quantitative determination of 7-dehydrocholesterol, in concentrations of 2 to 14 γ per cc., by measuring the ultraviolet absorption in the Beckman spectrophotometer for different concentrations at 322 mμ. Under these conditions, there is a linear relationship between the optical density and concentration of 7-dehydrocholesterol. Neither cholesterol, ergosterol nor calciferol interfere. By eluting 7-dehydrocholesterol from the known position on the chromatogram, the conversion from cholesterol or other precursors can be detected.

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[CONTRIBUTION FROM THE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Paper Chromatography of Steroids[1]

BY DAVID KRITCHEVSKY AND MARTHA R. KIRK

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The preparation and use of "Quilon" (stearato chromic chloride) impregnated paper for the reverse phase paper partition chromatography of steroids is described. The R_f values for a number of steroids in a variety of solvents are reported. Separation of cholesterol from epicholesterol, ergosterol and 7-dehydrocholesterol has been achieved. Several other separations are also reported.

Through the use of impregnated filter paper it has been possible to achieve separation of various steroid mixtures by paper chromatography. The corticosteroids have been separated using papers treated with propylene glycol,[2,3] formamide[2,3] or alumina[4,5]; the estrogens on paper treated with alumina,[4,5] glycerol,[6] ethylene glycol[6] or capryl alcohol[6]; and the androgens on papers impregnated with alumina[4,5] or a silicone.[7] Recently, Neher and Wettstein[8] have reported the separation of weakly polar steroids on paper treated with phenyl cellosolve. The successful separation of cholesterol and cholestenone on paper impregnated with "Quilon" (stearato chromic chloride) already has been reported.[9] This method also has been used for the separation of vitamins D_2 and D_3 from a mixture of sterols.[10] This report covers the results obtained by application of this method to a number of steroids. The R_f values obtained with twenty-one steroids using a variety of solvents are tabulated in Tables I, III, IV and V. Each R_f value represents the average of at least six separate chromatograms.

In the case of the weakly polar steroids, the separation of cholesterol from 7-dehydrocholesterol, ergosterol and epicholesterol has been accomplished. Stigmasterol and ergosterol have also been separated. Any two steroids whose R_f values are sufficiently far apart may be separated by this system, and in the case of a mixture of cholesterol and testosterone separation has indeed been carried out. The separations are summarized in Table II.

In the case of the corticosteroids and the androgens, the R_f values are reproducible but no resolution could be achieved with the solvent systems used. The salient feature of the data concerning these compounds is that the addition of water to the anhydrous solvent gives a higher R_f value rather than the lower one which might be expected. Thus, in methanol the corticosteroids all exhibit R_f values in the neighborhood of 0.75 and in methanol–water 9:1 the R_f values are approximately 0.80. The same variation was observed with various androgens and progestational hormones. It is possible that the systems under investigation are exceedingly sensitive to small changes in solvent composition and that the mixtures we have used have bracketed the area of greatest sensitivity. For example, the R_f values for cortisone in methanol–water 95:5, methanol–water 9:1 and methanol–water 85:15 are 0.81, 0.86 and 0.80, respectively. Other steroids exhibit similar variations. Several of the weakly polar steroids exhibit the expected lowering of R_f upon dilution of the methanol.

Addition of ammonia or formic acid to the methanol–water solvent system caused a change in the R_f values (usually higher than for methanol

TABLE I

R_f VALUES OF THE WEAKLY POLAR STEROIDS

	CH_3OH	CH_3OH-H_2O 9:1	C_2H_5OH	C_2H_5OH-H_2O 8:2
Cholesterol	0.56	0.31	0.92	0.52
Epicholesterol	.80	..	..	.97
Cholestanol	.63	..	..	.56
Stigmasterol	.52	0.27	..	.53
Sitosterol	.65	..	..	.54
Cholestenone	.82	..	0.97	.86
7-Dehydrocholesterol	.88	..	..	.94
Ergosterol	.84	0.90	..	.95

TABLE II

SEPARATIONS IN METHANOL

Compounds	R_f Values
Cholesterol/ergosterol	0.52/0.84
Cholesterol/7-dehydrocholesterol	.54/ .88
Cholesterol/epicholesterol	.56/ .81
Cholesterol/testosterone	.54/ .77
Stigmasterol/ergosterol	.47/ .85

