FRACTIONATION AND DETERMINATION OF THE LIPID AND STEROID CONSTITUENTS OF THE ADRENAL GLANDS OF RATS BY MEANS OF THIN-LAYER CHROMATOGRAPHY

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The determination of very small quantities of steroids in the tissues is often difficult owing to the presence of lipids. AXELROD AND HERNANDEZ[1] and GOLDZIEHER, BAKER AND RIHA[2] have recently described the use of silicic acid columns for the extraction and purification, in the presence of lipids, of steroids obtained from tissues and blood.

Particularly for the determination of corticosteroids in the adrenal glands of small animals or human and in the incubation media of the adrenals a micro-technique is required.

Paper-chromatographic techniques have been used for the determination of corticoids in the plasma of rats[3] and in the incubates of the adrenal glands of rats[4].

DYRENFURTH[5] made use of the same technique for the determination of corticoids in the incubates of human adrenal glands. A silica gel column followed by paper chromatography has been used by DAVIGNON[6] for the study of steroids in adrenal tumours in man, and by DE ROOS[7] for the study of the *in vitro* production of chicken adrenocorticoids.

The aim of the present work was to investigate the lipids and steroids of the adrenal glands of normal rats and of rats suffering from a deficiency of pantothenic acid, and to study the basic constituents of these glands or of their incubates by subsequent tests under various conditions.

In the course of previous investigations of the *in vitro* synthesis of the adrenocorticoids of normal rats and of those deficient in pantothenic acid, either in the presence or in the absence of ACTH, one of us had identified corticosterone amongst the steroids in the incubate. However, owing to the difficulties encountered in the purification of the extracts, paper chromatography using BUSH's toluene–methanol–water system did not permit a reliable identification of other adrenocorticoids[10].

The recent developments in the use of thin-layer chromatography for the analysis of lipids by MALINS AND MANGOLD[11] and the use of this technique by CAVINA[8,9] in the analysis of steroids in adrenocortical extracts led us to try a micro-technique for the fractionation and purification of the corticoids present in quantities of adrenal tissue, as small as 20 adrenal glands of rats, or the incubates of these glands.

This method, which also permits the isolation of the lipid components of the extract, involves the following steps:

(1) Preparation of the extract of the organ using the method described by FOLCH, LEES AND SLOANE STANLEY[12].

(2) Thin-layer chromatography on silica gel G (Merck). The thickness of the layer varied between 0.250 and 1.0 mm, according to the quantity of lipids used (20–60 mg). The solvent systems was benzene–ether–acetic acid (70:30:1), and the dimensions of the plates were 20 × 20 cm. The mixture was resolved into the following zones; cholesterol esters, triglycerides, fatty acids, cholesterol, and phospholipids. The corticoids remained in the phospholipid fraction, which was the least mobile.

(3) Colorimetric determination of the fractions obtained by elution of the various zones with suitable solvents. For free cholesterol and cholesterol esters[13] zinc chloride and acetyl chloride were used. Triglycerides were determined as glycerol by oxidation and reaction with chromotropic acid[14], fatty acids by the titrimetric method described by DOLE[15], phospholipids as phosphorus[16], corticoids by U.V. spectrophotometry and colorimetrically with tetrazole blue[17].

(4) Thin-layer chromatography for the purification and analysis of the corticosteroids. Plates measuring 20 × 20 cm coated with a 0.250 mm layer of fluorescent silica gel G were used for the first purification. The solvents used was chloroform containing 0.2 % ethanol. The zone between the start and the deoxycorticosterone introduced as a standard was removed and eluted. The eluate was fractionated on 20 × 40 cm plates using chloroform–methanol–water (90:10:0.25) as the solvent. Corticosterone, aldosterone and small quantities of 11-dehydrocorticosterone and 11-deoxycorticosterone were thus separated and identified. The first two steroids could be determined directly in the eluates by U.V. spectrophotometry and by colorimetry using tetrazole blue.

EXPERIMENTAL*

Animals

Male Wistar rats with a mean initial weight of 65 g were fed on the following purified diet[10] (with or without pantothenic acid): 67 % sucrose, 18 % casein without water-soluble components, 9 % butter, 4 % Osborne-Mendel saline mixture, 2 % cod-liver oil. The following quantities of vitamins were incorporated per kg of the diet: 10 mg thiamine hydrochloride, 20 mg riboflavin, 10 mg pyridoxine, 100 mg nicotinic acid, 100 mg *p*-aminobenzoic acid, 100 mg ascorbic acid, 200 mg choline hydrochloride, 200 mg inositol, 50 mg menadione sodium bisulphite, 100 mg α-tocopherol acetate, 10 g linoleic acid, and 100 mg calcium pantothenate (only for the controls).

