Methods of Paper Chromatography of Steroids Applicable to the Study of Steroids in Mammalian Blood and Tissues

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Chromatography has already been widely applied to the steroids, both for preparative and analytical work. Both adsorption and partition methods have been used, although the lipoidal solubility properties of these compounds have made the latter method difficult to apply. Burton, Zaffaroni & Keutmann (1950) have overcome these difficulties by using filter paper soaked in propylene glycol or formamide, and a hydrocarbon mobile phase, with good results. Earlier work with steroid Girard derivatives only gave separations according to the number of ketone groups available for condensation with Girard's reagent (Zaffaroni, Burton & Keutmann, 1949).

The present paper describes some of the results obtained during an attempt to devise paper chromatographic methods suitable for the analysis of steroids in small amounts in body fluids and biological media. The emphasis throughout has been on methods that are reasonably quick and simple, so as to be suitable for routine analytical work. In large measure these ends have been achieved, though as yet the methods described have only been used semi-quantitatively. Two types of system will be described: (1) an adsorption method using alumina-impregnated paper (Datta, Overell & Stack-Dunne, 1950; Bush, 1950*a*); (2) partition systems using aqueous methanol as stationary phase. Special methods have been developed for preparing the extracts from biological material and applying them to the papers and these are also described.

METHODS

Chromatography on alumina paper

Preparation of papers. Sheets of 20 × 45 cm. Whatman no. 54 or no. 4 were immersed for 5–10 sec. in a solution of $Al_2(SO_4)_3$ (A.R., 300 g./l.) made just cloudy with 2N-NaOH, at 60–70°, taken out, hung double over glass rods and suspended vertically in a large glass tank. The lids were immediately closed and 15 min. allowed to elapse. NH_3 solution (sp.gr. 0·880) was then pumped into the tank to a depth of 4–6 cm. by way of a tube and rubber bung near the bottom of the tank. The papers were left hanging in the vapour for 18 hr. On removal they were placed in another tank and immersed in about 25 l. of a solution of $CaCl_2$ (Becker's fused granulated $CaCl_2$, 0·2 g./l.). This product contains much lime and gives an alkaline solution. The solution was continually aspirated with a stream of air to remove excess NH_3. The papers were removed after 24 hr. to dry at room temperature.

Solvents. The papers were run in glass cylinders by the 'ascending' technique, usually to a height of 22–30 cm. The solvents used were benzene (A.R.), benzene-$CHCl_3$ (4:1, 3:1, 2:1), and 5% (v/v) acetone in benzene.

Detection of spots. After thorough drying the chromatograms were dipped quickly into a saturated solution of iodine in light petroleum (b.p. 60–80°). After drying, the background iodine sublimed and spots of steroid showed up yellow or brown on a faintly coloured background.

This reaction is evanescent, non-specific (Brante, 1949) and repeatable. It is prevented or greatly reduced in sensitivity by traces of water, organic solvents, or acid. For permanent records reflex photography in ultraviolet light, or spraying with a concentrated starch solution was used.

pH *of papers.* All papers were tested before use by dabbing with 'Universal' indicator (British Drug Houses Ltd.). When just dry the colour given corresponded with a pH of 7·0–7·5. Papers not treated with the $CaCl_2$ solution are very acid and of low adsorptive activity. Graded activity can be obtained by varying the concentration of $CaCl_2$ used.

This procedure was the routine one evolved from many variants. It was used for the detection of 17-hydroxycorticosterone in adrenal venous blood (Bush, 1950*b*), the examination of adrenal extracts, and of a number of blood samples from sheep, horse, and human subjects. It gave the most reproducible results and optimum sensitivity to the iodine reaction.

The activation with alkaline calcium chloride solution (other solutions such as 0·05N aqueous ammonia or very hard tap water, can also be used) was the key to reproducible results. Without this treatment the activity was low, R_F values varied greatly with the amount of steroid present, and some irreversible adsorption occurred at the origin of the chromatogram. After activation R_F values varied only slightly with amount of steroid and there was no residue at the origin.

The form of the spots varied. Oestrone and oestradiol gave symmetrical spots as on a partition chromatogram: an ion-exchange mechanism is probable for these phenolic compounds. The other steroids gave sharp-fronted, comet-shaped spots, with small tails.

Cortical steroids must be acetylated before flowing on alumina (Reichstein & Shoppee, 1949).

Table 1. *R_F values of various steroids on activated alumina paper*

(These values are taken from medium activity papers with a surface reaction to 'Universal' indicator (British Drug Houses Ltd.) between pH 7·5 and 7·8. Solvents: (*a*) benzene + $CHCl_3$, 3:1 (by vol.); (*b*) benzene + $CHCl_3$, 2:1; (*c*) benzene + $CHCl_3$, 1:1; (*d*) benzene + acetone, 19:1.)

