-5-pregnene	0.75	Corticosterone	0.87
Testosterone-17-methyl ether	0.85	17-Hydroxycorticosterone	0.87

* Compounds so marked were also chromatographed mixed with cholesterol. In no case was an effect on R_F observed.

References p. 416.

TABLE II

COLOR REACTION ON PAPER OF STEROIDS AND OTHER COMPOUNDS WITH PHOSPHOMOLYBDIC ACID SOLUTION (8% ALC.) AND PHOSPHOTUNGSTIC ACID SOLUTION (15% ALC.)

Compound	*Phosphomolybdic acid*	*Phosphotungstic acid*
5-Cholestene. Cholestan-3-one	NST	Neg. (*s,w*)
4-Cholesten-3-one	Neg. (*c,w*)	Neg. (*c,w*)
Cholestan-3α-ol and -3β-ol	+, ++ (*c,w*)	Light tan (*c,w*)
3β-Acetoxycholestane	+ (*c,w*)	Neg. (*s,h*)
7-Cholesten-3β-ol. 7,9-Cholestadien-3β-ol	++ (*c,w*)	Very light tan (*c,w*)
Progesterone. 4-Cholestene-3,6-dione	Neg. (*c,w*)	Neg. (*s,h*)
17α-Hydroxyprogesterone	Neg. (*c,w*)	NST
3β-Methoxy-20-keto-5-pregnene	+ (*c,w*)	NST
Testosterone	+ (*c,w*)	Neg. (*s,w*). Brown to gray (*s,h*)
Testosterone-17-methyl ether	Neg. (*c,w*)	NST
Testosterone acetate	+ (*c,w*)	NST
17α-Methyltestosterone	+ (*c,w*)	NST
4-Androstene-3,17-dione	Neg. (*c,w*)	Light brown-green (*s,h*)
Corticosterone. 17-Hydroxycorticosterone	Neg. (*c,w*)	Light green (*s,h*)
11-Desoxycorticosterone	NST	Neg. (*s,w*)
Cholesterol*, the acetate, the palmitate	+ (*c,w*)	Pink (*c,w*). Purple (*s,h*)
7-Ketocholesterol	Neg. (*c,w*)	Very light pink (*c,w*)
7-Ketocholesteryl acetate	NST	Pink (*s,w*)
Sitosterol acetate	NST	Pink (*s,w*). Purple (*s,h*)
Stigmasterol	+ (*s,w*)	Pink (*s,w*). Purple (*s,h*)
Ergosterol. 7-Dehydrocholesterol. (Omitted due to unresolved question of purity.)		
Diosgenin	+ (*s,w*)	Pink-orange (*s,w*). Red (*s,h*)
5-Pregnen-3β-ol-20-one	+ (*c,w*)	Pink (*c,w*)
5-Pregnen-3β, 17α-diol-20-one	NST	Pink (*s,w*)
5-Androstene-3β, 17β-diol	+ (*c,w*)	Pink (*s,w*)
5,6-Dehydro*epi*androsterone	+ (*c,w*)	Pink (*s,w*)
17α-Methyl-5-androstene-3β, 17β-diol	+ (*c,w*)	Pink (*c,w*)
4-Cholesten-6β-ol-3-one	Neg. (*c,w*)	Light tan (*s,w*)
4-Cholesten-3β-ol-6-one	+ (*c,w*)	Light yellow (*s,w*)
17α-Methyl-4-androstene-3β, 17β-diol	+ (*c,w*)	Orange 1 min RT, red 10 min 37°, purple after hours RT
17α-Methyl-4-androstene-3α, 17β-diol	+ (*c,w*)	Orange 1 min RT, purple 1 min 37°
4-Cholesten-3β-ol and the acetate	+ (*s,w*)	Pink (*s,w*). Brick-red (*s,h*)
Estrone	+ (Standing RT)	Neg. (*s,h*). Tan (48 h RT)
Estradiol. Estriol	+ (Standing RT)	Neg. (*s,h*). Tan-pink (48 h RT)
Morphine	Tan (1 min RT) Blue (Standing RT)	Light yellow-pink RT. Little effect of heat. (*s,h*)
Codeine hydrochloride	Tan (1 min RT) Tan (Standing RT)	(Same as morphine)
*iso*Octanol	+ (*l,h*)	Light brown (*l,h*)
n-Amyl alcohol	+ (*l,h*)	Light tan, pink hue (*l,h*)
Allyl alcohol	+ (*l,h*)	Brown-purple (*l,h*)
Methallyl alcohol	+ (*l,h*)	Yellow-pink (*l,h*)
Glycerol. Propylene glycol	Neg. (*l,h*)	Neg. (*l,h*)

