THE PLASMA CORTICOSTEROIDS

A Thesis submitted for the Degree of

Doctor of Philosophy

in

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by

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And having thus got rid of the
foolishness of the body we shall
be pure and know of ourselves
... the light of truth.

The Phaedo.

(Love of truth is) not entertaining
any proposition with greater
assurance than the proofs on which
it is based.

Locke.

.... our obligation to seek truth
is part of a general obligation to
do what pays.

William James.

Guts, humours, ventricles, nerves, fibres
and fat - the arterial labyrinth, body's hell.
Still, it was the first thing my mother gave me.

Christopher Fry.

SECTION 1.

INTRODUCTION.

The current intense interest in the secretions of the adrenal cortex is shared by physiologist, physician and pharmacologist alike. Such attention is not surprising in the case of a gland which is immediately essential to life, which can produce syndromes as varied as precocious puberty, virilization, Addison's disease, Cushing's syndrome, and the newly described hyperaldosteronism syndrome of Conn (1955), a gland which has been implicated in the pathogenesis of diabetes (Hoet and Lukens, 1954; Jackson, 1955), hypertension (Sapeika, 1948, 1955), pre-eclampsia, and atherosclerosis, and which profoundly affects so many of the metabolic processes of the body.

The need for accurate measurement of adreno-cortical function has therefore been accentuated in recent years. The purpose of this study was to find out as direct a method as possible for determining the rate of secretion of this gland, and to define with this method the norms and normal variations. One has no diffidence in adding to the galaxy of existing techniques, for none is entirely satisfactory in respect of sensitivity, specificity and especially convenience.

Without any pretence to completeness, it is appropriate to review existing adrenal function tests briefly, in order to define the problem with which one was faced when

beginning this study. The tests fall into three groups:

1. Those based on the effects of corticosteroids (CS) on the organism.

The measurements are several degrees removed from the rate of secretion of the hormones. Examples are the circulating eosinophil count (Thorn, Forsham, Prunty and Hills, 1948), the water-loading test (Soffer and Gabrilove, 1952), and the urinary sodium and potassium excretion. Techniques are on the whole simple, but not so the interpretation of results. Many of the metabolic effects of injury to intact animals occur also in adrenalectomised animals given a constant maintenance dose of cortisone. Ingle has coined the term "permissive action" to describe this property of some adrenal hormones. Sodium and potassium balance, and eosinophil counts (Thorn, Jenkins and Laidlaw, 1953) may fluctuate independently of detectable changes in CS level. None of the variables measured is exclusively under the influence of the adrenal cortex.

2. Chemical or biological assay of the urinary excretion of CS and their metabolites.

This group of tests is the most extensively used, and is further discussed in Section VI. The main justification for urine studies has been the relatively large quantities of steroid present. It has yet to be shown that a precise correlation exists between the secretion of CS and the excretion of CS metabolites, nor is it always clear which steroids these metabolites correspond to. No complete solution has

been found to the problem of hydrolysing conjugates, in which form some 95 per cent of steroids are excreted; neither acid nor enzymic hydrolysis alone appears to provide full recovery. In Section VI a comparison is described of plasma and urine studies at various levels of adrenal activity.

In favour of urine studies is the fact that they can measure a rate of excretion, whereas estimations on blood reflect CS metabolism at one point in time. Urine samples, if collected over a 24-hour period, "smooth out" such fluctuations in CS secretion as the diurnal variation.

3. Determination of CS in peripheral blood.

Such measurements are affected by less variables than the above, but nevertheless indicate the resultant of several factors; they represent the balance between release of CS from the adrenal gland and possibly the placenta, and elimination by hepatic, renal and perhaps other processes such as tissue protein binding. The main technical obstacle has been the minute quantities of CS present in a blood sample of reasonable size, varying from 0.01 - 1 μg. In the absence of reactions specific for these hormones, rigorous purification is essential and most methods stand or fall by the success which attends isolation of CS (Bongiovanni and Eberlein, 1955a). Chromatography has made possible the resolution of CS from each other and from contaminants, while a variety of methods of photometric determination (absorptiometry of coloured products, fluorimetry, and ultraviolet spectrophotometry of the

steroids themselves) possess sufficient sensitivity for the estimation of the more abundant CS.

Numerous techniques are now available for the determination of CS and their metabolites in plasma. These have provided valuable information, but in many cases simplicity and specificity have borne an inverse relationship. The majority depend on a reaction using phenylhydrazine in sulphuric acid (Porter and Silber, 1950), which determines steroids possessing a dihydroxyacetone side chain. Other carbonyl compounds, of course, may react with phenylhydrazine; endogenous keto-acids and some commonly used drugs may give falsely high values, and must be removed or avoided. Nelson and Samuels (1952), who first used the reaction to determine plasma 17 hydroxycorticosteroids (17-OHCS), indicated that the Porter-Silber chromogen in their extract was cortisol, but Bayliss and Steinbeck (1953a, 1953b), who modified this technique to the extent of running a more reliable blank, believe that ½ - ⅓ of the chromogen is cortisol, and that part of the remainder is the inactive derivative tetrahydrocortisone. Neither method attempts resolution of CS, and recoveries in the former vary from 55 - 132 per cent. The techniques depend on an adsorption chromatogram to separate CS from contaminating lipids.

Subsequent modifications (Bondy and Altrock, 1953; Kassenaar, Molenaar, Nijland and Querido, 1954; Porter and Silber, 1954) dispense with chromatographic purification and

rely on a single operation distribution to remove less polar contaminants. Bongiovanni and Eberlein (1955a) have had erratic results with this type of procedure, which is assessed with unfavourable results in experiments described in section III.

Among the earlier techniques was that of Morris and Williams (1953), whose method must rank as one of the most refined. A somewhat lengthy extraction and purification procedure culminates in reversed phase partition chromatography, after which CS are resolved on two partition columns. Polarographic determination follows, its sensitivity being such that 50 ml blood are required. These workers have recently modified the method (1955); they now resolve CS on a single column using the principle of gradient elution, the mobile phase being changed automatically; determination is by spectrophotometry at 235 mμ and absorptiometry of the diformazan produced by reduction of blue tetrazolium (Chen and Tewell, 1951; Mader and Buck, 1952). The latter reaction is specific for the α-ketol side chain possessed by all CS, and has been used in conjunction with the Porter-Silber reaction by Weichselbaum and Margraf (1955) in yet another technique for plasma CS determination.

Sweat (1955) has published a method of great interest. After purifying the plasma extract by partitioning between 70 per cent methanol and hexane, he resolves cortisol and corticosterone by adsorption chromatography on silicic acid, determining these steroids by fluorimetry (Sweat, 1954a), the

fluorescence being induced by sulphuric acid. This reaction is of remarkable sensitivity, a "suitable range" being 0.05 - 5 μg cortisol. Unfortunately, Sweet's elution diagram shows failure of his adsorption column completely to resolve the steroids mentioned; Weichselbaum and Margraf, using Sweet's method, separated them only twice in twenty attempts.

The methods of Morris and Williams and of Sweet give higher results for corticosterone than other procedures, and have been criticised in consequence by Bush (1955). This difference of opinion has led to re-investigation in the present study, results being analysed in Section VIII.

It seemed to the writer that there was still a pressing need for a method simple enough for routine use on a large scale, and by technicians possessing moderate qualifications; to be of value, such a method would have to be sensitive, to determine individual CS reproducibly, and to be immune to interfering substances. The use of paper chromatography in particular appeared worthy of study, in view of the technical difficulty of column work and the excellent resolving power of available paper methods. These investigations comprise Section III.

A serious criticism of plasma determinations has been that the level is so labile in health. Some factors believed to affect the rate of CS secretion have been investigated, and the conclusion is reached that the plasma

cortisol at least is more stable than had been suspected. This misconception stems largely from current hypotheses concerning "adaptation to stress"; the failure of the plasma cortisol to rise in response to some environmental variations is relevant in this connection.

In view of the paucity in the literature of detailed qualitative studies on the CS in peripheral plasma, two large pooled samples were worked up, the CS being identified by Rf values in several solvent systems, Rf values of the products of acetylation, and selective fluorescence and colour reactions (Section II).

The technique of preparing paper for chromatography, described in Section III, has permitted the use of paper instead of column chromatography for purifying and resolving the CS prior to quantitative analysis; this has greatly facilitated the handling of large numbers of samples, and has made available, in the paper strip, a microcolumn of extremely high reproducibility. The use of paper chromatography in routine plasma CS determination has hitherto been precluded by the excessive blank values obtained.

A reversed-phase paper-chromatographic method has been introduced for final purification of CS extracts from plasma; it is both rapid and simple, and provides effective "defatting" of CS with 98 - 100 per cent recoveries of cortisol.

The method ultimately evolved has been tested for specificity both by qualitative studies on pooled plasma

(Section II) and by the effect of ACTH, cortisol, and cortisone administration. In severe adrenal insufficiency negligible concentrations of cortisol are detected (Section V). Cortisol and corticosterone recoveries are reported.

Serial hydrolysis with β-glucuronidase, sulphatase and mineral acid was used to elucidate the nature and extent of conjugation of cortisol and 17-hydroxycorticosteroids in plasma, and the effects of cortisol and ACTH were observed (Section IV). It was concluded that the free plasma cortisol was a more satisfactory index of CS secretion than the conjugated plasma 17-OHCS.

CS are partly degraded and conjugated by the liver, and by analogy with the oestrogens may undergo an enterohepatic circulation. The rate of conjugation is reflected in observations described in Section IV.

Lastly, the method has been applied to a variety of endocrine and other disorders. The results, some of which are analysed in Section V, have in no case been inconsistent with clinical and other biochemical findings. It is felt that this has been the acid test of the technique of cortisol estimation.

The description has been omitted of attempts to estimate aldosterone in small samples of plasma. This work is of course proceeding, but sufficient reproducibility has yet to be achieved.

Some parts of the investigation have been submitted

for publication to the Biochemical Journal and to Acta Biochimica Biophysica.

SECTION II

QUALITATIVE STUDIES ON HUMAN PERIPHERAL PLASMA.

As a prelude to quantitative studies, an investigation was carried out into the nature of the corticosteroids in human peripheral blood. The most thorough analysis of circulating adrenocortical hormones is that of Bush and Sandberg (1953), whose subjects frequently received ACTH. Their method was to observe the paper-chromatographic behaviour of the CS, as well as their acetates and oxidation products; spots were identified by reactions specific for certain configurations characteristic of CS. Bush (personal communication, 1955) has lately confirmed this earlier work, avoiding ACTH administration. Cortisol was the most plentiful CS; corticosterone was present in about one case in five. Morris and Williams (1953) relied on chromatographic behaviour in a single solvent system, obtaining further evidence from the form of the polarographic wave. Sweat's (1955) findings were based on Rf values of CS on a rather inefficient adsorption column coupled with a fluorescence reaction of only moderate specificity.

Using quite different procedures, these workers found corticosterone to be far more plentiful than did Bush; Morris and Williams found the cortisol/corticosterone ratio to be 1.35, and in a later study (1955) to be 0.9. Sweat's

corticosterone levels were rather lower, the ratio being 2.23.

Unlike peripheral blood, adrenal venous blood has undergone detailed investigation. Some data are difficult to interpret, as the blood was collected during adrenalectomy for Cushing's syndrome. Hudson, Romanoff and Pincus (1953), and Hudson and Lombardo (1955) have reported on samples from patients undergoing adrenalectomy for advanced breast or prostatic carcinoma; ACTH was administered to some patients, and the endogenous ACTH level was presumably increased in all by the surgical procedure; but there was no reason to suspect any qualitative abnormality in adrenal function. Crystalline cortisol and corticosterone were obtained, and infra-red spectrophotometry was among the means employed to establish the identity of these steroids. Aldosterone and cortisone were not detected, but the presence of the androgen Δ^4 andro-stene-11β ol-3 : 17 dione was proved. Several unknown substances were present.

Bongiovanni and Eberlein (1955b) have isolated tetrahydrocortisone from peripheral blood, and characterised it by infra-red spectrophotometry. In urine, and probably in plasma, it is present largely in conjugated form.

In the present analysis, peripheral venous blood was drawn from normal subjects, one pint from each. ACTH was not administered. Four male students were the donors in experiment I, and a male and a female in experiment II.

Coagulation was prevented by heparin, and plasma was separated within 15 - 30 minutes.

I. The 1200 ml pooled plasma was worked up as in the quantitative procedure (Section III F) by ethylene dichloride extraction and alkaline and acid washing of the extract, which was evaporated to dryness and then taken up in acetone. The conventional distribution between aqueous methanol and petroleum was omitted to avoid any possible discrepancy between the qualitative and quantitative analyses. "Defatting" was carried out by reversed phase paper chromatography on a 6 cm wide dichlorodimethylsilane-treated paper strip; 85 per cent methanol was used, the run being 10 cm. The polar fraction lying within 3 cm of the solvent front was eluted with methanol. (This "defatting" procedure is analysed in Section III). The eluate was divided into four parts and chromatographed on Whatman no. 4 paper which had been washed in ethanolic sodium hydroxide (Section III):-

Fraction 1 was chromatographed by the ascending method in benzene/50% methanol (the B5 system of Bush, 1952) at 30°C, the technique adopted in the quantitative procedure.

Fraction 2 was chromatographed in toluene/propylene glycol (Burton, Zaffaroni and Keutman, 1950) at 21°C for 54 hours.

Fraction 3 was acetylated. It was taken to dryness; five drops of anhydrous pyridine and five of acetic anhydride were added, and the stoppered tube left at room temperature

for 24 hours. The sample was again taken to dryness in vacuo, then chromatographed in toluene-light petroleum/methanol-water 5:5:7:3 v/v (Bush's system B1).

To fraction 4 was added 10 μg each of authentic cortisol, cortisone and corticosterone. Mixed chromatography was performed, two-dimensionally, in benzene/50 % methanol followed by Bush's C system, toluene-ethyl acetate/methanol-water 9:1:5:5 v/v.