After about 60 days on this diet, when a state of deficiency had set in, as shown by the cessation of growth and by cutaneous symptoms, rats of the "deficient" group together with normal rats of the "control" group were decapitated after a 12 h fast. The adrenal glands were quickly removed, freed from the surrounding fat, and weighed on a torsion balance.

*Extraction of the adrenal lipids***

The adrenal glands (10 to 45) were ground with a little quartz sand (0.22 to 0.36 mm diameter), which had been washed and degreased with ether. A chloroform–methanol mixture (2:1) was used as the extraction solvent (15 ml/100 mg of fresh

* The experimental work was carried out in collaboration with A. MOLLICA.

** Extraction was by a slightly modified version of the method of FOLCH, LEES AND SLOANE STANLEY[12].

tissue), the whole of the solvent being divided into 4 equal portions. The adrenal tissue was triturated with each of these for a few minutes, and the samples were then allowed to stand for about 10 min. The solvent was carefully removed with a Pasteur pipette and transferrred to a cylinder through a G3 filter. Twenty per cent water was added and the solution was agitated and allowed to stand for a few hours, after which the top layer of the two phases was removed. The bottom layer was washed thrice with chloroform–methanol–water (3:48:47) solvent for the top layer and about 1 ml of methanol was finally added to clarify the solution. The lower chloroform layer was transferred to a flask and evaporated in a rotary evaporator at about 40° and then evaporated to dryness in an atmosphere of nitrogen. The residue was redissolved in a few ml of chloroform–methanol (2:1) and transferred into small weighing tubes (tare 6–7 g). The solution was then evaporated again in a current of nitrogen and the residue was kept under vacuum in a desiccator until constant weight was reached, the desiccator having previously been flushed several times with nitrogen to remove all oxygen. The samples was finally redissolved in chloroform–methanol (2:1), placed in small test tubes fitted with ground stoppers, and stored in the refrigerator.

Chromatographic separation of the lipid constituents of the extract

The lipids were fractionated by thin-layer chromatography on silica gel G (Merck) which had previously been washed with chloroform. The thickness of the layer varied between 0.25 and 1.0 mm according to the quantity of lipids used (20–60 mg). Plates measuring 20 × 20 cm were used, with benzene–ether–acetic acid (70:30:1) as solvent. Zones corresponding to the following constituents were resolved, from front to start: cholesterol esters, triglycerides, fatty acids, free cholesterol (which migrates together with the diglycerides); the corticosteroids remained in the least mobile fraction, namely the phospholipids. The zone nearest to the start was covered, and the remainder of the plate was sprayed with dichlorofluorescein, Na salt, 0.05 % in ethanol and viewed under a U.V. lamp.

Each lipid fraction was scraped off, transferred to a Soxhlet thimble, and extracted for 3 h with peroxide-free ether in a Kumagawa type extractor (in which the substance to be extracted can be kept at the same temperature as the solvent). The resulting extracts were evaporated to dryness and redissolved in 10 ml of chloroform. The following colorimetric determinations were carried out on these solutions: free cholesterol and cholesterol esters were determined with zinc chloride and acetyl chloride by the method of HANEL AND DAM[13], triglycerides as glycerol by VAN HANDEL AND ZILVERSMIT's method [14] and fatty acids by DOLE's[15] titrimetric method. The zone containing the phospholipids and the corticosteroids was marked (Fig. 1) and sucked into a suitable percolator through which 10 ml of anhydrous acetone were passed. The corticosteroids passed into solution, while the phospholipids remained practically insoluble. The total crude corticosteroid content was determined on aliquot portions (about $^1/_{10}$) of this solution, using the colorimetric method with tetrazole blue[17,9]. The phospholipids were determined directly on a small portion (about $^1/_{100}$ to $^1/_{200}$) of the whole adrenal lipid extract before chromatography, using the method described by BERENBLUM AND CHAIN[16]. Alternatively, the lipid extract was divided into two portions, giving two "phospholipid + corticosteroid" zones, one of which was extracted with acetone as described above, and the other with warm chloroform–methanol (2:1) in the extractor. The extract containing the