Compounds	Solvents (*a*)	(*b*)	(*c*)	(*d*)
Progesterone	0·75	0·95	1·0	0·8
Testosterone	0·40	0·60	—	—
Oestrone	0·30	0·40	0·9	0·6
Oestradiol	0·05	0·15	0·5	0·25
Pregnenolone	—	0·80	—	—
Dehydroandrosterone	—	0·41	—	—
Androsterone	0·25	0·39	—	—
11-Deoxycorticosterone 21-acetate	0·70	0·85	—	0·70
11-Dehydrocorticosterone 21-acetate	0·35	0·60	0·95	0·40
11-Dehydro-17-hydroxycorticosterone 21-acetate	0·05	0·10	0·40	0·10
17-Hydroxycorticosterone 21-acetate	0·02	0·06	0·30	0·05
Corticosterone 21-acetate	—	0·15	0·67	0·20

They are then quite stable. Although the acetates of 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone flow with different R_F values they are only separated by long flows in benzene-chloroform (3:1) or 5% acetone in benzene. R_F values are given in Table 1.

The iodine reaction gave very good results on alumina papers just alkaline to neutral, but not on acid papers or plain filter paper. Prolonged immersion and warming the iodine solution to 35° were necessary for the latter. On the standard, slightly alkaline, alumina papers, the lower limit of sensitivity was about 0·5 μg./sq.cm. for the two oestrogens, and 1 μg./sq.cm. for a variety of αβ-unsaturated ketosteroids. The reaction with neutral steroids lacking the αβ-unsaturated ketone group was much weaker.

This reaction is similar to Brante's reaction for nitrogenous compounds (Brante, 1949), but his explanation is difficult to apply to the steroids. However, the fact that traces of moisture and other solvents remaining in the paper can almost completely stop the colour reaction, and that the colours given are what might be expected from iodine in solution in the steroid, suggests that the 'reaction' is simply a partition of the iodine between petroleum and steroid, the latter taking up an appreciable amount of iodine from the saturated solution. Estimation of the iodine taken up by steroid spots showed that four to seven atoms of iodine could be held per steroid molecule. However, the rapid evaporation of the iodine did not make this a suitable method of estimating the steroids quantitatively.

One peculiar phenomenon is not explained by this simple theory. 11-Dehydro-17-hydroxycorticosterone (cf. Burton, Zaffaroni & Keutmann, 1950) and 11-deoxy-17-hydroxycorticosterone give a yellow colour on alkaline papers (pH about 7·5), green on neutral papers (pH, 7·0), and a bright blue on acid papers (pH about 6·5). The 21-acetates behave similarly. All other steroids tried gave colours in the range yellow to red-brown.

This method can give useful separations with short quick flows (15–40 cm.) and simple apparatus, with maximum sensitivity of the iodine reaction. It has several disadvantages, however, inherent in an adsorption method, which preclude its use in many investigations. In the first place, R_F values still show a slight variation with amount of steroid, even on papers of high activity and, in the second, all extracts of biological materials must be carefully purified before chromatography, otherwise impurities may produce disastrous displacement effects. These disadvantages have been overcome in the partition methods described below.

Paper partition systems for steroids

With all common organic solvents water is not a satisfactory stationary phase for steroid chromatography. The steroids move either with the solvent front, or as streaks. However, the streaky chromatograms were greatly improved by adding small amounts of *iso*propanol to the system (hydrocarbon/water). In view of this it seemed worth while to try to find systems using hydrocarbon solvents and aqueous methanol (Cornish, Archibald, Murphy & Evans, 1934; Butt, Morris & Morris, 1949) which would work on paper, particularly since the type of mobile phase used flows very quickly on filter paper.

The ordinary 'descending' technique is used, and only those points peculiar to the present method are described.

Apparatus. All tanks were enclosed in a thermostatically controlled box and maintained while in use at an elevated temperature, usually between 30 and 38°. In order to avoid the worst effects of current variation in mains supply, three-quarters of the heating elements (in terms of power) were run through a Simmerstat (J. W. Towers and Co. Ltd., Widnes) adjusted to keep the box 1–2° below the desired

temperature. The thermostat (Sunvic TS 3 air type, Sunvic Controls Ltd., London, W.C. 2) was set to the desired temperature and controlled the remaining heaters. The air in the box was circulated by a fan suspended from the lid, with the motor outside and the shaft passing through the lid. Tests with six simultaneously recording thermocouples showed a maximum spatial temperature difference of 0·5–1·0°. Without the fan operating this difference was usually 4–8°. Over periods of time up to 6 days the temperature could be kept within 0·5° of the mean.

Glass tanks. Owing to the great volatility of the solvents used, proper equilibration is much more difficult than with the usual chromatographic solvents. Saturation of the atmosphere is achieved by covering the walls of each tank with wads of filter paper continually soaking up the two phases used. Two wads dip into about 2 cm. depth of mobile phase covering the bottom of the tank, and a third into a small basin containing about 600 ml. stationary phase.

Hanging the chromatograms. The solvents are all fast-running, and to control the initial rush of solvent on to the paper it is essential to have the starting line at least 6 cm. from the trough edge, and to have the first 4 cm. of the sheet horizontal.

If a slower run is desired the horizontal stretch can be increased, or the trough suspended so that the first 8 cm. of the sheet rises at an angle of about 60°, before bending over a glass rod.