All papers were treated with the combined blue tetrazolium - 10 % aqueous sodium hydroxide fluorescence methods in the detection of spots.

Rf values or absolute rates of migration are shown in Tables 1 and 2.

TABLE 1

	Benzene/50 % methanol		Acetates in Bush's B1 system		Mixed chromatogram with cpds. F,E,B. Benzene/50 % methanol	
	Plasma	Ref.	Plasma	Ref.	Plasma	Ref.
Tetrahydrocortisone?	0.10	-	-	-	0.09	-
Cortisol	0.29	0.29	0.27	0.26	0.29	0.28
Aldosterone	0.39	0.40	-	-	0.41	0.39
Cortisone	0.46	0.45	0.36	0.35	0.45	0.45
Corticosterone	0.78	0.80	0.60	0.63	0.78	0.81

	Mixed chromatogram with cpds. F,E,B. Bush's C system		Acetates in benzene/formamide	
	Plasma	Ref.	Plasma	Ref.
Tetrahydrocortisone?	0.14	-	-	-
Cortisol	0.36	0.35	-	-
Aldosterone	0.38	0.40	0.35	0.33
Cortisone	0.63	0.64	0.40	0.42
Corticosterone	0.92	0.95	-	-

Ref. indicates reference compound.

TABLE 2.

TOLUENE/PROPYLENE GLYCOL
(54 hours development, 21° C)

	Cm from starting point.	
	Plasma	Reference
Tetrahydrocortisone ?	1.5	-
Cortisol	5.2	5.1
Aldosterone	-	10.8
Cortisone	10.0	10.2
Corticosterone	36.5	35.9

Reference strips were run with each chromatogram, using authentic cortisol, aldosterone, cortisone and corticosterone or their fully acetylated derivatives.

The benzene/50 % methanol and toluene/propylene glycol strips contained spots with Rf values (or in the latter, distances migrated) agreeing to within 2 % with the reference steroids cortisol, cortisone and corticosterone. The third chromatogram produced spots migrating at the same rate as the acetates of these three steroids, with errors of 4 % or less. The two-dimensional sheet showed four homogeneous spots with appropriate Rf values for cortisol, aldosterone, cortisone and corticosterone in both solvents.

All the spots referred to reduced blue tetrazolium, and on drying displayed yellow fluorescence. The former reaction is shown by α-ketols, and the latter by α-β unsaturated ketones. Both groups are present in all C3.

A comparison of the size and intensity of the spots

with serial dilutions of cortisone, developed in the same way, indicated the following approximate plasma concentrations, but slight variation is known to exist between CS with respect to intensity of colour produced by equimolecular quantities with blue tetrazolium.

Cortisol	7 μg /100 ml
"Aldosterone"	0.15 - 0.3 μg /100 ml
Cortisone	2 μg /100 ml
Corticosterone	1 μg /100 ml

Each paper showed in addition a small highly polar blue tetrazolium-reducing spot, probably tetrahydrocortisone; unfortunately the authentic steroid was not available for comparison. There was also a white fluorescence at the solvent front and a faint blue fluorescence between cortisol and cortisone, the nature of which was quite unknown.

The lower limit of sensitivity of the method of detection used was such that CS present at concentrations below 0.15 μg /100 ml would not have been observed.

Further confirmation of the presence of cortisol and (less convincingly) of corticosterone was obtained by employing the sulphuric acid-induced fluorescence reaction described by Sweat (1954a), a method possessing a fair degree of selectivity in that cortisol, corticosterone, their epimers and tetrahydro-derivatives, and oestradiol give strong fluorescence at 550 mμ , while other steroids fluoresce weakly or not at all. A 10 ml sample of normal peripheral plasma was

worked up as described in the quantitative procedure (Section III) as far as the stage of paper chromatography in benzene/50 % methanol. The 36 cm strip was divided into 2 cm sections; each was eluted and determined by a modification of Sweat's method (Section IIID). The elution diagram, corrected for the paper blank, is shown in Figure 1, with a comparable diagram obtained by chromatographing 2 μg each of authentic cortisol and corticosterone.

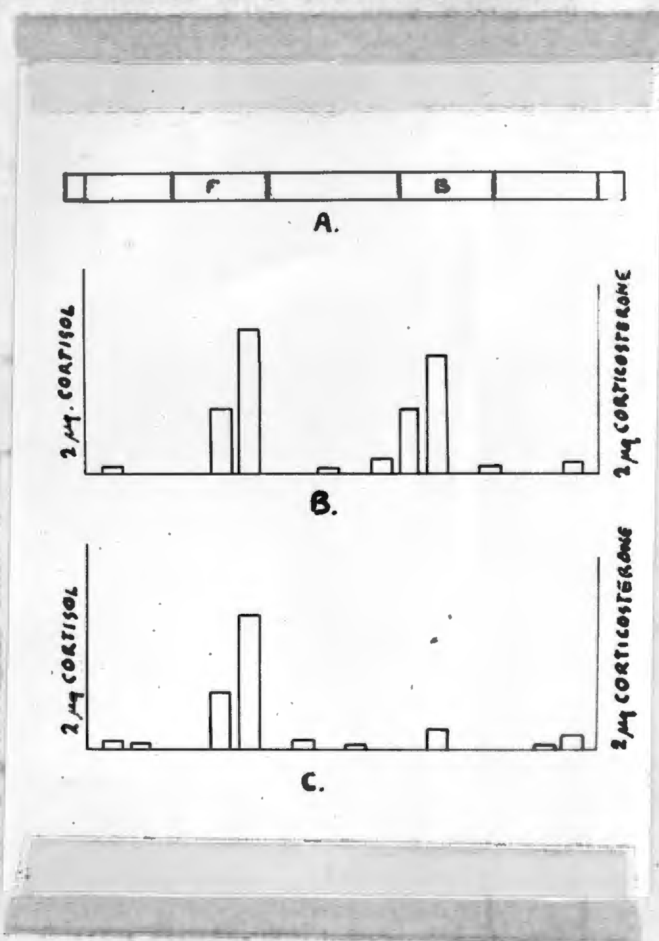


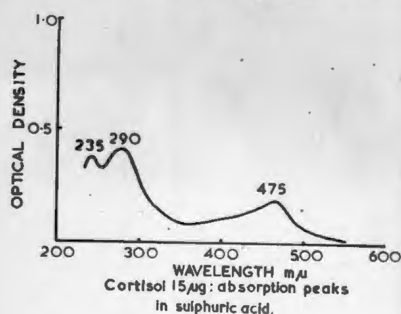
Figure 1 A. Regions eluted from benzene/50% methanol chromatogram in cortisol and corticosterone determination.

B. Elution diagram of chromatogram of authentic cortisol and corticosterone, 2 μg each.

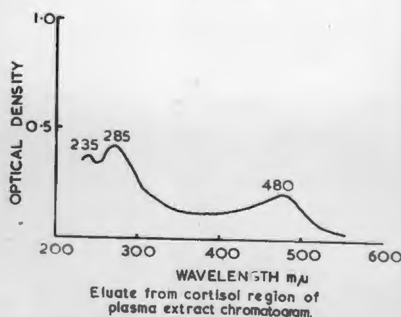
C. Elution diagram of chromatogram of plasma extract.

II. The possible presence of aldosterone in the two-dimensional chromatogram made further studies imperative. A second pooled plasma sample of 560 ml. was worked up in the same way as the first; CS were chromatographed in benzene/50% methanol on a 40x5 cm paper strip, at 30°C. Reference compounds were run as previously. Sections 4 cm in length, corresponding to cortisol, aldosterone (including cortisone), and corticosterone, were eluted with methanol, the eluates being further examined as follows:

(i) The cortisol zone eluate was examined by spectrophotometry in sulphuric acid (Zaffaroni, 1953), by fluorimetry in sulphuric acid, and in potassium tert butoxide (Abelson and Bondy, 1955), and by absorptiometry of the diformazan



Cortisol 15μg: absorption peaks in sulphuric acid.

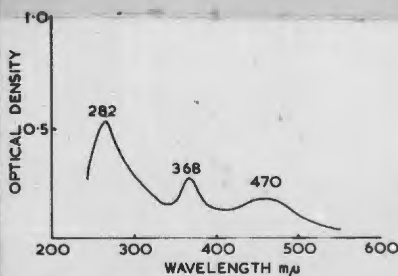


Eluate from cortisol region of plasma extract chromatogram.

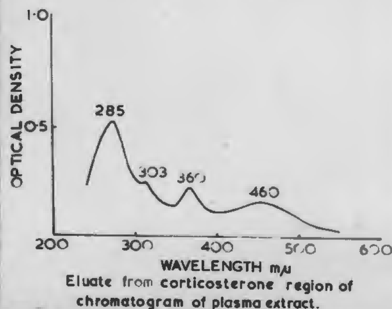
Figure 2.

formed with blue tetrazolium (Nowaczynski, Goldner and Genest, 1955). The absorption spectrum in sulphuric acid is compared in Figure 2 with that of authentic cortisol. The sulphuric-acid induced fluorescence reaction appears to depend on the presence of a hydroxyl group at 11, and an α-β unsaturated ketone in ring A. Yellow fluorescence in the presence of alkali is specific for α-β unsaturated ketones. Tetrazolium salts are reduced by α ketols. The three methods gave plasma levels of 10.9, 10.1 and 11.1 μg/100 ml respectively.

(ii) The corticosterone zone eluate, as determined by sulphuric acid fluorimetry, indicated a plasma level of 0.8 μg/100 ml. There was not enough material, therefore, for as many studies as were performed on the cortisol fraction. The ultraviolet absorption spectrum in sulphuric acid was determined (Figure 3).



Corticosterone: absorption peaks in sulphuric acid.



Eluate from corticosterone region of chromatogram of plasma extract.

Figure 3.

(iii) Assuming that the plasma level of the aldosterone-like substance was similar in the two pooled samples, the eluate from the aldosterone zone was calculated to contain about 1μg. One third was determined by the potassium ~~test~~ butoxide fluorescence reaction and contained 0.29μg of an α-β unsaturated ketone, equivalent to a plasma level of 0.18μg/100 ml. (The estimate based on spot size in the other sample was 0.15 – 0.3μg/100 ml). The remainder was acetylated by the method described and chromatographed in benzene/formamide (Burton, Zaffaroni and Keutman, 1950). The paper was sprayed with blue tetrazolium – sodium hydroxide reagent (Nowaczynski, Goldner and Genest, 1956) using 20 % sodium hydroxide. Two small spots were detected, corresponding to the cortisone acetate and aldosterone diacetate spots on the reference strips. The Rf of the former was 0.40 and of the latter 0.85. Both showed a blue colour; this reached a maximum in 9 – 10 minutes. The aldosterone spot was estimated to contain roughly 1μg on the basis of colour, and of fluorescence intensity on drying the paper.

The evidence for the presence of cortisol, aldosterone, cortisone and corticosterone in the pooled samples was therefore:

<u>Cortisol</u>:

1. Correct Rf value to within 2% in benzene/50% methanol and in toluene/propylene glycol.

2. Correct Rf value of the acetate to within 4% in

toluene-light petrol/50 % methanol.

3. Correct Rf values in mixed chromatography in benzene/50 % methanol and in toluene-ethylacetate/50 % methanol.

4. Presence of an α-β unsaturated ketone configuration.

5. Presence of an 11-hydroxyl group.

6. Presence of an α-ketol group.

7. Absorption spectrum in sulphuric acid closely similar to authentic cortisol. (Fig. 2).

8. As will be shown in Section V, the plasma level increases 3 - 4 times on administering ACTH or cortisol, and falls to 0 - 0.5 μg % in adrenalectomised or Addisonian patients.

9. Consistent results with three methods of determination possessing different (though overlapping) specificities.

10. In an elution diagram obtained by determining 2 cm sections of a benzene/50 % methanol chromatogram of plasma extract, using sulphuric acid-induced fluorescence, a peak similar in Rf to that of authentic cortisol is noted. (Fig. 1).

11. Solubility in lipid solvents, and chemical neutrality.

<u>Corticosterone</u>:

Points 1 - 6 and 11 apply.

Absorption spectrum in sulphuric acid resembles

corticosterone. (Fig. 8).

Small peak of correct Rf in benzene/50 % methanol elution diagram. (Fig. 1).

Cortisone:

Points 1 - 4 and 6 and 11 apply. In addition, appropriate Rf of acetate in benzene/formamide. The absence of a cortisone peak in the elution diagram obtained by sulphuric acid fluorimetry of a benzene/50 % methanol chromatogram is consistent with the presence of cortisone, as the reaction gives strong fluorescence only with 11-hydroxyl compounds, among the neutral steroids.

Aldosterone:

1. Appropriate Rf values in benzene/50 % methanol and toluene-ethyl acetate/50 % methanol.

2. Presence of an α-ketol group.

3. Presence of an α-β unsaturated ketone formation.

4. Correct Rf in benzene/formamide of the product of acetylation with excess acetic anhydride and pyridine.

5. Reasonable agreement of plasma levels as determined by fluorimetry in potassium tert butoxide and by the intensity of the diformazan colour and yellow fluorescence on paper.

6. Absence of a spot in the toluene/propylene glycol chromatogram is consistent with the known inability of this system to resolve aldosterone and cortisone.

7. Absence of a peak in the elution diagram referred

te; this would be expected in view of the small quantities present and the feeble fluorescence of aldosterone in sulphuric acid (Section III D).

8. Solubility in lipid solvents; chemical neutrality.

SUMMARY.

The presence of cortisol, aldosterone, cortisone and corticosterone in extracts of peripheral plasma has been confirmed. The evidence includes chromatographic behaviour in several solvent systems, Rf values of acetylated derivatives, colour and fluorescence reactions. Approximate relative quantities are described. These steroids were extracted without prior hydrolysis of conjugated derivatives, and without any procedure designed to release protein-bound CS. There is no evidence that any process involved can lead to artefact formation.

The subjects were physically normal, and did not receive ACTH. Not more than one pint was drawn from each.