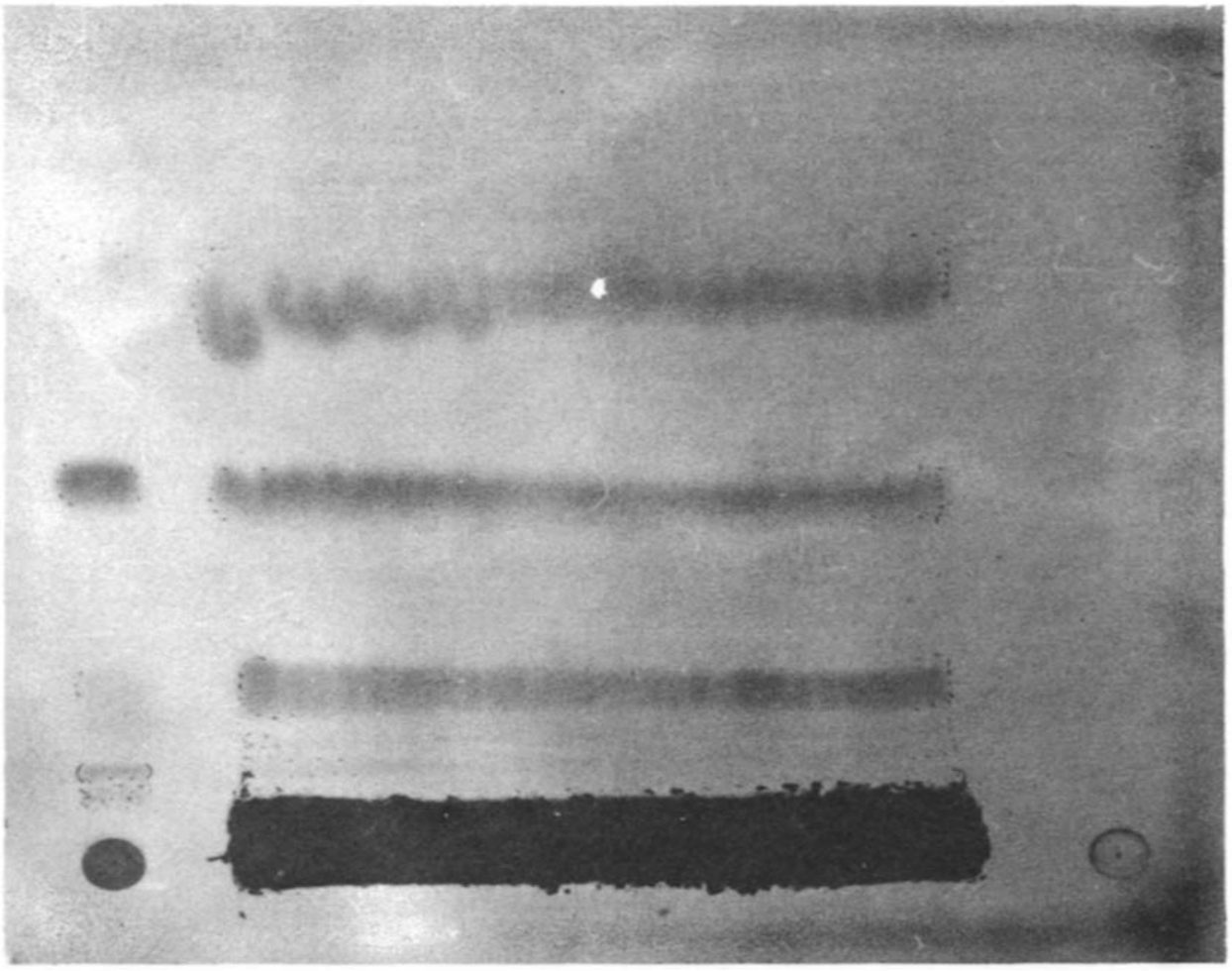

Fig. 1. Chromatographic separation of the lipid constituents of the adrenal extract. Solvent: benzene–ether–acetic acid (70:30:1). Detection with iodine vapour. Plate coated with a 0.5 mm layer of silica gel G. Left: reference lipid mixture. Right: cortisone and deoxycorticosterone used as reference standards. Middle (from the bottom): (1) removed band (phospholipids + corti costeroids); (2) two slight bands of diglycerides; (3) cholesterol; (4) triglycerides; (5) cholesterol esters.

phospholipids was used for the determination of the lipid phosphorus and for the preparation of the methyl esters of the fatty acids for gas chromatography.

Chromatographic purification and fractionation of the steroid constituents of the extracts

The acetone extract containing the corticosteroids (usually 50–200 μg) was evaporated to dryness, and the residue was deposited in a short strip (2 to 3 cm) on a 20 × 20 cm plate coated with a 0.25 mm layer of fluorescent silica gel GF_{254} (Merck), which had previously been washed with chloroform. Cortisone and deoxycorticosterone were used as reference standards. After development with chloroform containing 0.2 % of ethanol the layer between the start and the zone corresponding to deoxycorticosterone was removed by sucking it into a suitable percolator and extracting it with 5–10 ml of ethanol. The various bands situated beyond deoxycorticosterone gave a positive test with tetrazole blue and absorbed in the U.V. region, but they were not corticosteroids. Several alcohol extracts were combined to give 70–200 μg ag-