Symbols: *c* = 50 μg compound chromatographed then sprayed. *s* = 50 μg compound spotted over 1 cm², sprayed. *l* = drop liquid on paper, sprayed. RT = *ca.* 22° C. *w* = color development at 37° for 15 min. *h* = color development over hot-plate. Neg. = no color. + = estimate of blue color. NST = not submitted to test.

* The cholesterol, from Prof. T. REICHSTEIN, had been purified via the dibromide and by sublimation. M.P. = 148.5° (corr.).

known companion, 7-cholesten-3β-ol[18-20]; in this case, a longer developing time or a modification of the solvent might be effective. The mobilities of ergosterol and 7-dehydrocholesterol are not given owing to questions of purity as yet unresolved.

In Table II are shown results of the reaction on paper of various compounds with phosphomolybdic acid. They are given not only to extend the list of KRITCHEVSKY AND KIRK[11] but to show that under the conditions of color development used here, a greater selectivity is possible. By warming the sprayed paper at *ca.* 37° for 10–20 minutes, the background color was kept to a minimum, the Δ^4-3-keto group was not positive, and neither was the α-ketol group of the corticoids. The most reactive groups generally were secondary hydroxyls of the steroids, the 3-hydroxyls being more reactive than 17-hydroxyls. A strange exception to these two generalizations was provided by 17α-methyltestosterone. Compounds with double bonds more distant from the 3-hydroxyl than the 5,6 position were not as reactive as those with a double bond at the 5,6 position. Indeed, they were not markedly more reactive than cholestan-3β-ol, which was somewhat more reactive than its epimer. The five esters tested were as reactive as the parent hydroxyl compounds, although in the case of the two methoxy compounds, the parent compounds were more reactive. The two Δ^4-3-ol steroids tested were, surprisingly, no more reactive than the single double bond Δ^5 compounds.

Special attention is drawn to 4-cholesten-6β-ol-3-one and 7-ketocholesterol. Contrary to expectation, neither compound gave the blue color with phosphomolybdic acid even though allowed to remain in the warm chamber for about half an hour. It may be that under the influence of the reagent water splits out of the ring to give a double bond conjugated with the ketone; thus, no hydroxyl remains to react with the color reagent in the typical manner. However, 4-cholesten-3β-ol-6-one did give the characteristic reaction although it was not quite as reactive as cholesterol. The primary aliphatic alcohols tested were positive only when heated somewhat more strongly than in the warming chamber, and the two unsaturated alcohols were not more reactive than the two saturated ones. The two polyols, glycerol and propylene glycol, in spite of the presence of secondary alcohol functions, were not positive even with fairly strong heating. The results with these aliphatic alcohols and with the steroids might suggest that oxygen functions vicinal to the secondary hydroxyl group affect the initial complexing of the large phosphomolybdic acid molecule and the organic compound. Whatever the reason, it is obvious that phosphomolybdic acid cannot be relied upon to indicate all hydroxylated steroids on paper chromatograms but should be used in conjunction with other reagents.

Some interesting differences and similarities are revealed by comparing the results of reactions with phosphomolybdic acid on paper with those, carried out by other workers, in acetic acid with phosphomolybdic acid[21] and with phosphomolybdotungstic acid[22]. In acetic acid the more chromogenic steroids in general were those either with a Δ^4-3-ketone group or an α-ketol group. Compounds containing both groupings, such as desoxycorticosterone, were about twice as chromogenic. Compounds such as 5-pregnen-3β-ol-20-one, cholesterol, and 7-cholesten-3-ol gave almost no color. These results are directly opposite those obtained on paper. The results found to be similar in both systems were those with 7-ketocholesterol, which had a much lower order of reactivity than expected, and with 17α-methyltestosterone, which had a higher order than expected. Also, in acetic acid 1-androstene-3,17-dione

References p. 416.

was found not to be reactive[21], whereas 4-androstene-3,17-dione was. This is somewhat similar to the finding that the color produced on paper by 1-cholesten-3β-ol with phosphotungstic acid was less than expected.