With the ordinary arrangement a 45 cm. flow takes 100–180 min. with the solvents used. This time can be varied at will by combination of the arrangements of the sheet described above.

Equilibration. The sheets were usually left hanging overnight at the desired temperature. With some solvents, however, this was not necessary and 3–4 hr. was quite sufficient.

Running the solvents. Solvents were left equilibrating overnight at the desired temperature and then poured into the troughs by holes and funnels in the lids of the tanks (Hanes & Isherwood, 1949).

The best results were obtained with aqueous methanol as stationary phase, although ethanol and *iso*propanol were suitable at low temperatures.

Solvent systems. The following systems have been used extensively and successfully. They were prepared by mixing the solvents at the operating temperature and allowing the two phases formed to separate. In all cases light petroleum was the fraction of b.p. 80–100°, methanol was absolute and water was distilled.

A. 1000 ml. light petroleum, 800 ml. methanol, 200 ml. water.

B. (1) 500 ml. toluene, 500 ml. light petroleum, 700 ml. methanol, 300 ml. water.

(2) 667 ml. toluene, 333 ml. light petroleum, 600 ml. methanol, 400 ml. water.

(3) 667 ml. light petroleum, 333 ml. benzene, 800 ml. methanol, 200 ml. water.

(4) 1000 ml. toluene, 500 ml. methanol, 500 ml. water.

(5) 1000 ml. benzene, 500 ml. methanol, 500 ml. water.

C. 900 ml. toluene, 100 ml. ethyl acetate, 500 ml. methanol, 500 ml. water.

Detection of spots. After running and drying, the sheets were pinned down over Ilford Reflex paper no. 50 and exposed briefly to ultraviolet light, using a lamp emitting nearly all its energy in the 254 mμ. region. With the lamp 60 cm. above the paper an exposure of 2–3 sec. was usual.

Despite the sensitivity of this photographic method, the presence in biological extracts of impurities which absorb ultraviolet light close to the 240 mμ. absorption peak of αβ-unsaturated ketosteroids often made it impossible to detect surely less than 10 μg. steroid/spot. This difficulty could be overcome by the following fluorescence reaction: the papers were simply sprayed with a solution of 10 g. NaOH in 100 ml. of 60% (v/v) methanol and dried at 60–100°. When quite dry the αβ-unsaturated ketosteroids gave a brilliant primrose-yellow fluorescence in ultraviolet light which faded after several days but could be developed again by a second treatment.

This reaction is very sensitive (2 μg./sq.cm.) and within the group of steroids examined seems to be specific for steroids with an αβ-unsaturated ketone grouping. Thus eleven steroids containing this group in ring *A* all gave positive reactions, whereas nine steroids with various substituent groups, but containing a 3-hydroxyl group, gave no fluorescence, except equilenin acetate and oestradiol which gave a blue fluorescence. All the active cortical steroids, and also testosterone, androst-4-ene-3:17-dione, androst-4-ene-3:11:17-trione, and 11-deoxy-17-hydroxycorticosterone gave positive reactions. The major advantage of this method is that no other compounds in natural extracts have been found to give this particular colour, so that the background colour is completely dark except for spots of blue or green fluorescence.

Other colour reactions can be used to check the nature of unknown ultraviolet-absorbing spots, but for many problems the photographic method is sufficient.

General procedure. The best results are obtained with medium or slow runs after equilibration overnight. Most of the systems, however, can be safely run after 3 hr. equilibration. A 'fast' chromatogram can thus be equilibrated, run, and examined within 5–5·5 hr. This has been done repeatedly with the last two systems of group *B*.

The stationary phase should be replaced every fortnight with fresh aqueous methanol, and with toluene the mobile phase also needs changing. Benzene and light petroleum only need replenishment.

Uses of the partition methods

The steroids so far studied have been largely those containing the αβ-unsaturated ketone group in ring *A*. For the purposes of chromatography they can be divided into three groups according to their polarity and solubility properties as follows:

Classification of steroids

(1) *Relatively non-polar.* Progesterone, androst-4-ene-3:17-dione, testosterone, and deoxycorticosterone; and the acetates of the last, 11-deoxy-17-hydroxycorticosterone, 11-dehydrocorticosterone, and corticosterone.

(2) *Relatively polar.* The known active cortical steroids (for review, see Reichstein & Shoppee, 1943), and the acetates of 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone.

Table 2. *R_F values of various steroids obtained with partition systems using aqueous methanol as stationary phase*

(The values given are taken from 44 cm. flows on Whatman no. 4 paper at 34°. With higher temperatures the values increase. Errors of more than ±0·05 are rare, and are usually due to faulty equilibration. Mixtures of compounds III and IV, or IV and V, can only be separated completely by over-running with system *A* or *B*(3). Systems; see text (p. 372) for details. The 'amorphous fractions' are those of Wintersteiner & Pfiffner, 1936.)