gregates. The sample was evaporated to dryness, and the residue was deposited in the form of a small strip 1–2 cm long, on a plate prepared as described above, but measuring 20 × 40 cm. The following standards were deposited on the plate alongside with the sample: aldosterone, corticosterone, 11-dehydrocorticosterone, deoxycorticosterone, and a bovine cortical extract. The plate was then developed with a chloroform–methanol–water system (90:10:0.25). Inspection under U.V. light (λ = 254 mμ) permitted the identification of corticosterone and aldosterone. The latter were removed from the plate by suction and eluted with 5 ml of ethanol. The remainder of the plate was sprayed with tetrazole blue (0.5 % in ethanol to which a 2 *N* solution of NaOH had been added in the ratio of 40:60), in order to identify and obtain a semi-quantitative determination of other steroids. If a larger quantity of steroids is available, two or more chromatograms can be developed side by side; one of these is then treated with tetrazole blue, the others are used for the analysis and estimation of purity by paper chromatography, using BUSH's B_1 and C systems, as described in detail in refs. 8 and 9.

Analysis of the steroid constituents of the extracts

The ethanolic eluates of the corticosterone and the aldosterone zones were analysed by U.V. spectrophotometry between 225 and 255 mμ, on the basis of the extinction at the maximum in the region of 240 mμ. A colorimetric determination using tetrazole blue, as described by NOWACZYNSKI *et al.*[17], but modified as indicated in one of our previous papers[9], was carried out on the same solution. This method was also used for the determination of the total content, in the corticosteroid sample, of substances which reduce tetrazole blue. Aldosterone and other steroids present in quantities of 1–5 μg were determined by the micro-technique using tetrazole blue described by DESGREZ *et al.*[18]; the measurements were carried out with the aid of a Beckman DU spectrophotometer using cells of 1 cm thickness. The results were expressed conventionally in terms of hydrocortisone.

Gas-chromatographic analysis of the fatty acids present in the cholesterol ester fraction

The eluates of the cholesterol ester zones were converted into methyl esters using the method of STOFFEL, CHU AND AHRENS[19], with the addition of hydroquinone to prevent the oxidation of the unsaturated fatty acids. The details of this technique were reported in one of our previous papers[20].

The methyl esters were separated on a column 0.125 in. in diameter and about 2 m long and filled with 60–80 mesh silanated Chromosorb W containing 20 % of polyethylene succinate. Nitrogen with a flow rate of 20 ml/min was used as the carrier gas. The initial column temperature of 150° was increased at a rate of 3.3°/min until it reached 200°. A flame-ionisation detector was used in a Perkin-Elmer 800 instrument.

RESULTS AND DISCUSSION

Analysis of the lipid components

Table I gives the analytical data for the individual lipid fractions from the adrenal glands of both normal and pantothenic acid deficient rats.

The composition of the mixture of phospholipids, triglycerides, cholesterol and cholesterol esters in the controls is seen to be very close to that in rat serum,

TABLE I

LIPID CONSTITUENTS OF THE ADRENAL GLANDS OF RATS

	Extract No.	Number of adrenals	Adrenal tissue (mg)	Total lipids (mg/100 mg adrenal tissue)	Triglycerides (mg/100 mg adrenal tissue)	Triglycerides (mg/100 mg total lipids)	Cholesterol: Free cholesterol (mg/100 mg adrenal tissue)	Cholesterol: Free cholesterol % total cholesterol
Control rats	25	8	176	12.8	1.9	14.9	0.3	5.4
	26	14	300	13.2	1.7	13.1	0.3	5.7
	29	10	189	16.1	2.0	12.7	0.3	5.0
	39	36	633	12.3	1.6	13.1	0.3	5.7
	41	14	229	13.1	1.7	12.7	0.2	3.3
	43	10	164	12.8	1.8	14.3	0.3	5.0
	45	10	148	13.7	2.0	14.8	0.4	6.2
Mean standard deviation				13.5 ± 1.2	1.8 ± 0.1	13.6 ± 1.0	0.3 ± 0.05	5.2 ± 0.9
Pantothenic acid deficient rats	19	17	257	12.0	2.2	18.4	0.3	7.9
	20	17	259	12.0	2.3	19.4	0.3	7.3
	21	18	298	12.4	2.4	19.2	0.3	7.3
	22	18	296	12.3	2.1	17.5	0.3	7.9
	24	12	211	11.9	1.8	15.0	0.2	7.1
	27	14	291	10.6	1.9	17.8	0.2	6.0
	28	25	370	14.0	1.7	15.2	0.3	6.9
	38	44	669	9.8	1.1	11.6	0.2	7.2
	40	22	346	10.5	1.3	12.3	0.3	8.0
	42	15	178	11.6	1.2	10.5	0.3	9.6
	44	10	155	12.6	2.4	18.8	0.3	7.1
Mean standard deviation				11.8 ± 1.1	1.8 ± 0.5	16.0 ± 3.2	0.3 ± 0.05	7.5 ± 0.9

* $P < 0.005$.
** $P < 0.001$.

taken under indentical conditions (after a 12 h fast) and analysed in the same way[20]. The quantity of free fatty acids is smaller than that in the serum (mean value: 1.5–1.8 as compared with 8.3–7.8 mg/100 mg of lipids).