The results with phosphotungstic acid are also shown in Table II. The course of color development with Δ^5,3β-ol steroids during 15 minutes in the warm chamber at 37° was light tan to yellow to pink. Of 13 such steroids tested, 12 gave exactly this series of colors. 7-Ketocholesterol (negative with phosphomolybdic acid) gave almost no color although its acetate was positive. When heated more strongly with phosphotungstic acid, as over a hot plate, cholesterol and stigmasterol, for example, gave a yellow then vivid lavender within one minute. The intensity of this lavender was decreased, however, if the paper was first warmed for some minutes prior to the stronger heating. Steroids not having the Δ^5,3β-ol structure did not give this series of colors.

Especially noteworthy are the results of the reaction of phosphotungstic acid with five steroids having allylic alcohol functions. 4-Cholesten-6β-ol-3-one (negative with phosphomolybdic acid) gave only a tan color with phosphotungstic acid, while its 3,6 isomer, 4-cholesten-3β-ol-6-one, progressed as far as yellow after warming at 37°. Thus, it seems again that the second oxygen function has played a role. Both 17α-methyl-4-androstene-3β,17β-diol and its epimer, 17α-methyl-4-androstene-3α, 17β-diol, gave highly distinctive color development quickly at room temperature, and the two compounds were sufficiently different in behavior to enable one to distinguish them. 1-Cholesten-3β-ol and its acetate both gave a light pink when warmed at 37° but quickly gave a strong and permanent brick-red color when heated over a hot plate. Presumably here there is an influence of the neighboring methyl group at carbon 10, and a higher temperature is necessary even though an allylic alcohol is present. In the course of the investigation, no other allylic alcohol steroids were available. However, morphine and codeine hydrochloride and the two primary alcohols, allyl and methallyl alcohol, were obtained. These proved to be rather more like the Δ^5,3-ol steroids than the two highly reactive allylic Δ^4,3-ol steroids. However, allyl alcohol did give a brown-purple on stronger heating.

Phosphotungstic acid is a sensitive reagent. When 2 micrograms cholesterol was applied over 1 sq. cm of Whatman No. 2 filter paper (1 μg on each side), sprayed, and developed over a hot plate, a yellow color with pink cast appeared. A larger area of even less concentration would probably be visible. A definite pink was given by 3 μg per sq. cm, and 5 μg per sq. cm gave a vivid lavender. This sensitivity makes the reagent useful in detecting impurities, for example, of cholesterol in cholestanol. It was found that Whatman paper can give a yellow-pink background but only after very strong excessive heating. In normal testing procedures background interference was never encountered.

An attempt was made to produce a color by heating an alcoholic solution of cholesterol and phosphotungstic acid. No color developed, and none developed after acidification with acetic acid. However, when the reaction was attempted in glacial acetic acid alone, a clear brilliant violet color was produced after heating in a water bath at 85°. Stigmasterol reacted similarly. Diosgenin gave a pink followed by a black precipitate. The possibility is evident that phosphotungstic acid in acetic acid might be a satisfactory reagent for a simple quantitation of compounds such as cholesterol.

References p. 416.

Certain precautions other than those already noted need to be observed in the use of phosphotungstic acid and phosphomolybdic acid. It was found that when the chromatography paper had been treated with formamide, there was a partial or complete prevention of color development. (Interference by formamide with the Zimmerman reaction (alkaline *m*-dinitrobenzene) has also been noted in some of our experiments.) Also, at one time tests were attempted with phosphotungstic acid on presumably clean paper which, however, had been stored in the same drawer with paper sprayed with Keddè reagent (alkaline 3,5-dinitrobenzoic acid). There was no color development whatsoever on these papers.

Tissue extracts. Adrenal tissue

The rather high capacity of this chromatography system for holding fat at the origin and for preventing compounds such as cholesterol from entering the prefrontal region is particularly important when one wishes to free tissue extracts from such material and recover the more polar compounds such as the known estrogens, androgens, and corticoids of the adrenal. Excellent initial separations into origin material, cholesterol-area material, and prefrontal compounds have been accomplished by applying as much as 35–40 mg of ethyl acetate-soluble material, which had been derived from 500 mg rat adrenal tissue, on a Whatman No. 2 paper 175 mm wide. It might be possible to chromatograph as much as 70 mg material on No. 3 paper if one is interested only in prefrontal material. To further test the resolving powers of this system, cholesterol-4-^{14}C (*ca.* 100,000 counts per minute) was mixed with the ethyl acetate-soluble material from 500 mg rat adrenal tissue. After chromatography and elution it was found that all the measurable radioactivity resided in the cholesterol area; there was none whatsoever associated with the material remaining at the origin. It had been considered possible that the large amount of non-mobile substance might prevent movement of a portion of the mobile material, but this was not the case.