S E C T I O N III

DEVELOPMENT OF A QUANTITATIVE METHOD FOR CORTISOL AND CORTICOSTERONE IN PLASMA

A. COMPARISON OF EXTRACTING SOLVENTS: EXTRACTION PROCEDURES.

No reaction specific for CS is known; one of the major problems in determining these compounds in blood is their isolation, which involves the separation of less than 20 µg CS/100 ml plasma from a gross excess of lipid, the mean figure for which, in man, is 566 mg/100 ml (Page, Kirk, Lewis, Thomson and van Slyke, 1935). Apart from conventional solvent extraction, dialysis has been used by Axelrod and Zaffaroni (1954), while Hechter et al (1950) have employed adsorption on charcoal. Dialysis in a three-phase system is claimed to isolate CS in sufficiently pure form to permit immediate partition chromatography, with satisfactory yields of the highly polar steroids at least; however, the apparatus is somewhat cumbersome, and the procedure requires 48 hours. Adsorption provides inadequate and variable yields, artefact formation is common, and the method is not, at least at present, of value (Levy and Kushinsky, 1954).

In the present study, only simple extraction into a water-immiscible solvent was investigated, selectivity of the solvent being a primary consideration. Methanol, ethanol and acetone are good steroid solvents, but their use merely necessitates an additional stage in the extraction procedure, as water-soluble substances in plasma must ultimately be

removed by partitioning between organic and aqueous phases. The use of solvents able to precipitate protein, including the three mentioned, does not result in higher values for plasma CS than those obtained when ethyl acetate or chloroform is employed; there is no evidence for firm protein binding of CS in blood. Dialysis produces good yields; tryptic digestion of precipitated plasma protein does not release detectable quantities of CS (Bongiovanni and Eberlein, 1955b).

The decision to use plasma rather than whole blood was based on the relative ease with which extracts of the former could be purified, and the smaller volumes involved. Bush (1954a) has shown that if the plasma is separated from the cells within 15 - 30 minutes of drawing the sample, the latter contain only a negligible fraction of the CS. Ultimately, CS enter the red cell phase until equilibrium is reached; this is delayed by refrigeration.

An ideal solvent would fulfil the following requirements:

(i) CS should have a highly favourable partition coefficient in it with respect to water and plasma.

(ii) It should be selective, extracting minimal quantities of neutral fat, cholesterol, phospholipid and pigment.

(iii) It should not form stable emulsions with plasma.

(iv) It should not decompose CS, nor give rise to substances which do so.

(v) It should have a low boiling point.

The solvents investigated were ethyl acetate, chloroform, methylene chloride, ethylene dichloride ($CH_2Cl.CH_2Cl$), and n-butanol. The latter was not very volatile, however, and this consideration barred the use of methyl isobutyl ketone. Methyl ethyl ketone is somewhat miscible with water, and is expensive. All the substances mentioned are good CS solvents.

1. Partition coefficients.

Cortisol ("free alcohol") was used after recrystallisation from absolute ethanol; this steroid was chosen because it is the most abundant CS in plasma (Section II). To a mixture of 25 ml distilled water and 25 ml of the solvent under investigation were added 10.0 mg cortisol; the system was shaken mechanically for 15 minutes at 21°C, and centrifuged briefly. The organic phase was taken to dryness at 40°C under reduced pressure, and the residue weight obtained. A blank was performed with the solvent and distilled water.

The solvents were distilled on the day of the experiment from reagent grade stock. Ethyl acetate was subsequently shaken with 0.1 N aqueous sodium carbonate to remove acetic acid; Bush (1955) has pointed out that prior saturation with water diminishes the tendency of ethyl acetate to emulsify with plasma.

The superiority of ethyl acetate to the other solvents is evident; there is little to choose between the remainder. (Table 3).

TABLE 3

	Residue	Blank	Recovery
	mg.	mg.	%
Ethylene dichloride	7.9	0.1	78
Methylene chloride	8.5	0.1	84
Ethyl acetate	9.2	0.2	90
Chloroform	8.4	0.0	84
n-Butanol	8.1	0.1	80

2. Specificity and tendency to emulsify.

Fresh pooled human plasma from four healthy donors was used, blood samples being heparinized, and centrifuged within a few minutes of being drawn. The plasma was at no time frozen. Aliquots of 25 ml were extracted three times for ten minutes each with equal volumes of the solvents studied. A mechanical shaker was used, at a rate of 80 cycles/minute. The mixtures were centrifuged at 2,000 R.P.M. for five minutes, and the organic phases separated. Ethyl acetate required a further 15 minutes centrifugation before this was possible, while n-Butanol was still largely emulsified after this procedure.

Aliquots of the extracts were diluted three times with the same solvents, and compared in the Klett colorimeter; they were then recombined with the extracts, which were taken to complete dryness before weighing.

Solvents were prepared as in (1) above.

Results are shown in Table 4.

TABLE 4

	Extent of emulsion after centrifuging for 2 mins.	Yellow pigment in organic phase (arbitrary units)	Weight of residue mg
Ethylene dichloride	nil	11	3.6
Methylene chloride	nil	16	7.0
Ethyl acetate	$\frac{2}{3}$ vol. emulsified	48	15.4
Chloroform	nil	18	7.8
n-Butanol	Almost complete	Insufficient obtained	-

In a further experiment with another plasma sample, residue weights for ethylene dichloride, methylene chloride, and ethyl acetate were 2.6, 6.0 and 16.8 mg respectively.

The halogenated solvents are superior to ethyl acetate in selectivity. The use of butanol is precluded by the stability of its emulsion with plasma, and ethyl acetate is unsatisfactory for the same reason. Other workers have experienced less difficulty with the latter solvent; Bush recommends dilution of the plasma before extraction, a manoeuvre preferably avoided in view of attendant problems in concentrating large volumes for chromatography. Thielen, Nysted and Mihina (1954) avoid emulsions by gentle swirling in a separating funnel, but possibly because this group worked

with large volumes of plasma one could not confirm this finding. They found isopropyl acetate superior to the ethyl ester, but this in our experience was no more satisfactory.

Chloroform has the disadvantage that it has to be distilled immediately before use; oxidation products may be responsible for the loss of CS which otherwise occurs, but phosgene itself is evidently not to blame, (Loftin, Marks and Leonard, 1955).

Of the solvents tested, ethylene dichloride is undoubtedly the most selective.

3. Washing with alkali.

This procedure is necessary to remove from the extract fatty acids, phospholipids, phenols and pigment, but alkali itself must scrupulously be removed before taking the extract to dryness; CS are unstable in the presence of alkali, particularly at elevated temperatures.

The more polar CS are moderately soluble in water, hence washing should be carried out with a minimal volume of aqueous alkali.

The residues obtained in (2) above were redissolved in 30 ml of the same solvents, extracted for one minute with 2 ml normal aqueous sodium hydroxide, and centrifuged.

It was noted that much pigment entered the aqueous phase. Almost all the colour was removed from the ethylene dichloride extract; methylene chloride and ethyl acetate yield most of the pigment, but chloroform retains the greater

part of the colour.

All phospholipid is not removed by this washing process; precipitation with acetone and mercuric chloride indicates that 20 - 25 % remain in the organic phase in the case of ethylene dichloride, chloroform and ethyl acetate.

After the alkaline wash, the extracts were shaken with 2 ml 2 % aqueous acetic acid. The reaction of the extracts were then checked by further washing with 2 ml distilled water; these washings were at pH 7 with all solvents except ethyl acetate, which gave a pH of 10. This may be explained by the partial miscibility of ethyl acetate with water.

Discussion.

The recovery of cortisol from aqueous solution is slightly less efficient with ethylene dichloride than other solvents tested. This appeared to be the solvent of choice, however, on account of its selectivity, its ready separation from plasma after extraction, and the effectiveness of an alkaline washing in removing pigment from it. Theoretically, three extractions with equal volumes of the solvent would provide over 99 % recovery of cortisol from aqueous solution, but less success would be anticipated when dealing with plasma; Bischoff and Pilhorn (1948) have shown that CS are more soluble in water when protein is present. The overall recovery of CS from plasma with the method of estimation developed, is given in Section III F.

4. The provisional method for extracting plasma was to shake for 10 minutes with two volumes of ethylene dichloride; the extraction was carried out three times. The pooled extract was washed with $\frac{1}{20}$ volume N aqueous sodium hydroxide, then with the same volume of 2 % acetic acid.

The extract has next to be taken to dryness; as CS decompose fairly rapidly at 50°C, this is done at 35°C under reduced pressure. To facilitate taking up the residue quantitatively, the flask is rinsed down with 1 ml ether, which is then evaporated without boiling; this rinsing is then repeated. The ether is conveniently introduced by means of a tuberculin syringe bearing a fine bent needle.

The extract is then taken up in a minimal volume of solvent; 2 x 0.1 ml acetone is suitable. This is made easier by having a small well blown in the bottom of the distilling flask. CS are highly soluble in acetone, but much of the remaining phospholipid and pigment remain undissolved.

Apparatus.

The following apparatus greatly facilitated the extraction of plasma samples.

(i) Mechanical shaker. To ensure uniform agitation, and to permit handling of several samples at once, the apparatus illustrated in Figure 4 was constructed. The heavy organic solvent was most easily dispersed by rocking the horizontal tube. The to-and-fro movement of the tube, carried out by most commercial shakers, did not disperse the ethylene

Figure 4.

Automatic shaker (above)

Micro-separating funnel (right)

Distilling flask (extreme right)

dichloride sufficiently unless violent agitation was produced; emulsification was the usual troublesome result. With this apparatus, the tubes are moved through about 45°, at a shake rate of 80 cycles per minute.

(ii) Separating funnels. Removal of the lower (organic) layer after extraction was most easily accomplished with the 40 ml separating funnels illustrated (Fig. 4). These were able to withstand centrifugation at 2,000 R.P.M. The stopcocks were lubricated with a heavy silicone grease, and supported by rubber bands.

Extraction was performed in these funnels, and after centrifugation for 1 - 2 minutes the extract was drawn off. (The extract was washed in a polyethylene-stoppered centrifuge tube).

(iii) Distilling flasks (Fig. 4). The construction of special glassware was again necessary. The flask has a capacity of 100 ml, and a well holding 1 - 2 ml is blown at the bottom. A ground (B 14) joint attaches the still head, which has a side-arm for connection with the water pump and a fine air bleed.

B. PURIFICATION OF THE EXTRACT.

As prepared by the above procedure, the residue of an ethylene dichloride extract of 10 ml plasma weighs 1 - 1.5 mg, of which the OS comprise about 2 μg. The total lipid in such a plasma sample is of the order of 60 mg, so that considerable progress has been made in purification.

The acetone-soluble part of the residue contains principally neutral fat and cholesterol, the latter largely esterified; these lipids are considerably less polar than all known CS.

(1) Many workers have defatted such extracts by partitioning between 70 % methanol and hexane, e.g. Sweet (1955). This procedure was investigated as follows:

The technique described was used to extract 25 ml plasma with ethylene dichloride; a duplicate sample was similarly extracted with chloroform. These extracts were taken to dryness after alkaline and acid washing. After weighing, the residue was taken up in 7 ml absolute methanol; 3 ml water and 10 ml hexane were added. Following 10 minutes shaking, the aqueous phase was separated and taken to dryness. (The addition of further methanol was necessary to permit removal of water from the residue).

A solvent blank was run with aqueous methanol and hexane.

TABLE 5

	Initial residue mg.	70% methanol residue mg.	Blank residue mg.
Ethylene dichloride	3.6	0.7	0.0(5)
Chloroform	7.2	1.1	0.0

From Table 5 it appears that a single distribution such as this does not adequately remove less polar contaminants; some 5 μg CS are still accompanied by 0.65 mg or more

of other lipids. It is true that more dilute alcohols may be used if two or more distributions are carried out (Weichselbaum and Margraf, 1955, partition the initial residue three times between 30 % aqueous methanol and hexane); such a procedure is relatively time-consuming.

Morris and Williams (1953) freeze out part of the contaminating material from 20 % aqueous ethanolic solution before partitioning between 50 % ethanol and carbon tetrachloride. Both this group and Weichselbaum and Margraf use chromatographic methods to achieve final purification, however.

A further criticism of methods which rely upon a single distribution is that the evaporation to dryness of a water-containing solution is always tedious at low temperatures.

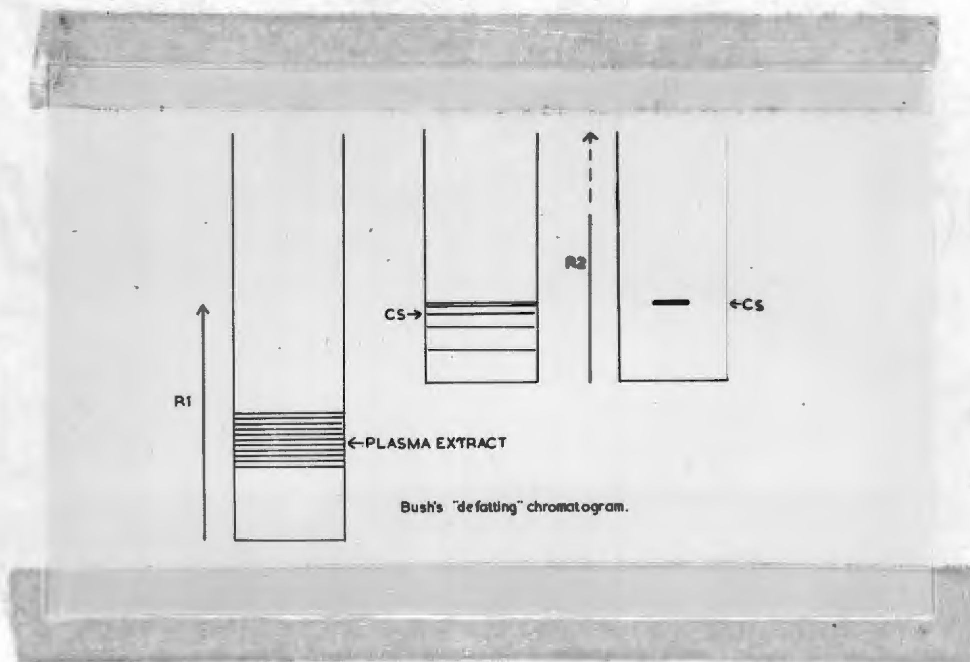
One consequence of inadequate purification of CS is that in paper chromatography 10 - 25 % of the CS may be retained at the starting point.