Adrenal lipid extracts are characterised by consistently high cholesterol ester contents, as had previously been observed by TEPPERMAN *et al.*[21], FIDANZA AND BONOMOLO[22] and POULTON AND REESE[23], using different techniques. Rats which were deficient in pantothenic acid exhibit a lower cholesterol content (4.0 mg/100 mg of adrenal gland, corresponding to 34.5 mg/100 mg of total lipids) than the normal controls (5.7 mg/100 mg of adrenal gland, corresponding to 42.4 mg/100 mg of total lipids). This reduction occurred in the esterified portion, confirming the data reported by FIDANZA AND BONOMOLO[22] and OSBORN, WEAVER AND ANDERSON[24].

On the other hand, an increase in the phospholipid content is observed (4.5 mg/100 mg of adrenal gland, corresponding to 37.9 mg/100 mg of total lipids) as compared with the controls (3.9 mg/100 mg of adrenal gland corresponding to 29.3 mg/100 mg of total lipids).

Table II shows the fatty acid composition as found by gas-chromatographic

sterified cholesterol		Total cholesterol		Fatty acids		Phospholipids		Recovery
ng/100 g *drenal ssue*)	% *total cholesterol*	(*mg/100 mg adrenal tissue*)	(*mg/100 mg total lipids*)	(*mg/100 mg adrenal tissue*)	(*mg/100 mg total lipids*)	(*mg/100 mg adrenal tissue*)	(*mg/100 mg total lipids*)	(*mg/100 mg total lipids*)
8	94.6	5.0	39.4	0.10	0.8	—	—	—
4	94.3	5.7	42.9	0.23	1.7	4.1	31.0	89.0
0	95.0	6.3	39.3	0.09	0.8	4.1	25.7	78.5
9	94.3	5.2	42.2	0.15	1.2	3.4	27.4	83.8
5	96.7	6.7	49.8	0.10	1.1	3.3	24.8	88.3
4	95.0	4.7	36.6	0.40	3.3	3.9	29.2	83.4
0	93.8	6.4	46.9	0.50	3.6	4.8	37.7	100.0
4**	94.8	5.7**	42.4**	0.22	1.8	3.9	29.3*	84.7
±0.8	±0.9	±0.8	±4.6	±0.16	±1.2	±0.5	±4.5	±8.4
.8	92.1	4.1	34.8	0.09	0.7	—	—	—
.2	92.7	4.5	37.6	0.11	0.9	0.9	—	—
.9	92.7	4.2	33.7	0.10	0.8	—	—	—
.8	92.1	4.2	33.8	0.09	0.8	—	—	—
.4	92.9	3.7	31.3	0.09	0.8	—	—	—
.8	94.0	4.0	39.5	0.13	1.2	—	—	—
.9	93.1	4.2	37.4	0.13	1.1	3.8	33.8	87.9
.9	92.8	3.1	31.4	0.20	2.0	3.9	40.0	85.3
.7	92.0	4.0	38.6	0.20	1.8	4.1	39.6	92.2
.1	90.4	3.4	29.5	0.30	2.9	5.5	45.8	88.7
.9	92.9	4.2	32.5	0.40	3.3	5.0	39.2	93.8
.7**	92.5	4.0**	34.5**	0.17	1.5	4.5	39.7*	89.6
±0.04	±0.9	±0.4	±3.3	±0.03	±0.9	±0.7	±4.2	±3.4

analysis of the cholesterol ester fraction. No appreciable variations exist between the deficient and the normal animals, apart from a moderate increase in poly-unsaturated fatty acids in the deficient animals, at the expense of the other fatty acids in the fraction, as also noted by FIDANZA, CONSTABLE AND WILSON[25,26], working on total lipids.

We believe that a more thorough investigation of these fractions in relation to the lipid composition of the diet would be useful. The fatty acid composition of the other lipid fractions of the adrenal glands will be reported in another paper and discussed in the light of the observations noted in the literature.