(1) The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

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TABLE III

R_f VALUES OF CORTICOSTEROIDS

	CH_3OH	CH_3OH–H_2O 95:5	CH_3OH–H_2O 9:1	CH_3OH–H_2O 85:15	CH_3OH–H_2O 8:2	C_2H_5OH	C_2H_5OH–H_2O 8:2	CH_3OH–NH_4OH 9:1	CH_3OH–NH_4OH–H_2O 85:5:15	CH_3OH–HCOOH 99:1	CH_3OH–HCOOH–H_2O 80:1:19
Cortisone	0.75	0.81	0.86	0.80	0.80	0.88	0.94	0.77	0.94	0.69	0.91
Desoxycorticosterone	.75	..	.85	..	..	.86	.95	.81	.97	.78	.92
17-Hydroxycorticosterone	.73	..	.86	..	..	.88	.89	.79	.94	.73	.92
Dehydrocorticosterone	.74	..	.83	..	..	.85	.95	.76	.95	.74	.92
Corticosterone	.79	0.83	.83	0.80	0.82	.83	.91	.76	.97	.69	.92

TABLE IV

R_f VALUES OF ANDROGENS AND PROGESTATIONAL HORMONES

	CH_3OH	CH_3OH–H_2O 9:1	CH_3OH–$CHCl_3$ 9:1	CH_3OH–$CHCl_3$ 95:5	CH_3OH–CCl_4 95:5	CH_3OH–C_6H_6 99:1	CH_3OH–C_6H_6 95:5	CH_3OH–$CHCl_3$ 1:9	CH_3OH–NH_4OH 95:5	C_2H_5OH	C_2H_5OH–H_2O 8:2	C_2H_5OH–H_2O 7:3	i-C_3H_7OH	Dioxane
Dehydroisoepiandrosterone	0.75	0.87	0.86	0.82	0.81	0.80	0.87	1.00	0.80	0.97	0.96	0.96	0.94	0.99
Dehydroisoepiandrosterone acetate	.76	.85	.87	.85	.86	.80	.88	1.00	.82	1.00	.93	.97	.95	.98
Testosterone	.78	.83	.87	.83	.81	.82	.84	1.00	.82	1.00	.91	.98	.93	1.00
Testosterone propionate	.81	.92	.87	.90	..	.81	.85	1.00	.79	1.00	.97	1.00	.94	0.96
Androsterone	.80	.94	..	..	..	..	..	..	..	1.00	.94	0.95	..	.99
Progesterone	.80	.95	0.97	..	..	..	0.86	0.95	0.78	..	..	.98	0.95	..
Pregnandiol	.78	..	..	..	..	..	..	..	..	..	..	..	..	..
Pregnenolone	.75	..	..	..	..	..	..	..	..	..	..	..	..	..

alone), but again no resolution was possible. Higher alcohols such as ethanol or 2-propanol gave R_f values close to 1.00 for the corticosteroids, androgens and progestational hormones.

The only compounds which did not show R_f values in the neighborhood of 0.80 when methanol was the solvent were compounds that had a long hydrocarbon side chain and no conjugation in the ring system. The only exception to this which we have observed to date is epicholesterol which exhibits an R_f value of 0.80 in methanol. Cholestenone, ergosterol and 7-dehydrocholesterol have a conjugated system in the A ring and all give R_f values similar to those obtained with the other classes of steroids used. In the case of ergosterol, chromatography in methanol–water gave higher R_f values than those obtained using pure methanol. Variations in the side chain do not appear to affect the R_f values as may be seen from the values for cholesterol and stigmasterol.

Small quantities of chromic chloride complexes with several other acids have been made available to us by the du Pont Company. The complexes of furoic, salicylic and *p*-aminobenzoic acids were applied to the paper in the usual way, but the methanol seemed to wash much of the material out of the paper, a wide green zone appearing at the front in every experiment. The R_f values obtained in these experiments were very erratic. Glucono chromic chloride gave a paper stable to the solvent and the R_f values obtained with several compounds are listed in Table V. The R_f value of cholesterol on this paper is considerably higher than that for paper impregnated with stearato chromic chloride. The other R_f values are in the range that they were with the original Quilon paper, but show a somewhat greater variation in values.

Investigations involving smaller increments of change in the methanol–water ratios are under way using papers treated with stearato- and glucono-chromic chloride, respectively.

TABLE V

R_f VALUES ON GLUCONO-CHROMIC CHLORIDE (METHANOL SOLVENT)

Compound	R_f
Cholesterol	0.90
Progesterone	.86
Cortisone	.76
Desoxycorticosterone	.80
17-Hydroxycorticosterone	.75
Dehydrocorticosterone	.85
Corticosterone	.83

Experimental

Impregnation of Paper.—Impregnation of the filter paper used (Whatman No. 1) could be carried out in either of two ways to give paper having the same chromatographic characteristics. A solution that was 2% in "Quilon" and 2% in the "neutralizer" solution suggested for use with this material[11] was sprayed on the paper and the paper dried at 100–110°, or the paper was dipped into a trough containing the same solution, allowed to drain and dried in a similar fashion. It was necessary to dip or spray the paper only until thoroughly wet; long immersion in the impregnating mixture did not change the characteristics of the paper and often made handling more difficult. At the temperature used, the drying time was generally about 5–10 minutes. Once the paper was thoroughly dry it was ready for use and further heating did not seem to enhance its chromatographic properties. In either of these operations the paper was supported on a rectangular wooden frame. Use of a solution that was 4% in "Quilon" and neutralizer gave a paper on which the R_f value of cholesterol was not altered. When a solution containing "Quilon" alone was used, the solvent seemed to wash the material from the paper and a green zone