	Partition systems						
Compounds	(*A*)	(B_1)	(B_2)	(B_3)	(B_4)	(B_5)	(*C*)
17-Hydroxycorticosterone (I)	0·0	0·03	0·10	0·01	0·15	0·32	0·4
11-Dehydro-17-hydroxycorticosterone (II)	0·01	0·05	0·20	0·02	0·30	0·50	0·7
Corticosterone (III)	—	0·23	0·65	0·15	0·70	0·85	—
11-Deoxy-17-hydroxycorticosterone (IV)	—	0·27	0·70	0·20	0·72	0·87	—
11-Dehydrocorticosterone (V)	—	0·36	0·75	0·25	—	0·96	—
11-Deoxycorticosterone (VI)	0·39	0·86	0·95	0·75	—	1·0	—
21-Acetate of I	—	0·25	—	—	—	—	—
21-Acetate of II	0·04	0·39	—	—	—	—	—
21-Acetate of IV	0·25	—	—	—	—	—	—
21-Acetate of VI	0·70	—	—	—	—	—	—
Testosterone	0·40	—	—	—	—	—	—
Androst-4-ene-3:11:17-trione	0·26	—	—	—	—	—	—
Androst-4-ene-3:17-dione	0·70	—	—	—	—	—	—
Progesterone	0·85	—	—	—	—	—	—
'Amorphous fraction' 1	0·0	—	—	—	—	0·25	0·3
'Amorphous fraction' 2	0·0	—	—	—	—	0·12	—
'Amorphous fraction' 3	0·0	—	—	—	—	0·03	—

(3) *Very polar.* The inactive polyhydroxy cortical steroids, such as *allo*pregnane-3β:11β:17β:20:21-pentol, and *allo*pregnane-3β:11β:17β:21-tetrol-20-one (Reichstein & Shoppee, 1943), and the unidentified components of the 'amorphous fraction' of the adrenal cortex (Wintersteiner & Pfiffner, 1936).

These three groups are conveniently handled by solvent systems *A*, *B* and *C*, respectively, where the aim is to examine an extract in a single run of 2–3 hr. If more time is available, better separations can be achieved by over-running with a system in which the steroids under study move with low R_F values.

Steroids less polar than progesterone would probably require a reversed phase system such as recently described by Kritchevsky & Calvin (1950).

Table 2 gives some R_F values obtained with the above type of steroid.

The steroids so far studied conform well to the postulate of Martin (1950) that, to a first approximation, in any given phase pair the partition coefficient of any compound in a series will be an additive function of its substituent groups. The properties and number of polar groups on an unknown steroid can be gauged fairly well by comparing their chromatographic behaviour with that of reference compounds in the above systems. The presence of acetylatable hydroxyl groups can be detected by the great increase in R_F value after acetylation, both on alumina paper and in partition systems.

Using temperatures of 15–25° equilibration is very slow (2–3 days) and with low concentrations of methanol streaky chromatograms result. The great improvement gained by using elevated temperatures and high concentrations of methanol is remarkable and probably related to the high molecular weight of the steroids combined with their low solubilities at room temperature and their strong adsorption on unwashed filter paper (Bush, 1950*a*), and on filter paper exhaustively washed by the method of Hanes & Isherwood (1949).

Concentration of extracts and spotting technique

This is always tedious with steroids, particularly since it is usually necessary to use the whole of a biological sample on one chromatogram. The problem is commonly to get all the steroid and a minimum of non-steroid material from *x* ml. of blood or urine on to 0·5 sq.cm. of filter paper. It is difficult to reduce any such extract to less than 1 ml. without running into serious volumetric errors when using volatile and mobile solvents, and the normal pipetting technique for paper chromatograms is very tedious with more than 20–40 μl. of solvent. Further, repetitive spotting with a pipette piles up the solutes as a ring on the paper, often dense enough to hinder dissolution and spoil a chromatogram.

The following techniques have therefore been devised to solve this problem.

Spotting with volumes of 40–100 μl.

The starting line is drawn well away from one end of the paper and marked, not with the usual spots, but with lanes, 5–10 mm. wide, running back from it at right angles (Fig. 1*a*). The solutions to be chromatographed are then pipetted

along, and within, their respective lanes and the solvent allowed to dry (Fig. 1*b*). If possible this is done in one operation so that an even deposition of solute occurs.

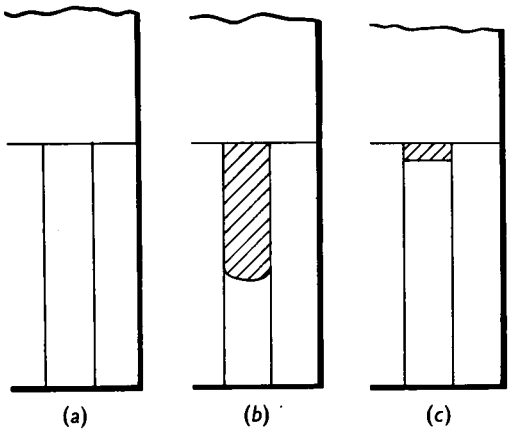

Fig. 1. Concentration of 0·02–0·2 ml. of extract for chromatography by a preliminary chromatogram (see text, p. 373).