(2) Chromatographic "defatting" has been employed by several groups. Morris and Williams use a reversed-phase partition column, achieving a high degree of purity. The adsorption method on silica gel (Bush and Sandberg, 1953) gives variable recoveries in humid climates (Bush, 1955 - personal communication). The "Florosil" (magnesium trisilicate) adsorption column introduced by Nelson and Samuels (1952) demands scrupulous care, but has found considerable popularity.

Paper strips are eminently suited to chromatography

of CS. The use of a paper method for separating CS from other lipids has been combined with chromatographic resolution of CS on the same strip (Bush, 1955 - personal communication), and is illustrated in Figure 5. The plasma extract is applied diffusely to the paper; a short ascending run (R1) using 85 % methanol carries the CS at or near the solvent front. The paper bearing the less polar lipids is cut away, the CS are concentrated on the starting line, and resolved by a second chromatogram R2.

Bush describes this as a reversed-phase technique, the stationary phase being the less polar lipids themselves; cortisol is partitioned between these lipids and the 85 %



Bush's "defatting" chromatogram.

Figure 5.

methanol. Whatever the mechanism, it has the great advantage of eliminating the necessity for careful packing of a column, development with precisely the correct volume of one or more solvents, and concentration of a more or less bulky eluate in preparation for spotting on a confined area of a paper strip.

In assessing this method, 10 μg cortisol was added to 20 ml plasma; an ethylene dichloride extract was prepared as in Section III, A(4); the residue was taken up in acetone and chromatographed on a 2 cm wide strip of Whatman no. 42 paper for a distance of 10 cm.

The paper was divided longitudinally into two parts. One was dried after spraying with 2N aqueous sodium hydroxide to locate cortisol; the other was dipped for 10 seconds into a saturated solution of Sudan Black in 70 % ethanol, and then differentiated in 60 % ethanol. The latter technique shows 10 - 20 μg /sq. cm of neutral fat or lecithin, and 50 μg /sq. cm of cholesterol ester, or free cholesterol in the presence of traces of other lipid. Osmium tetroxide vapour is far less sensitive.

Cortisol was found to run largely at the solvent front, but to tail considerably, extending to within 3 cm of the starting line. Most sudanophil material and all the pigment do not migrate, but some lipid travels rapidly and reaches the solvent front. This sudanophil material is not cortisol, for it is seen when plasma extracts are run without addition

of cortisol.

(3) Several such experiments were performed, varying the technique with regard to area of spotting, distance of the starting line from the edge of the paper; time of equilibration, type and width of paper, and length of run. On no occasion was cortisol completely resolved from other lipids. Nor could "artificial" mixtures of 10 μg cortisol and 200 μg cholesterol be separated.

It is surmised that less polar CS such as corticosterone would have fared worse than cortisol as regards purification by this procedure.

On running the chromatogram with 60, 75 and 90 % methanol no greater success was attained. Addition of ethyl acetate to the solvent (methanol 70, ethyl acetate 10, water 20 v/v) improved resolving power, but not sufficiently for confident use in quantitative work.

(4) Tailing is frequently encountered in paper chromatography when a solvent with a high water content is used. In view of the failure of conventional methods to resolve CS from less polar lipids, reversed-phase paper chromatography was next investigated. Lipids, having distribution coefficients greatly in favour of organic solvents as compared with water, are often best resolved by chromatography with a mobile aqueous phase.

Paper strips were immersed in a 5 % solution of Dow Corning silicone 550 in cyclohexane, blotted, and dried at

100° for 30 minutes. Ascending chromatography of mixtures of cortisol with plasma lipid extracts was carried out. The stationary solvent was hexane (applied to the paper in the vapour phase), and 70 % methanol formed the mobile phase. Admirable resolution was noted; cortisol ran compactly at the solvent front, while other lipids moved with Rf 0 - 0.15.

(5) Could the same silicone-treated strip be used to resolve the purified CS? Kritchevsky and Tiselius (1951) have separated steroid mixtures by reversed-phase paper chromatography; using their procedure, cortisol and cortisone were found to migrate rapidly, but rather close together; corticosterone and 11-dehydrocorticosterone were likewise not sufficiently separated. Resolution was adequate for qualitative work, but the method did not possess the resolving power of conventional paper chromatography as used by Bush (1952) and Burton, Zafferoni and Keutman (1950, 1951).

(6) An attempt was made to use the lower end of a paper strip for reversed-phase "defatting", the CS being subsequently resolved by a conventional method on the untreated remainder of the paper after cutting away the siliconed part. This was unsuccessful, for it was observed that the silicone (which renders the strip translucent) spreads rapidly up the paper during equilibration.

(7) The next step was to employ dichlorodimethylsilane to render the paper non-wettable. Unlike silicone oils which simply coat the paper mechanically, the halogenated silane

reacts with the hydroxyl groups of cellulose, eliminating hydrochloric acid and forming a hydrophobic surface of methyl groups. The silane is thus firmly bound to the paper.

To treat part of the paper strips with the reagent, the apparatus shown in Figure 6a was constructed. Papers are firmly clamped between the accurately-fitting halves of the glass lid, preventing escape of the vapour. The papers were exposed for two hours, at atmospheric pressure.

The lower 5 cm of the strips were thus treated. The solution to be chromatographed was applied to the centre of this part of the paper as a band 1 cm in width (heavy shading in Figure 6b).

On such papers, cortisol-plasma lipid mixtures were satisfactorily resolved in the reversed-phase partition system 70 % methanol/hexane, the latter being applied in the vapour phase. Rather simpler was the use of liquid paraffin as a stationary phase, applied as a 10 % solution in petroleum ether and permitted to dry for 15 minutes before spotting. The mobile phase was equilibrated with paraffin before use.

(8) Finally, it was found that equally satisfactory results were obtained when the stationary phase was omitted and 85 % methanol (E1) used as solvent. (Figure 6b). Cholesterol and neutral fat (studied separately and in plasma extracts), and almost all pigment extracted from plasma remain at the starting point. Cortisol, and the less polar OS corticosterone, move compactly at the solvent front on the

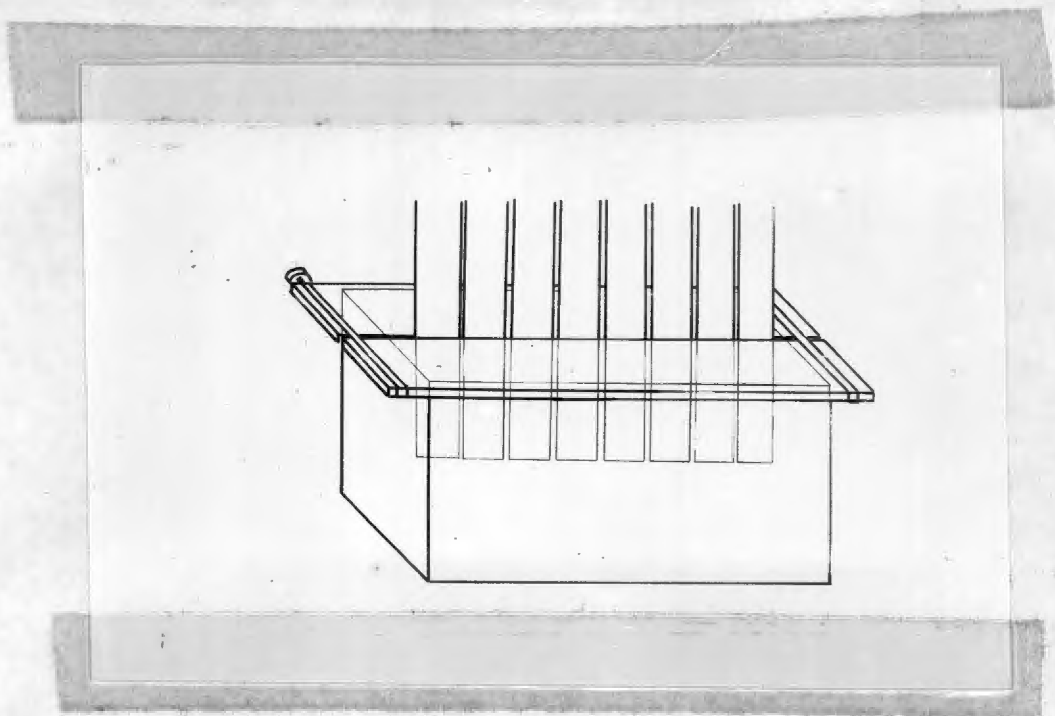


Figure 6a. Apparatus for exposing lower ends of paper strips to dichlorodimethylsilane vapour.

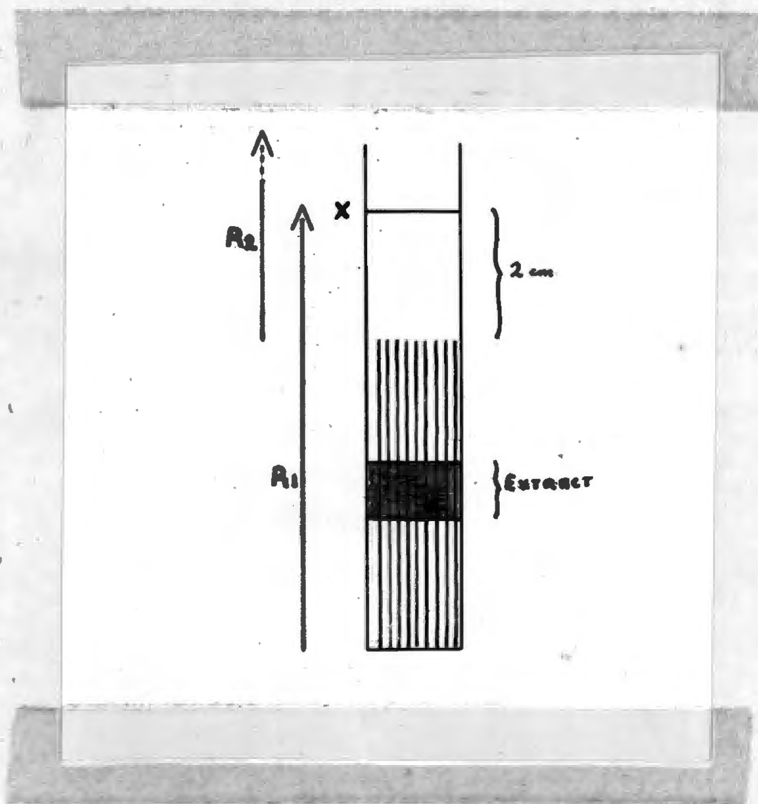


Figure 6b. Reversed phase defatting chromatogram, $\frac{3}{4}$ scale. Fine hatching indicates silane-treated region.

non-wettable part of the paper, but tail slightly once the untreated part is reached. A faint trace of pigment runs at the front.

The solvent is permitted to reach line X, 2 cm above the silane-treated part of the paper. The paper is removed from the tank and dried, after which the non-wettable part of the paper, bearing the weakly polar lipids, is cut away.

Fifteen to thirty minutes equilibration is sufficient, the bottom of the tank being covered with 85 % methanol, which is replaced weekly.

Tailing of CS on the untreated paper is minimised by keeping the run as short as possible.

The method is satisfactory even when 40 μg cortisol is spotted, an amount far in excess of any likely to be encountered in an average-sized blood sample. However, ethylene dichloride extracts from more than 50 ml plasma should not be applied to the usual 1.5 cm wide strip, as mechanical obstruction is offered by the lipids to the advancing solvent; if necessary, wider strips should be employed.

The mechanism of this "defatting" procedure is not clear; it appears to involve one or both of the following processes:

(i) Adsorption chromatography, the less polar lipids being adsorbed on to the methylated surface of the paper.

(ii) Elution from the solid phase, CS being extracted by the advancing solvent.

The fact that CS move at the solvent front suggests that only a single distribution operation is taking place, favouring the latter mechanism.

(9) Although most cortisol and corticosterone run within 5 mm of the solvent front, a more compact region is desirable if CS are to be resolved by subsequent chromatography on the same strip. The "concentrating chromatogram" of Bush (1952) is useful for this purpose; in this laboratory the solvent was modified to the extent of replacing chloroform by ethylene dichloride; this was to exclude the possibility of loss of CS due to oxidation of the former. The composition was ethylene dichloride 45, ethyl acetate 45, methanol 10 parts v/v.

Figure 7.

Concentrating the CS after the defatting chromatogram.

After the 85 % methanol chromatogram R1 has carried the CS up to or near line X, the silane-treated part of the paper is cut away. The CS are now concentrated at line X by slowly pipetting the solvent on to the lower edge of the strip (Figure 7), while the paper hangs vertically. The CS are carried at the solvent front and come to lie compactly on line X.

(10) Recoveries of cortisol and corticosterone in this reversed-phase chromatographic procedure were next determined. Cortisol or corticosterone were spotted together with 200 cholesterol on the siliconed part of the paper strips and chromatographed as described. Drying, cutting away the silane-treated part, and concentration of the CS were carried out. The strip of paper from 1 cm below line X to 1 cm above it was cut out, eluted as described in Section III C(4), and determined fluorimetrically as in Section III D. Recoveries are shown in Table 6, corrected for the mean of three blanks on which cholesterol alone was chromatographed.

TABLE 6.