Mention was made earlier of the general desirability of freeing the paper of chromatography solvents without heating or long drying periods and of eluting the compounds as quickly as possible to increase recovery. When one is dealing with unknown compounds, this is of some considerable importance for yet another reason as indicated by the following results. When adrenal extracts were chromatographed, generally there was an ultraviolet-absorbing area at the prefront, definite but not dark, while the material at the origin showed little or no absorption. When the paper was allowed to hang at room temperature for about 17 hours and then viewed again in the Haines scanner, both the origin and prefront areas were noted to be considerably darker, that is, the UV absorption had increased. When the paper was hung in a nitrogen atmosphere for even longer periods of time, such a change did not occur. Thus, there are indications that an oxidation had taken place. These phenomena were observed several times with both dog and rat adrenal extracts. It was considered that perhaps a hydroxyl group with an α,β double bond had been oxidized to an α,β unsaturated ketone. This may have been the case; however, when 17α-methyl-17β-hydroxy-4-androsten-3β-ol was tested under similar circumstances, no UV-absorbing compound was formed. Indeed, there was no evidence that steroids were involved. No further investigation of this problem was carried out, but it indicates a possible source of artifact formation.

References p. 416.

Testis tissue

This system was found to be quite useful in studying the products resulting from incubation of rat testis tissue with various steroids in buffer and bovine serum. The incubation mixture was extracted with ether and the extract was applied directly to the chromatogram. Ether-soluble material from as much as 2.7 g rat testis tissue and 15 ml bovine serum has been chromatographed on one paper 175 mm wide. As shown in Fig. 1, most of the developed chromatograms had two definite ultraviolet-absorbing bands in the prefront region. The faster running band contained the mixture of compounds to be further resolved in other systems. It was desirable to get rid of the material running just behind the most mobile material since it interfered with subsequent resolution in the BUSH systems[13]. Use of this reversed phase chromatography system was found to be superior to silica gel chromatography columns since this large amount of unwanted material, thought to be cholestenedione, was difficult to separate from the small amounts of the compounds desired, whereas on the paper separation was good, and the areas were easily delineated in the scanner and then cut for elution.

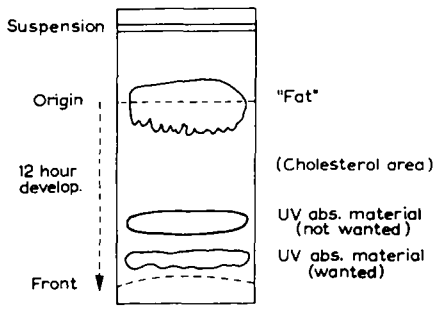

Fig. 1. Replica of reversed phase chromatogram. Stationary phase: Odorless kerosene. Mobile phase: 60% aq. *n*-propanol. Resolution of ether-soluble material from incubation of 2.7 g rat testis tissue in 15 ml bovine serum with 0.5 μmoles progesterone. The most mobile band of material contained progesterone, testosterone, androstenedione, etc.

One obvious major disadvantage of this particular chromatography system is the long equilibration time and the rather long development time. It is conceivable that the equilibration time might be decreased somewhat by adding a small amount of the stationary phase (kerosene) to the solution of the material to be applied to the paper; thus, the displacement of the kerosene on the paper by the solvent would not be so great. However, in doing preparative paper chromatography one cannot decrease the equilibration time beyond a certain point because the relatively large amount of material applied must have time to become distributed and associated with the required amount of stationary solvent before the developing solvent advances over the origin area. It is particularly important in a system where the stationary phase must retard the movement of a large amount of material as is the case here.