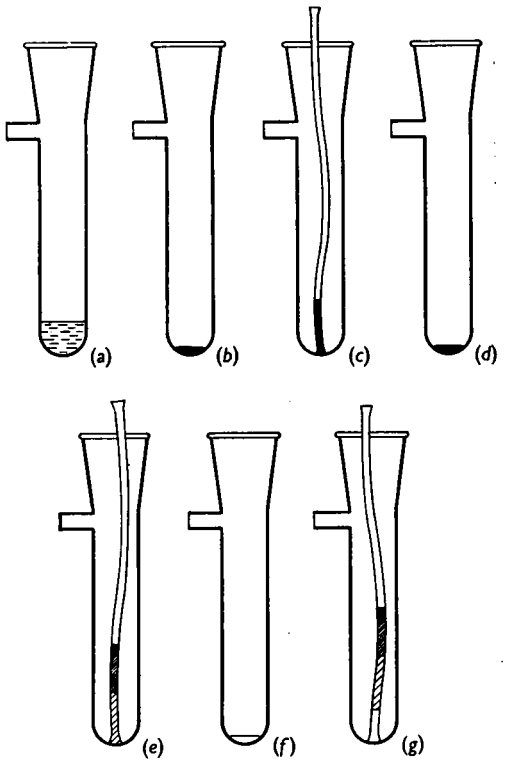

Fig. 2. Concentration of an extract for total deposition on a chromatogram (see text, p. 374).

The sheet is then hung in a small tank specially kept for the purpose with the lower edge dipping into a mixture of $CHCl_3$-ethyl acetate (1:1, v/v) containing a small addition of methanol. An ascending chromatogram is thus run with a solvent in which all the steroids move with the front, which is allowed to ascend to the starting line. The result, when dried, is that all the steroids are compressed into small rectangles on the starting line and are evenly deposited (Fig. 1*c*). This preliminary chromatogram takes 5–10 min.

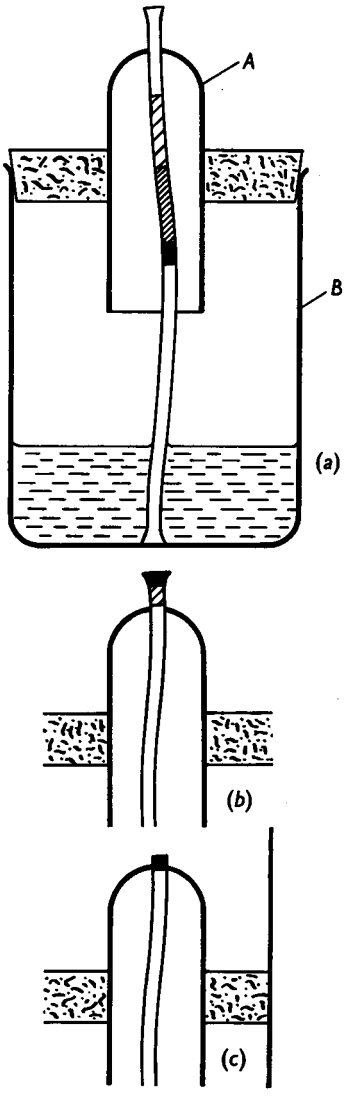

Fig. 3. Concentration of an extract for total deposition; concentration by a 'wick chromatogram' (see text, p. 375).

Total deposition of extracts (1–2 *ml.*)

The extract is made light pink by adding a minimal amount of Sudan Red and then evaporated *in vacuo* in a test tube with side arm to a small red drop at the bottom of the tube (Fig. 2*a*, *b*). This drop is taken up on to a three to five strand lamp wick, previously cleaned by 8 hr. Soxhlet

extraction with 90% ethanol (Fig. 2*c*). The tube is rinsed down with about 0·1 ml. methanol-ethyl acetate (1:1, v/v) and taken up on the wick. At the third wash the drop will be colourless (Fig. 2*f*) indicating complete recovery of the dye and lipoid solutes in the original extract.

The wick is then suspended in a glass cowl (Fig. 3*a*, *A*) as shown, with 1–2 cm. emerging. The beaker (*B*) is filled with a little ethyl acetate and this is allowed to rise up the wick. In about 15 min. the dye is concentrated on the emerging zone (Fig. 3*b*). Immediately before preparing the chromatogram the wick is drawn down till only 2–3 mm. emerge (Fig. 3*c*). When concentrated on this small zone, as indicated by the dye, it is simply painted on to a lane and treated as described above.

These methods have saved much time in the analysis of blood samples for steroids.

Extraction of steroids from blood samples

The following methods have been used for extracting cortical steroids from blood.