Analysis of the steroid components

The analytical data for the corticosteroids are listed in Table III. The quantities of substances which reduce tetrazole blue (expressed as hydrocortisone) are 26.7 μg/100 mg of adrenal gland from the controls, and 32.2 μg/100 mg from the deficient animals, corresponding to 0.25 and 0.2 % of the total lipids, respectively. Thus the difference is not significant.

TABLE II

GAS-CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACIDS PRESENT IN THE CHOLESTEROL ESTER FRACTION

Number of C atoms of the fatty acids	*Pantothenic acid deficient rats*	*Control rats*
12	trace amounts	trace amounts
14	5.7	8.8
16	22.4	24.3
16:1	8.6	10.0
17	0.7	0.7
17:1	0.9	0.9
18	5.8	5.6
18:1	29.1	31.2
18:2	2.0	1.9
18:3	11.5	8.2
20	3.8	2.3
20:4	7.9	4.9
21	trace amounts	0.6
22	1.6	0.6
Saturated fatty acids	40.0	42.9
Mono-unsaturated fatty acids	38.6	42.1
Poly-unsaturated fatty acids	21.4	15.0

The principal steroid component is always corticosterone. This observation clearly corroborates the earlier results[28,29].

Aldosterone has been detected beyond doubt in all the extracts analysed; the quantities present are sufficiently large to permit its determination by the use of larger quantities of extracts than were used in the present work. Fig. 2 shows that traces of 11-dehydrocorticosterone (compound A), and probably also deoxycorticosterone, can be detected in several extracts. Non-steroids, which give a positive test with tetrazole blue, are also observed; some of these remain at the start, whilst others migrate to the front. The presence of these substances explains the fact that the total corticoid contents (cautiously referred to as "substances which reduce tetrazole blue") obtained from the extract to be chromatographed are higher than the values obtained after chromatography. In fact, the combined amounts of corticosterone and aldosterone as obtained after chromatography are about $^1/_6$–$^1/_7$ of the value found for the substances which reduce tetrazole blue (in μg/100 mg of adrenal gland). Unidentified components migrating with the front may be eliminated, as has been mentioned before, by preliminary chromatography of the extract with chloroform containing 0.2 % of ethanol. This operation is not absolutely essential but it is advisable, since otherwise the "tails" of these impurities, if the latter are present in large quantities, could be contaminate the zones of the active steroids and thus lead to positive errors in the determinations.

Check on the recovery and on the chromatographic separation

The substances which react with tetrazole blue and which are situated near the front are accompanied by other, slightly more mobile, components which absorb in the U.V. region, but have not yet been identified; these substances do not interfere in any way with either the chromatographic separation or the recovery of corticosterone and aldosterone.

TABLE III

CORTICOSTEROIDS OF THE ADRENAL GLANDS OF RATS

	Extract No.	Number of adrenals	Adrenal tissue (mg)	Total lipids (mg)	Substances which reduce T.B.* (µg/100 mg adrenal tissue)	Pool No.	Amount analyzed (µg)	Corticosterone (µg/100 mg adrenal tissue)		Aldosterone (µg/100 mg adrenal tissue)	
								U.V.	T.B.*	U.V.	T.B.*
Pantothenic acid deficient rats	19	17	257	30.9	27.1						
	20	17	259	31.9	34.7						
	21	18	298	37.1	37.2	1	200	5.1	4.2	0.89	0.9
	22	18	296	36.6	37.8						
	24	12	211	25.1	31.2						
	27	14	291	31.0	27.5						
	42	15	178	20.7	35.7	2	85	3.6	4.4	—	1.2
	44	10	155	20.0	30.0						
	28	25	370	42.0	27.3		73	2.0	2.9	—	1.6
	38	44	669	66.2	33.8		68	6.6	4.6	2.98	2.7
	40	22	346	36.6	62.1**		129	3.2	4.1	—	1.0
Mean standard deviation					32.2 ±3.6			4.1 ±1.7	4.0 ±0.7		
Control rats	12	36	597	87.2							
	13	14	213	39.0	38.0	3	100	2.8	2.9	0.54	—
	14	20	418	40.3							
	17	10	222	25.5	17.3	4	68	3.0	3.2	0.24	—
	18	10	224	24.0	20.7						
	25	8	176	22.5	22.0						
	39	36	633	78.2	18.6	5	83	4.7	4.8	—	1.8
	43	10	164	21.0	36.6						
	26	14	300	39.7	26.4						
	29	10	189	30.5	36.4	6	115	3.0	3.6	2.40	0.9
	45	10	148	20.3	24.2						
	41	14	229	30.9	65.5**		66	1.6	2.5	—	1.2
Mean standard deviation					26.7 ±8.2			3.0 ±1.1	3.4 ±1.0		

* T.B. = tetrazole blue.