Steroid applied, μg	μg recovered	% recovery
Cortisol 0.5	0.49	98
Cortisol 0.5	0.49	98
Cortisol 3.0	2.97	99
Cortisol 3.0	3.01	100
Corticosterone 0.5	0.49	98
Corticosterone 0.5	0.48	96
Corticosterone 3.0	2.94	98
Corticosterone 3.0	2.90	97

C. PAPER-CHROMATOGRAPHIC RESOLUTION OF CORTICOSTEROIDS. PREPARATION OF PAPERS FOR QUANTITATIVE CHROMATOGRAPHY.

There are several chromatographic methods for the resolution of CS mixtures, employing columns (Morris and Williams, 1953, 1955; Sweat, 1954b; Haines, 1952) and paper (Burton, Zaffaroni and Keutmann, 1950, 1951; Bush, 1950, 1952; Kritchevsky and Tiselius 1951; Pechet, 1953). The chemical neutrality of CS thus seems to be no bar to their separation by chromatography. Bush (1954b) has reviewed the subject lucidly and thoroughly.

Adsorption and partition methods, both on columns and paper, have been advocated. Adsorption procedures are rather less valuable, being prone to displacement effects and tailing, associated with saturation of a limited adsorbing surface; column partition methods are exceedingly versatile, and it is possible in some cases to alter resolving power almost at will.

Column chromatography, indispensible research weapon though it is, presents technical difficulties which limit its use in routine investigations. It is not easy to pack a column evenly and reproducibly, a variation of 6 - 10 % being claimed by practised chemists; this necessitates the separate determination of numerous fractions, which have often to be taken to dryness first. The cost of automatic fraction collectors is such that the number of analyses which can be carried out at one time is severely limited; fraction cutters,

in one's experience, require careful maintenance.

In contrast, paper strip chromatography has much to recommend it. Techniques of high resolving power and great versatility are available. Methods are relatively simple, and involved apparatus is not required. In distinction to the difficulty of packing columns, paper strips form microcolumns of excellent reproducibility; on papers prepared as described, the Rf values of CS have been found to vary by less than 2 % in successive as well as simultaneous runs with some of the volatile solvent systems of Bush (1952). In addition, a 36 cm ascending run takes only 90 - 100 minutes with these systems. Six or even twelve papers are easily chromatographed at the same time, a burdensome undertaking with column techniques but essential to any method of CS determination to be used routinely on a large scale.

Despite the availability of admirable paper methods for qualitative work, these have not been exploited in true quantitative analysis, although semiquantitative procedures based on spot size and intensity have provided valuable information (Bush, 1952; Bush and Sandberg, 1953; Neher and Wettstein, 1955). The reason is undoubtedly the extremely high blank values encountered in paper work with a variety of methods of determination, notably sulphuric acid fluorimetry (Sweat, 1954a), potassium tert butoxide fluorimetry (Bondy, 1955, personal communication), formazan absorptiometry, spectrophotometry at 240 mμ, and reduction of arsenomolybdate

(Schwarz, 1953).

Investigations were accordingly carried out with a view to preparing papers in a form suitable for quantitative work.

(1) Strips of Whatman no. 4 "paper for chromatography" 1.5 cm wide and 50 cm in length, were cut with the greater dimension in the machine direction of the sheets; they were washed in various ways. After drying, an 8 cm piece was cut from near the top, and another from near the bottom, and eluted with 0.2 ml absolute ethanol (Section III C,(4)). The eluates were compared with cortisol standards, ethanol blanks being employed, by spectrophotometry at 240 mμ, fluorimetry in sulphuric acid (Sweat, 1954a) and blue tetrazolium chloride (BT) reduction (Mader and Buck, 1952).

(2) Washing techniques:

(a) Papers were washed with redistilled absolute ethanol for 72 hours in a Soxhlet apparatus.

(b) Papers were suspended in a large descending chromatography tank and washed by downward syphoning with glacial acetic acid for 24 hours, followed by distilled water until the eluate was free from acid.

(c) Similar to (b), but using normal ethanolic sodium hydroxide (in redistilled 95 % alcohol) for 24 hours, followed by distilled water until the eluate was free from alkali, and lastly 95 % ethanol for three hours.

(d) Washing in running tap water for 24 hours, then

soaking in distilled water for one hour.

TABLE 7

	(a)	(b)	(c)	(d)	unwashed
Spectrophotometry at 240 mμ	17	34	7	42	240
Cortisol 10 μg standard	24	28	9	49	215
Sulphuric acid fluorimetry	10	33	3	31	104
Cortisol 1 μg standard	14	39	5	36	> 110
BT reduction; absorptiometry	8	22	3	27	48
Cortisol 10 μg standard	11	26	3	26	40

Method (c) appeared to reduce blanks to a workable level in fluorimetric work and in formazan absorptiometry. The method was further investigated, three 8-cm segments from each of ten papers being eluted and determined fluorimetrically.

The range of paper blanks was 2 - 10% of the fluorescence produced by 1 μg cortisol, mean 4.5, S.D. 1.3

There was no tendency for the values to be higher in segments taken from the lower parts of the strips (referring to the position of the papers during washing).

In view of the somewhat wide scatter of results, it was decided that a paper blank should be included whenever quantitative chromatography was performed.

Method (c) is to be published shortly. (Lewis, 1956a).

(5) Chromatographic resolution of CS mixtures.

It is clear that no single solvent system is ideal for resolving all CS mixtures; Bush has published seven

(Bush, 1952), Burton, Zafferoni and Keutman (1951), two, and Pechet (1953, 1955), several. Only partition methods are suitable for quantitative work; the use of alumina-impregnated paper for adsorption chromatography requires conversion of CS to their acetates, and is like all adsorption methods, prone to displacement effects due to saturation of the adsorbing surface; slight impurities in the material chromatographed may lead to this.

Zafferoni has found that excellent resolving power is possessed by systems using propylene glycol or formamide as the stationary phase, and his methods have had widespread application. However, the even impregnation of paper with these non-volatile solvents is not easy; the use of a wringer to remove excess stationary phase could easily contaminate papers. The most serious objections are the long running time for polar steroids (24 - 72 hours), and the difficulty in removing these solvents after chromatography, prior to elution of CS; even with cumbersome vacuum apparatus this procedure requires some hours. Rf values for polar CS are not obtainable, as it is necessary to overrun the strips with the solvent.

It is technically difficult to achieve compact spotting on papers previously dipped into the stationary phase, as described by Pechet (1955). His toluene-butanol-petroleum-ethanol-water system was used on five occasions, to resolve mixtures of 10 μg each of cortisol, cortisone, corticosterone

and 11-dehydrocorticosterone. Cortisol and cortisone were adequately separated, but the less polar steroids showed considerable tailing. Nor were Rf values sufficiently consistent, the cortisol figure varying by 10 %.

When application of the stationary solvent in the vapour phase was attempted (24 hours equilibration), all steroids gave diffuse overlapping spots; equilibration and running at 35° C improved results, but the spots were still large. Pechet's method was therefore not adopted.

Reversed-phase partition chromatography (Kritchevsky and Tiselius, 1951) was employed to separate the same steroids. Even application of the silicone oil was achieved by using a solution in cyclohexane. Cortisol and cortisone ran considerably faster than corticosterone and 11-dehydrocorticosterone, but the former pair lay uncomfortably close to one another. In view of experience with siliconed paper (Section III B, 6), it seemed likely that elution with ethanol would remove some of the silicone; it was shown that elution of an 8 x 1.5 cm strip by downward syphoning of 5 ml ethanol left the paper wettable. The possibility of resolving CS on dichlorodimethylsilane-treated paper was not explored.

The volatile-solvent partition systems introduced by Bush were classified into groups suitable for less polar, moderately polar, and very polar steroids. The Rf values published suggested that the CS of peripheral

plasma (Section II) would be separated most widely by members of the second group, the systems B4 and B5, consisting of toluene 1000, methanol 500, water 500 and benzene 1000, methanol 500, water 500 parts by volume, respectively.

Bush made the valuable discovery that tailing with volatile systems could be prevented by running at an elevated temperature (30 - 40 ° c).

The running time with these volatile solvent systems was 90 - 120 minutes, and the initial rate of development with the descending technique was so great that Bush laid his papers over a glass rod in such a way as to keep the first ten centimetres horizontal.

The ascending technique was preferred in the experiments to be described; apparatus was greatly simplified; it was possible to run several strips at once, and in these the rates of development were practically identical. Little supervision was required.

Temperature control was achieved by placing the tanks in a large incubator which was maintained at 30°C. A fan circulated the air in the incubator. The temperature inside a tank varied from 29.7° to 30.2° during a 100 minute run with this apparatus, despite the incubator door having been opened several times to take readings.

Battery jars 45 x 20 x 20 cm, or large rectangular specimen jars 48 x 15 x 8 cm were used as tanks. The lids were made of $\frac{5}{16}$" plate glass, and each had a small central

slit through which the mobile solvent was added down a long thistle funnel after equilibration. An airtight seal for the lids was ensured by using minimal quantities of a paste prepared by mixing 9 parts by weight of soluble starch with 33 of glycerol, and heating with stirring to 140° C.

Stainless steel racks were constructed (Figure 8); the height could be varied, and papers were supported separately by crocodile clips, giving further flexibility of adjustment.

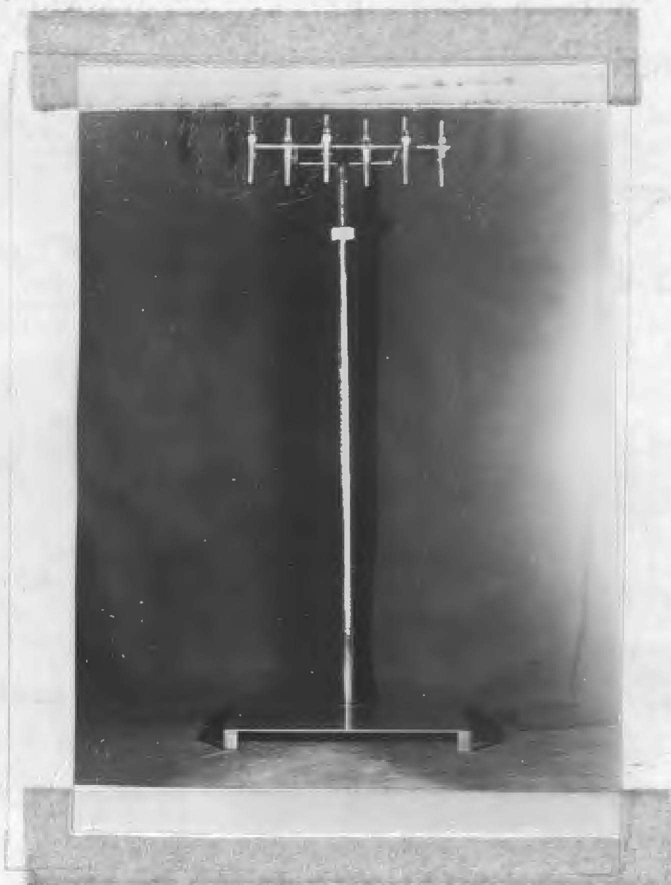


Figure 8. Rack for ascending paper chromatography.

The bottom of the tank was covered with the mobile solvent; two 250 ml beakers in diagonally opposite corners each contained 100 ml of the stationary phase. Volatilization was hastened by scrolls of filter paper in the beakers, and by lining three sides of the tank with the same material. Overnight equilibration was the rule.

The paper strips were cut in the machine direction of the sheets, Whatman no. 4 being used after washing as described in Section III (E). The usual width was 1.5 cm, and a run of 36 cm was usual, taking 90 - 100 minutes.

Mixtures of 10 μg each of cortisol, cortisone, corticosterone and 11-dehydrocorticosterone were spotted in a 5 mm band, 2.5 cm from the lower edge of the strips. They were chromatographed in toluene or benzene/50 % methanol in the apparatus described; after drying the strips they were sprayed with 2 N aqueous sodium hydroxide and dried for 15 minutes in a 90° oven (Bush, 1954c). Bright yellow fluorescence in short-wave ultraviolet light has been shown by Bush to be specific for α-β unsaturated ketones, and to be highly sensitive (0.25 μg/sq cm). Compact spots 2 - 2.5 cm in length were seen, visible tailing being absent.

In connection with amino-acid chromatography on filter paper, the disquieting suggestion has been made that tailing may occur in sufficient degree to invalidate quantitative work, but not be evident with the ninhydrin reaction on paper (Kofranyi, 1955).

To exclude this possibility in steroid work, known amounts of cortisol and corticosterone were chromatographed in the benzene system; the strip was divided into 2 cm sections, and these were eluted (Section III,C,4) and determined by the highly sensitive sulphuric acid-induced fluorescence reaction (Section III, D). The elution diagram (Figure 1, page 16) corrected for the paper blank, shows no evidence of serious tailing, though the corticosterone spot is less compact than the cortisol one.

The relative merits of benzene and toluene as mobile phase were next investigated. Five steroids were used; nine observations were made on each steroid in the course of three separate experiments. The Rf values are shown in Table 8.

TABLE 8

	BENZENE		TOLUENE	
	Present study	Bush (1952)	Present study	Bush (1952)
Cortisol	0.28-0.29	0.32	0.14-0.15	0.15
Aldosterone*	0.39-0.40	-	-	-
Cortisone	0.45-0.47	0.50	0.27-0.28	0.30
Corticosterone	0.77-0.80	0.85	0.68-0.70	0.70
11-dehydro-corticosterone	0.88-0.91	0.96	0.74-0.76	-

(*) Aldosterone was run in the benzene system only, on three occasions.

The discrepancy between these data and those of Bush (1952) may be attributed to:

(a) The lower temperature in the present experiments (30° cf. 34° in Bush's technique).

(b) A difference in the distance of the starting line from the edge of the paper, which has a remarkable influence on the rate of migration, possibly by the mechanism of frontal analysis of the mobile solvent

(c) A difference in the length of run (44 cm in Bush's experiments, 36 cm in the present ones).

(d) Use of the ascending instead of descending technique.

Variations of 3 % were seldom exceeded in the present experiments. Bush reported that errors greater than 5 % were rare.

No displacement effects were observed when 5 μg of each of four steroids were run on the same 1.5 cm wide strip. The same values were obtained when 25 μg quantities were run.