(*A*) The blood was stirred gradually into 4 vol. absolute methanol. After 15 min. the protein was filtered off at the pump with Whatman no. 1 paper, and the precipitate washed twice with 15 ml. methanol warmed to 45°. The filtrate was distilled to about 1 ml. *in vacuo* at 45°, cleared with minimal ethanol and added to 50 ml. acetone. After adding 2–3 drops saturated $MgCl_2$ in ethanol the acetone was chilled for 30 min. and then filtered through Whatman no. 1 at the pump. The precipitate was washed twice with 10 ml. cold acetone and then distilled (as above) to near dryness. The residue was taken up in 50 ml. of light petroleum (b.p. 40–60°) and the flask washed with 50 ml. 60% methanol. This methanol was used in equal fractions to extract the petroleum solution three times. The methanol extracts were combined and washed once with 15 ml. petroleum before distilling down to about 10 ml. aqueous solution. This was made up to 50 ml. 0·1 N-Na_2CO_3 with water and 0·5 N-Na_2CO_3 chilled in ice. The alkaline solution was immediately extracted with three portions of 15 ml. ethyl acetate. The combined ethyl acetate extracts were washed once with 10 ml. 0·1 N-HCl and once with 10 ml. water. The washings were back-extracted with 4 ml. ethyl acetate, and the combined ethyl acetate fractions dried over anhydrous Na_2SO_4.

(*B*) The blood was diluted with 1 vol. of water and shaken up well with 6 vol. of $CHCl_3$. The resulting emulsion was then cleared by chilling in ice and adding an excess of well powdered anhydrous Na_2SO_4. The clear $CHCl_3$ solution was filtered and distilled to 2 ml. at 45° *in vacuo* and added to 30 ml. acetone, saturated $MgCl_2$ added, and placed in ice for 0·5 hr. After filtration the acetone solution was distilled (as above) down to a gum which was taken up in 70% ethanol (30 ml.) and washed three times with 10 ml. light petroleum. The ethanol solution was then distilled to near dryness and taken up in minimal methanol, to which ten times its volume of ethyl acetate was then added. After 30 min. in ice the solution was centrifuged and the supernatant drawn off. The precipitate was then redissolved in methanol and reprecipitated by ethyl acetate.

The final extracts were then concentrated for chromatography by one of the methods described earlier (p. 373).

These methods are based on the work of Pfiffner & Vars (1934), Pfiffner, Wintersteiner & Vars (1935) and Grollman (1939). Recoveries have not been quantitatively checked, but chromatographic examination of all the rejected fractions in the above schemes showed no detectable cortical steroid. Using samples yielding 30–40 μg. of corticosterone or 17-hydroxycorticosterone in the final extract, and with a limit of detection at 5–7 μg. per chromatogram spot, this indicated that losses by co-precipitation or partition were less than 10–20%. Losses by destruction remain unknown, but there has been no indication that they are large.

Both methods yield final extracts which are clean enough for immediate partition chromatography when using the whole extract of up to 40 ml. blood. The first method is the more thorough and was generally used.

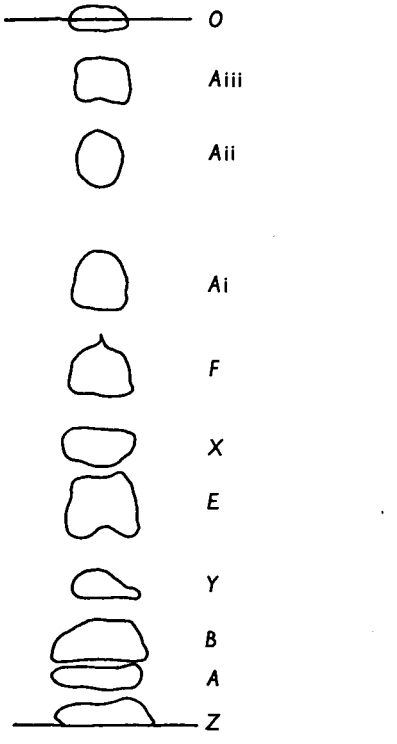

Fig. 4. Whole cortical extract run with system *B*5 on Whatman no. 4 at 34°. Photograph of the fluorescence developed by spraying with methanolic NaOH. *O*, starting line; *A* i, *A* ii, *A* iii, 'amorphous fraction' components; *A*, 11-dehydrocorticosterone; *B*, corticosterone; *E*, 11-dehydro-17-hydroxycorticosterone; *F*, 17-hydroxycorticosterone; *X*, unidentified blue fluorescence; *Y*, *Z*, unidentified spots; *Z*, solvent front.

RESULTS

In all the work so far only rough estimates of quantity have been made by comparing the density and area of spots (photographic) with spots containing known amounts of a reference compound. The spots have usually contained 5–20 μg.

Extracts of adrenal gland

The main object of study has been a semi-processed extract of 900 lb. (410 kg.) ox adrenals from Allen and Hanbury's Ltd. Results so far indicate the presence of appreciable amounts of all the three substances absorbing strongly in the ultraviolet, reacting with 2:4-dinitrophenylhydrazine and 2-hydroxy-3-naphthoic acid hydrazide (Camber, 1949), and falling in group 3 have been found on chromatograms of the 'amorphous fraction', with R_F values identical with similar substances from the above glandular extract (*A* i, *A* ii and *A* iii, in order of increasing polarity).