** The mean does not include these two values obtained from chloroform–methanol hot-extracted samples. Nevertheless their contents of corticosteroids is similar to the others after purification.

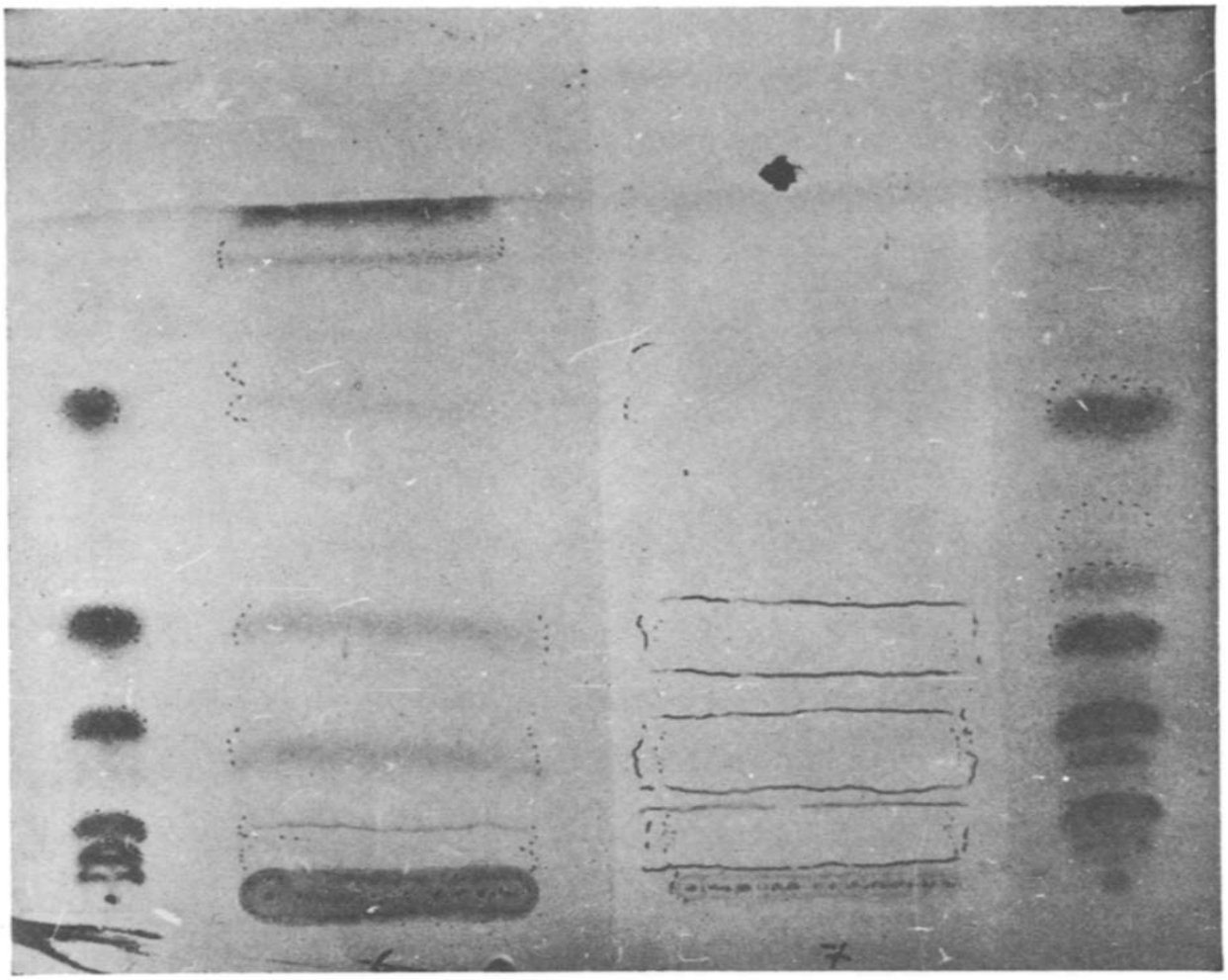

Fig. 2. Chromatographic fractionation of the steroid constituents of the adrenal extracts after their separation from lipids. Solvent: chloroform–methanol–water (90:10:0.25). Detection with tetrazole blue spray. Plate coated with a 0.25 mm layer of fluorescent silica gel G. From left to right: 1st track (from the bottom): THF, THE, cortisol, cortisone, corticosterone, 11-dehydrocorticosterone used as reference standards. 2nd track: crude corticosteroid extract sprayed with tetrazole blue. (It is possible to identify aldosterone, corticosterone, compound A and probably deoxycorticosterone (close to the front).) 3rd track: crude corticosteroid extract protected from the tetrazole blue spray; the bands are later removed. 4th track: bovine adrenocortical extract containing the principal steroids: THF, THE, cortisol, aldosterone, cortisone, corticosterone, compound S, and compound A.