In conclusion, it should be stated that no attempt was made to prove that this system is a reversed phase system in the strict sense of the term[6]. It is probable that the 60% aq. propanol would elute and move the compounds from the origin of an untreated paper in somewhat the same order as from the paper impregnated with kerosene, and it is not possible to say how much the hydrocarbon impregnation masks the polar character of the paper. It can only be said that the odorless kerosene is necessary for a satisfactory system, and that the order of chromatography is reversed from that usually obtained.

References p. 416.

ACKNOWLEDGEMENTS

The author expresses his sincere appreciation to Dr. LEO T. SAMUELS, Head of the Biochemistry Dept., University of Utah, and to Dr. IAN E. BUSH, Radcliffe Infirmary, Oxford, England, for valuable help and suggestions. Special thanks is given to Prof. T. REICHSTEIN and to Dr. CH. TAMM for providing some of the compounds and for allowing some of the work to be carried out in the laboratories of the Institute of Organic Chemistry, Basle, Switzerland. Gratitude is expressed for financial help, as previously indicated.

SUMMARY

A system of paper chromatography has been presented in which the stationary phase is a high-boiling hydrocarbon mixture (odorless kerosene) and the mobile phase is 60% aq. propanol. This system is not only useful for separation and identification of the cholesterol class of compounds but may be conveniently used to free tissue extracts of so-called fat. The use of the system in preparative chromatography has been discussed.

Special application of two color reagents, phosphomolybdic acid and phosphotungstic acid, has been described. Proper use of the former can make it fairly specific for some steroids with secondary alcohol functions. Phosphotungstic acid was shown to give a characteristic pink color with many steroids having Δ^5, 3β-ol functions if warmed gently and a vivid lavender if heated more strongly. Certain steroids with allylic alcohol functions were also very chromogenic but with varying results. Precautions and limitations in the use of the reagents were discussed.

REFERENCES

1 W. J. HAINES, *Recent Progr. Hormone Research*, 7 (1952) 255.
2 D. KRITCHEVSKY AND M. CALVIN, *J. Am. Chem. Soc.*, 72 (1950) 4330.
3 T. H. KRITCHEVSKY AND A. TISELIUS, *Science*, 114 (1951) 299.
4 G. F. LATA AND C. S. VESTLING, *Anal. Chem.*, 24 (1952) 208.
5 R. NEHER AND A. WETTSTEIN, *Helv. Chim. Acta*, 35 (1952) 276.
6 E. HEFTMAN, Partition chromatography of steroids, *Chem. Revs.*, 55 (1955) 679.
7 E. G. HARRISON, *Paper Chromatographic Study of Adrenal Cholesterol*, Thesis, University of Illinois, 1949.
8 R. P. MARTIN AND I. E. BUSH, *Federation Proc.*, 14 (1955) 252.
9 J. S. MILLS AND A. E. A. WERNER, *Nature*, 169 (1952) 1064.
10 E. KODICEK AND D. R. ASHBY, *Biochem. J.*, 57 (1954) xii.
11 D. KRITCHEVSKY AND M. A. KIRK, *Arch. Biochem. Biophys.*, 35 (1952) 346.
12 L. DOUSTE-BLAZY, J. POLONOVSKY AND P. VALDIGUIE, *Compt. rend.*, 235 (1952) 1643.
13 I. E. BUSH, *Biochem. J.*, 50 (1952) 370; *Recent Progr. Hormone Research*, 9 (1954) 321.
14 R. SCHÖNHEIMER, *J. Biol. Chem.*, 110 (1935) 461.
15 R. SCHÖNHEIMER, H. v. BEHRING AND R. HUMMEL, *Z. physiol. Chem.*, 192 (1930) 93.
16 S. BERGSTROM AND O. WINTERSTEINER, *J. Biol. Chem.*, 145 (1942) 327.
17 E. SCHWENK, N. T. WERTHESSEN, H. ROSENKRANTZ, *Arch. Biochem. Biophys.*, 37 (1952) 247.
18 L. F. FIESER, *J. Am. Chem. Soc.*, 73 (1951) 5007.
19 P. R. MOORE AND C. A. BAUMAN, *J. Biol. Chem.*, 195 (1952) 615.
20 D. R. IDLER AND C. A. BAUMAN, *J. Biol. Chem.*, 195 (1952) 623.
21 S. BURSTEIN, *Anal. Chem.*, 25 (1953) 422.
22 R. D. H. HEARD AND H. SOBEL, *J. Biol. Chem.*, 165 (1946) 687.

Received April 4th, 1957