No steroid was detected at the starting point after running the chromatogram.

The rate of ascent of the solvent was remarkably constant in any one experiment.

Both systems give Rf values for cortisol, cortisone, corticosterone and 11-dehydrocorticosterone, which vary by no more than 3 %. The more polar steroids are rather more widely separated by the benzene system; in addition, toluene is known to deteriorate, possibly by an auto-oxidation

mechanism, the product apparently destroying CS. Benzene was therefore adopted for routine chromatography of plasma CS. Chromatographic solvents are prepared freshly at monthly intervals from redistilled reagents.

Aldosterone was shown to run a little slower than cortisone, overlapping slightly.

(4) Elution of CS from paper.

Methods have been described for determining substances, notably amino-acids, by spot area and intensity. Those which depend on visual comparison are at best semi-quantitative, though improved procedures using photo-electric cells, often with some scanning apparatus, have been used for amino-acids and for paper strip electrophoresis of proteins. Nevertheless, none as yet possess high reproducibility; in the present technique CS are eluted before determination. Steroids are highly soluble in all polar organic solvents, but the technique of elution imposed certain problems in extracting the CS quantitatively in a minimal volume of solvent. A method in which the solvent syphons downward through the paper is clearly preferable to one in which the strip is merely soaked in two or more changes of solvent, obviating the tedious operation of concentrating the eluate with the attendant possibility of excessive blank values.

Initial experiments were carried out by spotting 10 - 50 μg cortisol or corticosterone on to an 8 x 1.5 cm strip, and after elution showing any residual CS on the paper

by the NaOH fluorescence reaction. Most CS was removed by methanol or ethanol, but if the solvent were merely allowed to drip from the end of the strip into a small test tube, some fluorescence always persisted at the lower edge of the paper until about 0.5 ml solvent had been used. If the eluate was collected from the tip of the paper in a horizontally mounted opsonic pipette, 0.1 ml methanol would quantitatively elute 50 μg cortisol or corticosterone. Ethanol was slightly less efficient; 0.1 ml eluted 20 μg cortisol completely. (A normal blood sample would contain of the order of 1 μg cortisol).

Sulphuric acid-induced fluorescence was used to determine recoveries more precisely. The steroid has to be dissolved in 0.1 ml ethanol for this reaction; fluorescence was found to be about 25 % weaker when this solvent was replaced by methanol. Cortisol or corticosterone in 4 μg quantities was applied diffusely to 7 x 1.5 cm strips, and eluted with 0.1 ml ethanol. In five such experiments with each steroid, the range of recovery was 99 - 101 %.

To facilitate the handling of numerous samples, the apparatus illustrated in Figure 9 was constructed in stainless steel (Lewis, 1956b). In place of pipettes, 1.5 mm bore glass capillary tubing is used to collect the eluate, one end being bent up at a right angle and flared to receive the tip of the paper strip. They are calibrated at 0.1 and 0.2 ml. Twelve such tubes are attached by clips to short pieces of

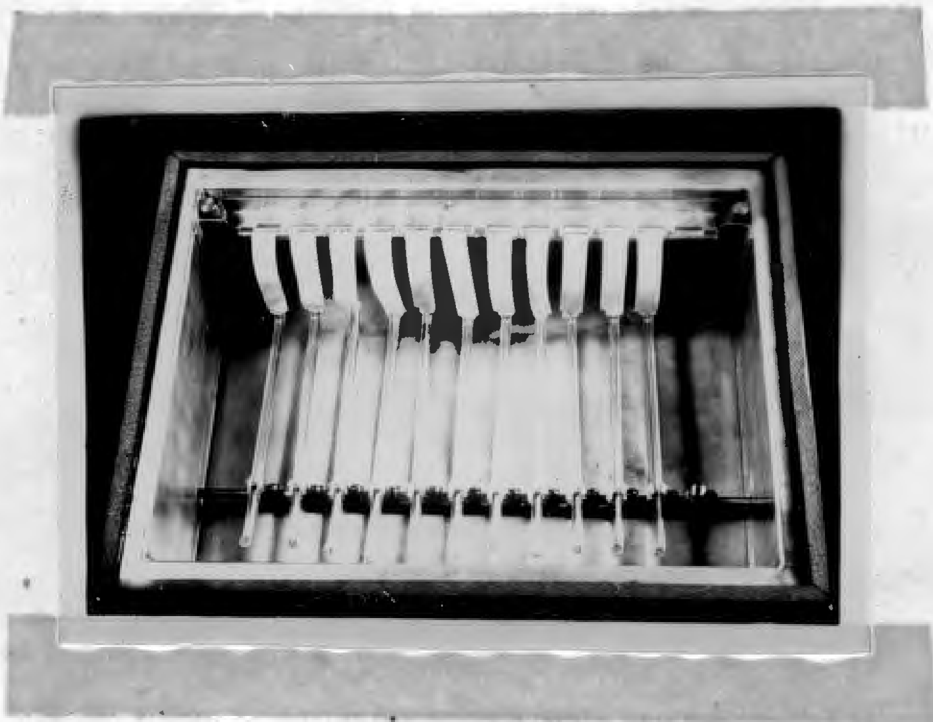


Figure 9. Apparatus for elution of paper chromatograms.

steel tubing, which are mounted in turn on a horizontal steel rod. The slope of each glass tube may be adjusted by slackening a fixing screw which holds the steel tubing to the rod. The tank is covered by a heavy glass lid which rests on a sponge-rubber rim.

The papers hang from a narrow trough, the height of which may be varied by turning the screws on which it is supported at either end. A thin sheet of glass rests diagonally in this trough, projecting 1 cm beyond its edge. The papers are held between this sheet beneath and small rectangles of ¼" plate glass above, the weight of which is sufficient to

hold the papers firmly in place.

The bottom of the tank is covered with ethanol, 10 ml of the solvent is added to the trough, and the lid placed in position. The ethanol rises by surface tension between the glass plates, and syphons down the papers into the capillary tubes. Elution with 0.1 ml requires 30 - 40 minutes; the rate does not vary from strip to strip.

D. DETERMINATION OF CORTICOSTEROIDS.

Several microchemical procedures are now available, and have been reviewed in the introduction. The most sensitive appeared to be (i) the colorimetric determination of the diformazan formed by reduction of blue tetrazolium by the α-ketol "side chain" of CS; (ii) fluorimetry in sulphuric acid; (iii) fluorimetry, the fluorescence being induced by potassium _tert_ butoxide. At the time when these experiments were being carried out, the formazan colorimetric methods published were less sensitive than the fluorimetric ones, but the former have been improved considerably and are currently being reinvestigated in this laboratory. In comparing the two fluorimetric techniques, attention was paid to simplicity; the sulphuric acid method is delightfully easy to use, though the alkaline procedure is not particularly involved; but the latter, in our experience, suffers from the drawback that potassium _tert_ butoxide is not readily prepared in sufficiently pure form to keep blank values down to a workable level. If the _tert_-butanol is redistilled on an efficient column

immediately before use, after having been dried by refluxing with magnesium and iodine followed by potassium metal, the reagent is satisfactory and the calibration curve is rectilinear between 0.025 and 10 μg cortisone; but within a week the sensitivity is grossly diminished by increasing blank values. This was found to be the case with batches of *tert*-butanol from two manufacturers.

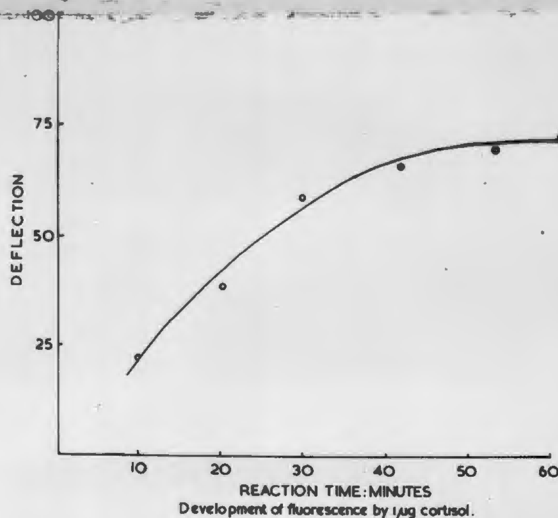
The *tert*-butanol method is apparently specific for α-β unsaturated ketones, into which category all CS fall; paper blank values are low. The sulphuric acid method gives strong fluorescence with only a few steroids, though several others show weak activity; paper blanks are not as low as with *tert*-butoxide, but are sufficiently reduced by the washing procedure described. The method is of no use in determining aldosterone or cortisone; strong fluorescence is given by cortisol, corticosterone and their epimers, oestradiol and compound E of Reichstein (Sweat, 1954 a).

The Farrand fluorimeter used by Sweat was not available; initial experiments were performed using the Hilger-Spekker fluorimeter, and employing 0.5 ml rectangular glass microcells.

(i) To 1.0 μg cortisol, dissolved in 0.05 ml absolute ethanol, was added rapidly 0.4 ml concentrated sulphuric acid; the mixture was placed in one fluorimetry cell, and a solvent blank (0.05 ml ethanol with 0.4 ml sulphuric acid) was added to a second, matched cell. Readings were taken at 10 minute

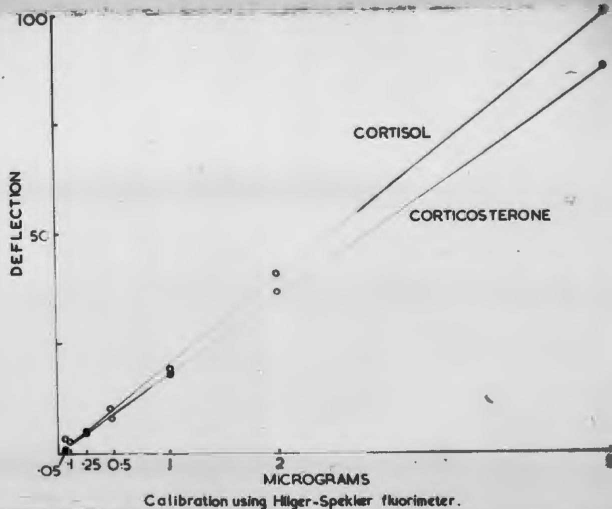
intervals for one hour. The experiment was repeated using 0.1 μg cortisol, and using 1.0 μg corticosterone. Room temperature was 21° (Figure 10). The readings increased rapidly up to 40 minutes after mixing, then remained approximately constant for the duration of the experiment. In subsequent measurements, readings were accordingly taken precisely 40 minutes after mixing. In Sweet's experience, using larger volumes, the rate of increase after 20 minutes was negligible.

Figure 10.



Development of fluorescence by 1 μg cortisol.

Figure 11.



Calibration using Hilger-Spekker fluorimeter.

(ii) The effect of temperature on the degree and rate of development of fluorescence was next studied. Ethanolic cortisol in 1 μg quantities was mixed with sulphuric acid in 4 x 1 cm test tubes and kept at 0, 21, 40 and 80°. Blanks were similarly prepared. The highest reading after 40 minutes followed incubation at 40°. (Table 9). Further investigation showed that this reading was attained after 20 minutes at this temperature. The difference on comparison with the room temperature experiment was not great enough to justify complicating the technique.

TABLE 9

Temperature °C	Reading
0	58
21	70
40	83
80	45

(iii) No fluorescence was recorded when cortisol was mixed with hydrochloric acid; phosphoric acid induced fluorescence, about 20% of that obtained with sulphuric acid.

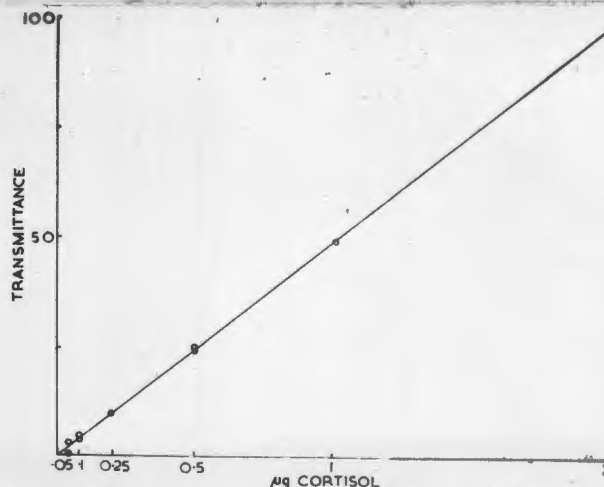
(iv) The use of other alcohols than ethanol was attempted, particularly as elution from paper is more effectively carried out with methanol than ethanol. Cortisol 1 μg was dissolved in 0.05 ml of the alcohol.

	Reading
Methanol	37
Ethanol	50
n-Butanol	10

Butanol was subsequently found to produce high blank values unless rigorously purified. Ethanol was the most suitable solvent.

(v) It was found more satisfactory to set the drum to 100 % transmission and record the galvanometer deflection than to null the apparatus by rotating the drum; more reproducible results were the rule; readings were more rapidly obtained, but slight wandering of the galvonometer was the rule.

(vi) Using the volumes referred to, and taking galvanometer readings 40 minutes after mixing, a calibration curve was prepared. (Figure 11). Duplicate samples were used; good reproducibility was evident between 0.1 and 5 μg cortisol, the graph being rectilinear in this range.



Lower range of calibration curve for cortisol, using the Beckman fluorimeter

Calibration graph for Beckman fluorimeter (see p. 65).

Subsequently a Beckman DU spectrophotometer became available(+). It was fitted with a photomultiplier, and with the Beckman fluorimetry attachment which uses 4 x 1 x 1 cm Corex cells. Unlike the Farrand and Hilger-Spekker instruments, which employ mercury arc lamps, the Beckman uses a tungsten filament; the latter emits a continuous spectrum and was found by Sweat to be less effective than the mercury arc in exciting fluorescence. This was surprising, for his previous studies had shown maximum fluorescence to result from an excitatory beam at 475 mμ, yet the mercury lamp emits most strongly at 436 mμ; he attributed the discrepancy to the fact that the intensity of the 436 mμ line was so great that its distance from the optimal wavelength was relatively unimportant.