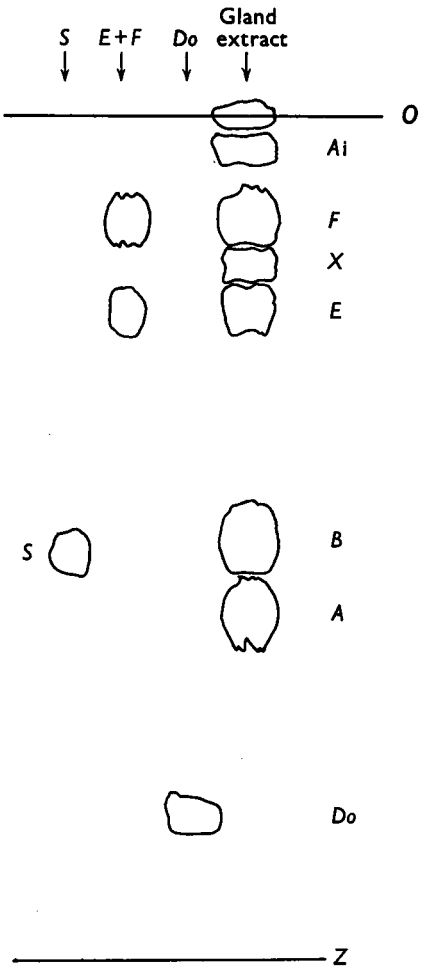

Fig. 5. Cortical extract and reference compounds run with system *B* 4. *A*, *B*, *E*, *F*, *O*, *X*, as in Fig. 4; *S*, 11-deoxy-17-hydroxycorticosterone; *Do*, deoxycorticosterone; *Z*, solvent front.

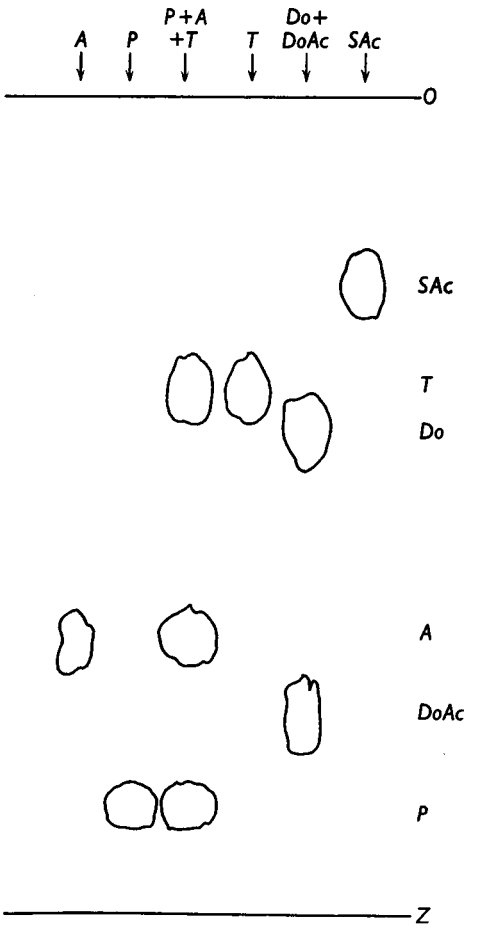

Fig. 6. Pure steroids of group I (see text) run with system *A*. *P*, progesterone; *T*, testosterone; *A*, androst-4-ene-3:17-dione; *Do*, deoxycorticosterone; *DoAc*, deoxycorticosterone acetate; *SAc*, 11-deoxy-17-hydroxycorticosterone acetate; *O*, starting line; *Z*, solvent front.

active cortical steroids previously described (Reichstein & Shoppee, 1943), except that deoxycorticosterone and 11-deoxy-17-hydroxycorticosterone have not yet been detected (cf. Zaffaroni, Burton & Keutmann, 1950). A large number of substances belonging to group 3 were found on chromatograms. By the great kindness of Dr O. Wintersteiner a sample of the original 'amorphous fraction' (Wintersteiner & Pfiffner, 1936) was available for comparison with these unknown substances. So far

Substance *A* i has been obtained substantially pure from such chromatograms and was assayed by Dr M. Vogt (Edinburgh) using the cold-exposure test of Selye & Schenker (1938), in which it was found to be very active. The sample was free of all known active cortical steroids but contained a trace of a fluorescent impurity.

These experiments are being further pursued.

Table 3. *Protocol of a typical experiment on the adrenal effluent of a dog*

(Dog, male (10·1 kg.) Nembutal anaesthesia. Continuous collection of adrenal venous blood from left gland. Blood pressure maintained by saline and 'Dextran' transfusion into femoral vein. Adrenal blood flow 2–5 ml./min. Each half-hour collection extracted and purified; one-tenth of each final extract used for chromatogram.)

Successive 30 min. collections	1	2	3	4	5
17-Hydroxycorticosterone on chromatogram (μg.)	8	18	16	18	18
Average secretion rate during each collection in mg./hr.	0·16	0·36	0·32	0·36	0·36

Extracts of adrenal venous blood

The presence of large amounts of 17-hydroxycorticosterone in adrenal venous blood has been demonstrated by isolation (Nelson, Reich & Samuels, 1950; Nelson, Reich & Zaffaroni, 1950) and by chromatography. Using the above methods, and the criteria of (1) chromatographic behaviour of the acetate with three different alumina-paper systems and three different partition systems; (2) ultraviolet absorption and fluorescence with sodium hydroxide; (3) ketone reaction with 2:4-dinitrophenylhydrazine and 2-hydroxy-3-naphthoic acid hydrazide; (4) iodine colour reaction on alumina paper, all being identical with pure 17-hydroxycorticosterone, as a satisfactory demonstration of this compound, it has been found as the chief steroid in adrenal venous blood in eleven dogs. The operative procedure was the same as that of Vogt (1943) except that nembutal was more commonly used as anaesthetic.