(11) du Pont Product Information Bulletin, "Quilon," January, 1950.

could be observed in the region of the solvent front. This paper gave erratic results.[12]

To determine the extent of impregnation of the paper by these methods, various samples of the treated paper were ashed and the residues assayed for chromium with the following results[13]:

Sample	Cr (mg./25 cm.²)
Dipped, soln. A	0.26; 0.23
Dipped, soln. B	.24; .24
Sprayed, soln. B	.24; .22

Solutions A and B refer to solutions 2% in both "Quilon" and neutralizer which were prepared at different times.

Chromatography.—Reagent grade solvents were used throughout. All ratios of solvents represent percentages by volume. In all cases 10 γ of material was applied to a spot 1.5 cm. in diameter on a strip of paper about 4 cm. wide. The papers were suspended from metal troughs in large test-tubes (7 × 50 cm.) which contained a few cc. of the solvent mixture and which were sealed with rubber stoppers during the course of chromatography. Generally two papers were suspended from each trough. Descending chromatography was used throughout, the solvent being allowed to run 25–35 cm. from the origin. The papers were allowed to dry thoroughly before testing for the presence of the material being chromatographed. R_f values were measured from the foremost point of the origin and the leading portion of the spot.

As the percentage of water in the solvent was increased the time required for the solvent to run the specified distance was also greatly increased. The upper limit of dilution for methanol is about 20%. Methanol–water 75:25 takes very long to run and at lower temperatures does not wet the paper. Methanol–water 7:3 will not wet the paper. The dilution limit is somewhat higher with the higher alcohols.

Detection of Steroids.—The methods available for the detection of steroids on "Quilon" treated paper have already been reported.[14] In general, iodine vapor was used for detection of the androgens and progesterone; silicotungstic acid for cholesterol, epicholesterol, cholestanol, stigmasterol and cholestenone; antimony pentachloride for sitosterol, ergosterol and 7-dehydrocholesterol; and triphenyl tetrazolium chloride[3] for the corticosteroids. In some cases, several methods were used for detection of the same steroids and the same R_f values were obtained. With cholesterol-4-C^{14} and epicholesterol-4-C^{14} the color tests were further confirmed by radioautography.[15]

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(12) The other chromic chloride complexes were all mixed with "neutralizer" prior to treatment of the paper.

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[CONTRIBUTION FROM THE CHEMICAL RESEARCH LABORATORY OF THE ALTON OCHSNER MEDICAL FOUNDATION AND THE DEPARTMENT OF BIOCHEMISTRY, TULANE UNIVERSITY SCHOOL OF MEDICINE]

Arylnitroalkenes: A New Group of Antibacterial Agents[1]

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It was found that β-nitrostyrene inhibits bacterial growth and that its effectiveness was only slightly reduced when the culture medium contained protein. The influence of structural variations on antibacterial activity was studied by preparing and testing a total of 55 compounds, including 20 arylnitroalkenes which had not been described before. 4-Hydroxy-β-nitrostyrene, previously prepared by a cumbersome route in poor yield, was obtained in 79% yield when a mixture of equimolecular amounts of aldehyde and nitromethane was heated in presence of aniline as catalyst. Most effective amongst the substances tested were 1-(3,4-dichlorophenyl)-2-nitropropene against *Micrococcus pyogenes* var. *aureus* in protein-free medium, 2,6-dichloro-β-nitrostyrene against the same organism in presence of albumin, and 1,4-bis-(β-nitrovinyl)-benzene against *Escherichia coli*. Intravenous administration to mice of a few selected compounds showed that these substances were not very toxic.

It was found that β-nitrostyrene in concentrations of 1 mg. or less per 100 ml. of culture medium inhibited the growth of *Micrococcus pyogenes* and of *Escherichia coli* and that its effectiveness was only slightly reduced when albumin was added to the culture medium. Earlier reports dealing with the biological activity of β-nitrostyrene stated that it had a detrimental effect on insects[2,3] and on the growth of fungi[2,4,5] and that it could be used for the protective treatment of textiles, leather and other organic materials.[2] A comparison of a few nitrostyrenes showed that there was no correlation between the physiological effect on man and fungistatic activity.[4] There was little difference, for example, in the fungistatic effectiveness of β-nitrostyrene and of 4-methoxy-β-nitrostyrene, but in man the first compound acted as a powerful sternutator (an irritant which provokes

(1) Presented before the Division of Biological Chemistry at the 121st Meeting of the American Chemical Society in Milwaukee, Wisconsin, March 31, 1952.

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