(vii) In calibrating the Beckman instrument a Corning 5113 primary filter was used; it is characterised by high transmission between 425 and 470 mμ. The secondary filter was Wratten gelatin 61, transmitting mainly between 530 and 560 mμ. The tungsten lamp and the instrument itself were permitted to stabilise for two hours before taking readings. A photomultiplier setting of 3 was suitable; the zero suppression control was set at the off position. To the steroid, dissolved in 0.1 ml redistilled absolute ethanol, 3 ml 'Analar' concentrated sulphuric acid were added rapidly; the mixture was stirred with a glass rod, and shortly before taking the

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(+) One acknowledges with gratitude the generous assistance of Professor J.F. Brock and Dr W.P.U. Jackson.

reading was decanted into a 4 cm Corex cell. The reading was taken (against an ethanol-sulphuric acid blank) at 40 minutes.

With the solvent blank in the light path and the transmission scale at zero, the galvanometer was nulled with the dark current adjustment; blank fluorescence was always low). The strongest solution was next placed in the light path, the transmission set at 100, and the instrument again nulled with the sensitivity control. These operations were then repeated, as the dark current had to be adjusted slightly at different sensitivity settings. The blank and standard solutions were always plased in the same cells. A correction factor was obtained for the remaining cells at 5 μg and 0.1 μg amounts of cortisol; this factor varied from 0.99 to 1.015. As the correction for a given cell was similar at high and low cortisol levels, a mean figure was derived for subsequent use.

The calibration graph was rectilinear between 0.1 μg and 10 μg cortisol or corticosterone, with identity of most duplicate readings above 0.1 μg.

For routine use of this method, a 2 μg cortisol standard is used; 0.5 μg quantities are used if low values are anticipated, and 5 μg rarely, if massive concentrations are expected.

The sensitivity of this modification is similar to that of the original method of Sweat, in which reproducibility was good at 0.05 μg and a rectilinear calibration was obtained

between 0.05 and 5 μg cortisol. It is important to note that the larger cell size does not materially impair sensitivity. The present technique uses 5 ml sulphuric acid; Sweat added 1 ml. There is no analogy with absorptiometry; in fluorimetry the number of molecules illuminated is the significant factor, not their concentration. Provided that the entire contents of the cell are exposed to the light source and that the photocell aperture is not smaller than the appropriate face of the cuvette, cell size should not exert a great effect on sensitivity. On the other hand, quenching of fluorescence at relatively high steroid concentrations is of importance; above 6.5 μg cortisol per ml ethanolic sulphuric acid, in Sweat's investigations, the calibration graph deviates from linearity due to self-absorption of the fluorescence.

(viii) Aldosterone, a potent mineralocorticosteroid, has been demonstrated in adrenal venous, and in peripheral blood. In the latter its concentration is about $\frac{1}{50}$ of that of cortisol. Chemically it differs from corticosterone in that the methyl group at 18 is oxidised to an aldehyde; (in solution this group and the 'side chain' at 17 undergo ring closure to form a hemi-acetal).

Aldosterone 2 μg gave a reading in the sulphuric acid-induced fluorescence method 6% of that of 2 μg cortisol, excluding the possibility of determining it by this procedure.

X. TECHNIQUE ADOPTED FOR PLASMA CORTICOSTEROIDS DETERMINATION.

On the basis of the foregoing experiments in Sections II and III, the technique finally adopted was as follows:

Reagents and apparatus.

Organic solvents are distilled monthly, with collection of the middle fraction.

Solvent systems for chromatography are prepared at monthly intervals.

Ethanol for fluorimetry is prepared from 95 % spirit by refluxing over quicklime before distillation.

Beckman DU spectrophotometer with fluorimetry attachment, photomultiplier, primary filter Corning 5113, secondary filter Wratten gelatin 61.

Mechanical shaker of rocking type (Figure 4, page 31).

Micro-separating funnels (Figure 4, page 31).

Distilling flasks (Figure 4, page 31).

Preparation of paper for chromatography: Whatman no. 4 "paper for chromatography" was cut into 50 x 1.5 cm strips, the greater dimension being in the machine direction of the paper. The strips were washed in large chromatography tanks by downward syphoning of the following:

(i) 2 N-sodium hydroxide in 95 % ethanol, for 24 hours.

(ii) Distilled water, until eluate is neutral to litmus.

(iii) 95 % ethanol for 5 hours.

The papers are dried; the lower 5 cm are exposed for two

hours to the vapour of dichlorodimethylsilane at atmospheric pressure, in the apparatus shown in Figure 6, page 40. The papers may then be stored for at least 3 months in an air-tight container; blank values do not change during this time.

Procedure.

Heparinised blood is centrifuged within 15 - 30 minutes of being drawn. A plasma sample of 5 - 10 ml is usually required, but a larger or smaller volume, (2 - 20 ml), may be used if a great deviation from the normal figure is anticipated. No loss of CS was detected in a sterile sample of plasma stored at 0°, aliquots of which were estimated weekly for one month.

The plasma is extracted three times, for periods of 10 minutes, with ethylene dichloride, using two volumes for each extraction. Brief centrifugation is needed to break the emulsion completely. The use of a mechanical shaker is preferable, to ensure uniformity of handling and to avoid over-violent agitation which may produce relatively stable emulsions. A shake rate of 80 per minute is suitable. Extraction may be carried out either in 50 ml centrifuge tubes with polyethylene or glass stoppers, or in small separating funnels.

The combined extract is washed by extraction with 1 - 2 ml normal aqueous sodium hydroxide, then with the same volume of 2% acetic acid. The aqueous layer is carefully removed. (Drying with anhydrous sodium sulphate is unnecessary; water has a negligible solubility in ethylene

dichloride.

The extract is taken to dryness under reduced pressure at 35 - 40°. Two flasks are conveniently connected to a single water pump, which maintains a pressure of 2 - 4 cm mercury. A generous stream of air through the bleed is helpful, and does not oxidise CS under the conditions of the experiment; it is unnecessary to use a stream of nitrogen. Two flasks may be taken to dryness in 10 - 12 minutes.

Ethylene dichloride does not splash excessively, as acetone or ethyl acetate are wont to; but it is essential to wash down the flask and air bleed into the well with the utmost care. This is conveniently done with 1 ml ether, delivered from a tuberculin syringe through a fine bent needle; after evaporation of the ether, the procedure is repeated.

The residue is taken up quantitatively in two 0.1 ml volumes acetone. It is applied to a 1 cm band in the centre of the silicone-treated part of the paper strip (Figure 6 b, page 40). A stream of nitrogen ensures compact spotting.

A short ascending chromatogram is now run (R1), using 85 % methanol. A paper blank is included at this stage. Thirty minutes equilibration in the tank is sufficient. The solvent front is allowed to reach a point 2 cm above the siliconed region (line X in Figure 2). The 4 - 5 cm run takes about 5 minutes. The strip is then dried at room temperature (15 - 20 minutes); the siliconed part of the paper is then cut away.

CS are now concentrated on line X by slowly pipetting on to the lower edge of the hanging paper a solvent of composition ethylene dichloride 45, ethyl acetate 45, methanol 10. This is allowed to dry, and the manoeuvre repeated. Concentration of the CS fraction requires about 1 minute per strip.

The CS are then resolved by ascending chromatography in benzene/50 % methanol. Details of technique are described on page 50. A reference strip is included. With a 36 cm run, the cortisol spot is centred about 10 cm from the start, and corticosterone about 27 cm from the start. The sections eluted are therefore from the 6th to the 13th and from the 22nd to the 29th cm respectively (Figure 12); a reference strip

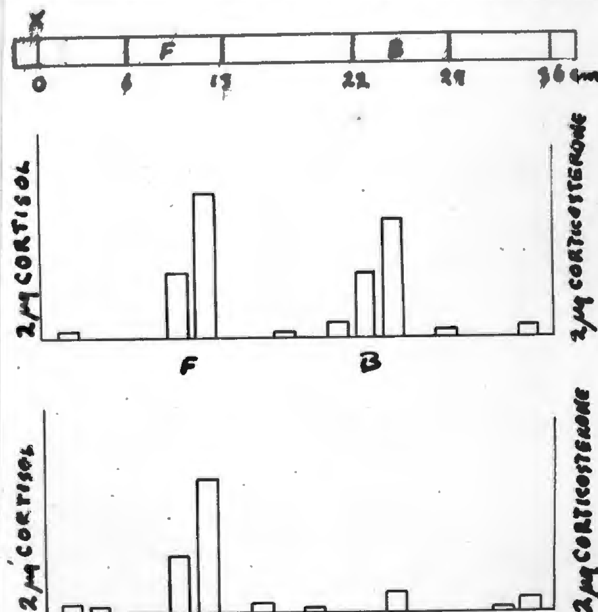


Figure 12.

Bands eluted for cortisol and corticosterone. Elution diagrams for pure steroid mixture (centre) and for plasma CS extract (bottom).

provides a worthwhile check, at least when using a new batch of paper or freshly-prepared solvents.

The papers are eluted with 0.1 ml ethanol, by allowing the solvent to syphon down the papers from which it is collected in glass capillaries (Figure 9, page 57). The eluates are transferred to 8 cm conical centrifuge tubes, and 3 ml concentrated sulphuric acid added to each at timed intervals. Fluorimetry is performed after 40 minutes. A solvent blank and standards of 2 µg cortisol and corticosterone are included, the paper blank value is determined and subtracted from the unknowns.

Viewing this procedure in the light of qualitative studies described in Section II, it may be stated that no CS other than cortisol and corticosterone would be expected in the regions eluted in amounts which would interfere materially with results. The pooled samples analysed contained cortisone, aldosterone and probably tetrahydrocortisone. Only aldosterone would lie (partly) in an eluted area, but like cortisone it does not fluoresce strongly in sulphuric acid.

F. <u>RECOVERIES</u>.

Data were obtained by estimating duplicate 10 ml samples of normal plasma; to one of each pair was added 0.5 or 2 µg of the CS studied. Cortisol recovery was determined twenty times, and corticosterone on ten occasions. For the former the range was 86-96 %, mean 92 %; the corticosterone recovery varied from 82-96 %, mean 87 %. The detailed results

appear in Table 10 and 11.

TABLE 10

CORTISOL

Normal	Added	Total	Recovery	
μg/10ml	μg	μg/10ml	%	
0.81	0.5	1.28	94	
1.14	0.5	1.60	92	
0.89	0.5	1.33	88	
0.72	0.5	1.17	90	
1.00	0.5	1.48	96	
1.05	0.5	1.51	92	
0.76	0.5	1.20	88	
0.68	0.5	1.34	92	
1.20	0.5	1.67	94	
0.93	0.5	1.41	96	
1.01	2.0	2.77	88	
0.78	2.0	2.66	94	
0.71	2.0	2.59	94	
0.96	2.0	2.68	86	
1.12	2.0	2.98	93	
0.84	2.0	2.73	94	
0.66	2.0	2.48	91	
0.89	2.0	2.67	89	
1.01	2.0	2.81	90	
1.12	2.0	2.98	93	Mean 92

TABLE 11

CORTICOSTERONE

Normal	Added	Total	Recovery	
μg/10ml	μg	μg/10ml	%	
0.03	0.5	0.46	86	
0.33	0.5	0.79	92	
0.00	0.5	0.41	82	
0.41	0.5	0.85	88	
0.00	0.5	0.42	84	
0.06	2.0	1.98	96	
0.01	2.0	1.67	83	
0.14	2.0	1.82	84	
0.22	2.0	2.00	89	
0.02	2.0	1.72	85	Mean 87

The values compare favourably with those obtained by other techniques for plasma CS determination. The Nelson and Samuels procedure for 17-hydroxycorticosteroids (1952) recovers 55 - 132 % of added cortisone; the Bayliss and Steinbeck modification (1953) is less variable (68 - 93 % of cortisol). In the technique of Morris and Williams (1953) 80 - 91 % of added cortisol, and slightly lower quantities of less polar steroids are recovered. Sweat (1955) does not report recovery data.

G. NORMAL VALUES.

These were determined on samples of venous blood from 30 apparently normal subjects between 15 and 42 years of age of whom 20 were males. The females were not menstruating at the time of drawing the blood. The subjects included students, laboratory assistants, hospital orderlies and medical practitioners. The possibility of racial variation was not investigated, all subjects in the series being of European stock.

Diurnal variation of plasma and urine 17-OHCS levels is known to occur, with maximal plasma levels at about 6 a.m. To minimise the effect of this variation, samples in this series of normal were drawn during the late morning, 10 a.m. to 12 noon; this policy was adhered to, as far as possible, throughout the present investigation.

The range for cortisol was 6 - 12 μg /100 ml plasma, mean 9.2, S.D. 1.5. Thus 95 % of the population studied

would be expected to have levels between 6.2 and 12.8 μg / 100 ml; this calculated range is in good agreement with the observed limits. Corticosterone levels varied from 0 - 6 μg/100 ml, mean 1.2; this steroid was frequently not detected. The cortisol/corticosterone ratio was therefore about 8.

In addition duplicate determinations of cortisol on twenty random samples of plasma, both normal and abnormal were made. The range was 7.6 - 29 μg/100 ml; the difference between duplicates varied from 0.0 to 2.2. At a probability level of 0.05, a difference of 2.7 is significant; at the level 0.02, the least significant difference is 3.7.

In the male subjects the mean for cortisol was 8.9, and in the ten females 9.9 μg /100 ml. There is no published evidence of a sex difference in CS levels (referring to non-pregnant, non-menstruating females).

In elderly individuals similar figures are obtained; five subjects between 60 and 79 years of age had cortisol and corticosterone levels in the range stated, the means being 8.1 and 1.6 respectively.

Two 24-hour infants had cortisol levels of 4.4 and 6.0 μg /100 ml, but the same subjects at birth had had 8 and 15.6 μg /100 ml of cord plasma. The latter figures probably reflect transfer of CS across the placenta, for women in labour have markedly elevated plasma CS concentrations.