In addition the two least polar components of the 'amorphous fraction' (*A* i and *A* ii) seem to occur in the adrenal effluent though in smaller quantity. The identity with *A* i and *A* ii is not yet completely proven.

In two dogs appreciable amounts of a compound fulfilling the above criteria of identity with corticosterone appeared in the adrenal effluent in one-quarter to two-thirds of the amount of 17-hydroxycorticosterone during different periods of collection.

The amounts found in the adrenal effluent were of the same order as those arrived at by Vogt (1943) using the Selye-Schenker assay (see Table 3).

DISCUSSION

The methods described above were evolved largely with cortical steroids in mind, but the partition systems can handle any steroid as polar as, or more polar than, progesterone. The alumina-paper method can deal with steroids less polar than progesterone but its disadvantages outweigh its advantages. However, it is extremely useful with oestrogens; oestrone and oestradiol can be separated (and oestriol left on the origin) in a 10–15 min. ascending run and detected in amounts of 0·5 μg. by the iodine reaction.

The applications that have been made so far are only semi-quantitative, and quantitative estimations would probably be better done on columns as described by Butt *et al.* (1949). The paper method, however, lends itself readily to the biologist or biochemist who often needs qualitative or semi-quantitative results on a large number of different samples.

The results so far obtained by studying the dog adrenal venous blood are interesting when compared with the numerous biological assays that have been made on this material. Hartmann, Brownell & Thatcher (1947) described a 'fat factor' in adrenal extracts, with no glycogenic or electrolyte activity, which Hartmann & Liu (1950) found in the adrenal venous blood of dogs. Eight out of fourteen animals secreted appreciable amounts, but the rate of secretion was not affected by adrenocorticotropic hormone (ACTH) injections even when the glycogenic assay of the blood (Brownell, 1950) was greatly increased. Spencer (1950) found the equivalent of 4 μg./ml. deoxycorticosterone acetate in the serum from dog adrenal venous blood, using a bioassay based on acute salt retention in mice.

Nelson and his co-workers (Nelson, Reich & Samuels, 1950; Nelson, Reich & Zaffaroni, 1950) and Hechter (1950*a*, *b*) have shown that 17-hydroxycorticosterone is the predominant secretory product of the adrenals of dogs and oxen, but it is not clear whether the secretion of this one compound can explain all the complex physiological actions of the adrenal cortex. The regulation of sodium chloride excretion is a particularly difficult problem. Certainly Spencer's (1950) results could not be due to deoxycorticosterone itself, for a concentration of 1 μg./ml. serum would have been detectable on the chromatogram of the blood samples usually taken, and in one case where a much larger sample of blood was used the concentration of deoxycorticosterone must have been less than 0·2 μg./ml. to escape detection (cf. however, Hechter, 1950*a*, *b*).

While the above experiments throw no light on the functions of the various secretory products, they do show that the dog adrenal can secrete large amounts of 17-hydroxycorticosterone and corticosterone, and also small amounts of two compounds probably contained in the 'amorphous fraction' of cortical extracts. It does not, therefore, seem likely that adrenal function can be explained entirely in terms of the secretion of 17-hydroxycorticosterone, and its stimulation by ACTH.

SUMMARY

1. The use of alumina paper for the chromatography of steroids is described. The elimination of displacement effects due to impurities demands tedious purification of most biological extracts.

2. Partition systems of the aqueous methanol/hydrocarbon type can be run very conveniently on paper if certain special procedures are adopted. Extracts sufficiently pure to be handled by these systems can be simply and rapidly prepared.

3. Special methods for quick concentration of these extracts and their even deposition on filter paper are described.

4. Some applications to pure substances, extracts of adrenal glands, and adrenal venous blood are described.

5. A preliminary examination of the 'amorphous fraction' obtained from adrenal extracts by Wintersteiner & Pfiffner (1936) indicates the presence of at least three very polar substances with strong ultraviolet absorption near 254 mμ., two of which appear in adrenal venous blood.

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Kinetics of 'Acid' Phosphatase Action

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Prostate 'acid' phosphatase is present in normal human seminal fluid in very high concentration (Kutscher & Wolbergs 1935). The optimum pH for acid phosphatase in sperm was found to be 4–6·2 depending upon the experimental conditions (Kutscher & Wörner, 1936; Lundquist, 1947). Lundquist computed the dissociation constant of the enzyme-substrate complex (Michaelis constant) from Kutscher & Wörner's experiments with β-glycerophosphate and on the basis of his own experiments with choline phosphate, phenyl phosphate and β-glycerophosphate.

Little attention has so far been paid to the reaction kinetics of the hydrolysis of phosphoric acid esters by acid phosphatase. In the present study reaction mechanisms are described which lead to simple