The recovery was checked in the following manner: an extract containing 9.9 mg of lipids per 100 mg of adrenal tissues was divided into 2 equal portions. One portion was used for the determination of the original values, whilst to the other 50 μg of corticosterone were added. The whole analytical procedure was then carried out on both portions, with the following results:

Original extract	corticosterone 12.3 μg/100 mg of clean tissues aldosterone 2.75 μg/100 mg of clean tissues
Original extract + 50 μg corticosterone	corticosterone 64.2 μg/100 mg of clean tissues aldosterone 2.37 μg/100 mg of clean tissues.

The difference between the corticosterone contents of the two portions was 51.9 μg.

As has been stated before, it was found convenient to combine several extracts to obtain larger quantities of steroids. The identity of the corticosterone and aldosterone zones, and the non-steroid character of the other zones which gave a reaction with tetrazole blue could thus be checked by paper chromatography, using BUSH's B_1 and C systems. The analytical results for aggregates Nos. 1, 3 and 4 have clearly established the following points: Zone (a) (start), extending between about —0.5 and +0.5 cm, contains an unidentified non-specific material showing extensive tailing and a weak orange-yellow coloration. Zone (b), between about 0.5 and 3 cm corresponding to the THF and the THE in our thin-layer chromatography system, contains no substances which give a positive test with tetrazole blue with the exception of only a small spot showing blue fluorescence. Zone (c), situated between 4.8 and 6.8 cm, corresponding to aldosterone in our thin-layer chromatography system, contains only one compound, which was identified in BUSH's C system as aldosterone. Zone (d), between 7.8 and 11.3 cm, corresponding to corticosterone in our thin-layer chromatography system, contains only one substance, which was identified in BUSH's C and B_1 systems as corticosterone. Zones (e) and (f), situated between 14.4 and 16.9 cm and 19.2 and 21 cm, resp. contain no components which react with tetrazole blue with the exception of small fluorescent spots in the "tail". These zones correspond in our thin-layer chromatography system to 11-dehydrocorticosterone and 11-deoxycorticosterone, respectively, which are present in trace amounts in some of the extracts. The final zone (g), which lies between 23.5 and 26.2 cm, close to the front, contains one component, which also migrates with the solvent front in paper chromatography, and which gives a weak orange-yellow color with tetrazole blue.

Paper chromatography using BUSH's C system was also carried out directly on the crude extract containing the corticoids obtained after the first chromatographic separation of the lipids. Corticosterone was identified but not aldosterone, since the latter migrates with the impurities which mask the tetrazole blue test.

CONCLUSIONS

The above results show the value of this micro-technique in determining the basic qualitative and quantitative patterns of the lipid and steroid composition of the adrenals, using small quantities of tissue, such as 300 to 400 mg. These determinations are much more difficult to achieve by other methods. However, the data available at present, do not permit any definite conclusions regarding quantitative differences between the steroid production in normal and pantothenic acid deficient rats. They do show, on the other hand, that there are no qualitative differences and that aldosterone is always present in appreciable quantities in the adrenal secretion of rats, together with corticosterone, which should be regarded, as is well known, as the precursor of the former. Cortisol and related metabolic products, such as cortisone and the tetrahydro derivatives of both cortisol and cortisone, are clearly absent, whilst the presence of small quantities of 11-dehydrocorticosterone and 11-deoxycorticosterone is explained by the catabolic and anabolic transformations of the two principal steroids. This micro-technique may be useful in the investigation of the cortical secretions of small animals, as well as in all other research work where only

small quantities of adrenal tissue are available. The chromatographic resolution of the adrenal lipids confirms what was discussed and amply illustrated in one of our earlier investigations.

SUMMARY

A method is described for fractionation and determination of the lipid and steroid components in very small amounts of substrates.

Samples as small as 20 suprarenal glands of either normal or pantothenic acid deficient rats were extracted with chloroform–methanol (2:1). The total lipid extracts were separated by thin-layer chromatograms, the solvent system being benzene–ether–acetic acid (70:30:1). Spots corresponding to cholesterol esters, triglycerides, fatty acids, cholesterol, and phospholipids were obtained; corticosteroids lag behind as the least mobile fraction, together with the phospholipids. The areas were eluted with appropriate solvents and the main components were determined.

Corticosteroids were estimated by a colorimetric method with tetrazole blue and by U.V. spectrophotometry.

A second chromatogram was then performed on the corticosteroid eluate; the solvent system was chloroform–methanol–water (90:10:0.25). A clear-cut separation of the principal corticosteroids present in the suprarenal glands of rats was obtained.

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