SUMMARY.

An analysis of methods of extracting CS from plasma is presented; quantitative data for various solvents are given. A simple reversed-phase chromatogram for purifying CS extracts has been developed. Paper chromatography has been used to resolve the plasma CS prior to determination; this advance in technique depended on finding a method for reducing the high blank values hitherto found in paper work. Cortisol and corticosterone are determined by fluorimetry in sulphuric acid. The full technique for plasma CS determination is described. Normal values and recovery data are presented. The latter compare favourably with those of published methods.

SECTION IV

CORTICOSTEROID CONJUGATES IN PLASMA

The level of cortisol in the blood stream represents a dynamic balance between the rate of release of this hormone from the adrenal cortex and its uptake, excretion or degradation by the tissues. The metabolism of cortisol is by no means fully understood, but reduction of ring A to give tetrahydrocortisol, "side-chain" degradation - partly to 17-ketosteroid, and 11-oxidation to cortisone derivatives are of importance; reduction at C20 gives rise to inactive steroids with a glycol side-chain; these have been isolated from urine; liver slices have been shown to perform some of these reactions in vitro. Conjugation at the 21-position with water-soluble substances, notably glucuronic acid and sulphuric acid, precedes renal excretion of all but a trace of the steroid hormones, forming a glycoside in the former case and an ester sulphate in the latter; (phenolic steroids give rise to ethereal sulphates). Recently it has been shown that the very rapid disappearance of intravenously-administered cortisol from the circulation is at least partly explained by tissue binding; the effect of temperature on this process indicates a physical rather than a biochemical reaction. Far less is taken up by depot fat than by protein-containing tissues, suggesting that protein binding rather than solubility in body fat is the underlying mechanism.

It is well known that the yield of CS and their derivatives from urine is greatly increased if extraction is preceded by hydrolysis, using either mineral acid or β-glucuronidase. Recently, similar findings have been published for plasma; acid hydrolysis doubles the yield of Porter-Silber chromogens (mainly cortisol and tetrahydrocortisone), and β-glucuronidase hydrolysis has similar results. In addition, oral administration of tetrahydrocortisone was followed by the appearance of massive amounts (2000 - 3000 μg/100 ml) of β-glucuronidase-hydrolysable Porter-Silber chromogen (Bongiovanni and Eberlein, 1955b).

Determinations on unconjugated plasma CS in Cushing's syndrome have on occasion given normal results (Mason, 1955).

It is therefore of major importance to know whether the adrenal secretory rate is satisfactorily indicated by the level of unconjugated CS in plasma, or whether a more reliable estimate is obtained by determining the total (free and conjugated) CS, including the major degradation product tetrahydrocortisone.

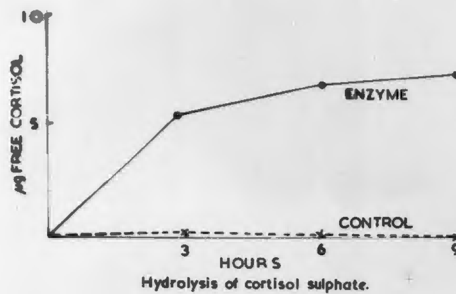
The plan of the experiment was to separate free cortisol from the various conjugates by extraction alternating with serial β-glucuronidase, sulphatase and acid hydrolysis, prior to fluorimetric determination of this steroid. The method of Bayliss and Steinbeck (1955b) was employed to determine 17-hydroxycorticosteroid (mainly cortisol and tetrahydrocortisone), in similarly treated plasma. These analyses

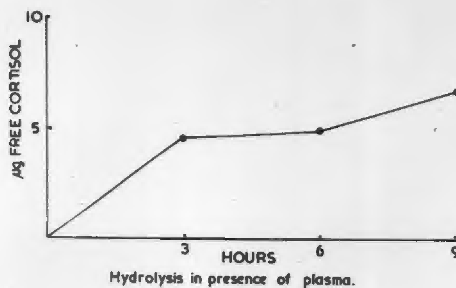
were made on normal plasma, and on samples from individuals receiving ACTH or cortisol.

A. PREPARATION OF A STEROID ESTER SULPHATASE.

Both alkyl and aryl steroid sulphates are found in urine. The possibility of finding CS sulphate in plasma had therefore to be investigated. Roy (1954) and Savard, Bagnoli and Dorfman (1954) have demonstrated sulphatase activity in extracts of the viscera of certain molluscs. There are evidently several sulphatases of high specificity present in crude extracts, some splitting aryl and others alkyl sulphates; in addition, β-glucuronidase activity may be present. Roy has worked with Patella vulgata, a limpet, and Savard with an African land snail Otala punctata.

Neither species is available in the vicinity of Cape Town, but a fairly closely related limpet P. granularis is abundant. An acetone powder was prepared from the visceral hump of this mollusc, and 100 g quantities extracted with 50 ml 0.1 M aqueous potassium chloride. Further purification such as that carried out by Roy was not pursued; the filtered extract was used immediately. Roy's steroid sulphatase had an optimum pH of 4.5, with a flat peak, and was inhibited by phosphate and sulphate. The characteristics of the P-granularis preparation were not investigated; 13 μg of cortisol 21-sulphate (equivalent to 10 μg cortisol) in 5 ml 0.5 M acetate buffer at pH 4.5 was mixed with 5 ml of the extract and incubated at 37°; an aliquot withdrawn at 3-hourly





<u>Figure 13.</u> (Above). Release of cortisol from 15 μg cortisol 21-sulphate by extract from P. granularia.

<u>Figure 14.</u> (Below). Same reaction in presence of plasma, showing lack of significant inhibition of steroid sulphatase.

intervals for cortisol determination (Figure 13).

To a 5 ml sample of plasma was added 0.1 ml 2% ammonium molybdate. The supernatant after centrifugation was extracted once with 15 ml ethylene dichloride. The plasma residue, in the light of subsequent experiments, contained of the order of 0.05 μg cortisol conjugated with sulphuric and glucuronic acid. The plasma was mixed with 15 μg cortisol 21-sulphate, 5 ml enzyme preparation and sufficient 0.5 M acetate buffer to bring the pH to 4.5. The mixture was incubated at 37°, and aliquots withdrawn as before. Cortisol release is shown in Figure 14, and is not materially retarded by the presence of the plasma preparation.

The limpet preparation thus contained an enzyme capable of hydrolysing cortisol 21-sulphate. It was decided that in subsequent experiments an attempt would be made to improve the yield by extracting cortisol from the reaction mixture after hydrolysis, then re-incubating with additional enzyme.

B. STEROID CONJUGATES IN PERIPHERAL PLASMA.

The nature and relative quantities of steroid conjugates were determined as follows: 20 ml normal plasma was extracted exhaustively, with 5 x 2 volumes ethylene dichloride; the free cortisol content was determined. The pH of the plasma was then adjusted to 4.5 with 0.5 M acetate buffer; β-glucuronidase (Warner-Chilcott Laboratories) was added to a final concentration of 1000 units/ml. Twelve hours

incubation at 37° followed, after which extraction with 3 x 2 volumes ethylene dichloride was performed. A further 1000 units/ml of the enzyme were added, incubation and extraction repeated. The extracts were separately estimated for cortisol.

To the aqueous layer 0.2 ml ammonium molybdate was added, and after centrifugation the supernatant was mixed with 10 ml of the P. granularis extract. The pH was re-adjusted to 4.5, and the plasma incubated for 12 hours at 37° before extraction with 3 x 2 volumes ethylene dichloride. Ten ml more of the sulphatase preparation were added, incubation and extraction being repeated; the extracts were again separately estimated.

Finally acid hydrolysis was performed as described by Klein, Papadatos, Fortunato and Byers (1955); an equal volume of 37 % hydrochloric acid was added to the plasma, and after 15 minutes shaking at room temperature, three ethylene dichloride extractions were performed.

A duplicate sample of plasma served as control, being treated with enzyme preparations inactivated by boiling, and extracted similarly.

It was unfortunate that an intravenous preparation of unesterified cortisol ("hydrocortisone free alcohol") was not available; it has been shown in Section V that oral cortisol free alcohol appears in the blood stream within one hour if the subject has fasted previously. This mode of administration was chosen in following the effect of exogenous cortisol

on free and conjugated plasma cortisol and its Porter-Silber-reactive metabolites.

A dose of 120 mg was given to a normal 22-year old male; at zero time, and 4 and 8 hours later, blood samples were drawn. These were worked up largely as described above; the steroid released by the two glucuronidase hydrolyses was pooled before estimation, and the same was done with the products of the sulphatase hydrolyses. No control was run. In addition, most extracts underwent the Bayliss and Steinbeck procedure for 17-hydroxycorticosteroids, with the modification that an ethylene dichloride extract was used in place of a chloroform one.

The effect of a 4-hour ACTH infusion at 10 units per hour was studied similarly; the venepunctures were timed from the beginning of the infusion.

Results.

The levels of free and conjugated cortisol and 17-hydroxycorticosteroids under basal conditions, after cortisol administration, and after ACTH stimulation, are shown in Tables 12, 13 and 14.

C. DISCUSSION.

Under basal conditions, the total cortisol level was about double the figure for the free steroid. Similar figures have been published for β-glucuronidase hydrolysis by Bongiovanni and Eberlein (1955b) and for acid hydrolysis by Klein et al (1955). In all determinations glucuronic acid was the

T A B L E 12.

CORTISOL CONJUGATES IN NORMAL PLASMA

	Cortisol μg/100 ml plasma	
	Experimental	Control (inactivated enzymes)
Ethylene dichloride - extractable	10.3	11.2
β-glucuronidase hydrolysis (i)	4.4	0
β-glucuronidase hydrolysis (ii)	0.6	0.2
Sulphatase hydrolysis (i)	2.3	0.5
Sulphatase hydrolysis (ii)	1.0	0
Acid hydrolysis	3.6	6.0
Total	22.2	17.9

T A B L E 13.

CORTISOL, ITS CONJUGATES AND METABOLITES AFTER ADMINISTRATION OF CORTISOL

	Cortisol μg/100 ml plasma			17-OHCS μg/100 ml plasma		
	Basal	+4 hrs.	+8 hrs.	Basal	+4 hrs.	+8 hrs.
Free	7.1	42	16	12.4	30	22
Glucuronidate	2.4	9.4	7.7	10.5	42	53
Sulphate	1.7	4.4	5.0	4.8	8.7	9.8
Acid-hydrolysable	1.1	2.9	2.4	1.5	3.1	3.9
Total	12.3	58.7	31.1	29.2	83.8	88.7

TABLE 14

EFFECT OF ACTH ON CORTISOL, ITS CONJUGATES AND METABOLITES

	Cortisol µg/100 ml plasma			17-OHCS µg/100 ml plasma		
	Basal	+4 hrs.	+8 hrs.	Basal	+4 hrs.	+8 hrs.
Free	9.2	37	25	11	29	32
Glucuronidate	4.2	8.1	7.0	14	52	75
Sulphate	2.9	2.1	3.0	3.3	4.1	7.8
Acid-hydrolysable	0.6	0.9	0.6	1.7	2.6	3.4
Total	16.9	48.1	35.6	30.0	87.7	118.2

predominant conjugating group. The yield after incubation with P. granularis extract was evidently due, not to its glucuronidase content, but to splitting of ester sulphate. Sulphate esters remain at low concentrations in all the experiments performed, and there would appear to be no justification for their determination in the measurement of adrenal function.

A relatively small fraction of circulating cortisol is conjugated, contrasting with the large proportion of 17-OHCS combined in this way. This discrepancy is most pronounced after ACTH or cortisol have been given.

Bayliss and Steinbeck (1953a) infer from paper-chromatographic studies that with their method of determination of 17-hydroxycorticosteroids (17-OHCS), tetrahydrocortisone

as well as cortisol is determined. The former is almost certainly a metabolic product of the latter. Unfortunately the 20-hydroxyl metabolites of CS are not determined. The rise in 17-OHCS after cortisol or ACTH is less dramatic than that of free cortisol, which appears to be the most sensitive index of immediate changes in the rate of addition of cortisol to the blood; a rise from 7.1 μg/100 ml to 42, within 4 hours of giving a moderate dose of cortisol by mouth, is as striking a response as one could wish to see. The only comparable increase was of glucuronidate-conjugated 17-OHCS, from 10.5 to 55 μg/100 ml in 8 hours, following ACTH.

The 17-OHCS peak after cortisol or ACTH occurs later than the cortisol peak, this applying particularly to total concentrations. This is in agreement with the contention of Bayliss and Steinbeck that their method partly determines cortisol degradation products.

The 17-OHCS conjugate peak appears still later than the free 17-OHCS maximum. It would seem that conjugation is preceded by reduction of cortisol to tetrahydrocortisone.

Bongiovanni and Eberlein (1955b) have suggested that CS secretion might best be measured by the total Porter-Silber chromogen after β-glucuronidase hydrolysis, and have shown that tetrahydrocortisone administration is followed by its conjugation with glucuronic acid; enormous concentrations of the glycoside were found in plasma. However, the present experiments do not support their contention, for the rise of free

cortisol was greater (and easier to determine), than that of conjugated tetrahydrocortisone; the conclusion is of course provisional only, for only simultaneous measurements in a variety of endocrine and non-endocrine diseases will resolve the difficulty.

One circumstance can be envisaged where total (free plus conjugated) 17-OHCS determination would be superior to free cortisol determination: in severe liver disease the ratio of free to conjugated CS rises (Klein, Papadatos, Fortunato, Byers and Puntereri, 1955); the rate of adrenal secretion would appear high on determination of free cortisol, on account of its tardy clearance from the circulation. The relative merits of the two tests would depend on whether one were primarily interested in the behaviour of the adrenal in such a patient, or in the extent to which his tissues were under the influence of circulating, active cortical hormones. That this influence may be excessive in liver disease was shown by Bongiovanni himself, when he described the association of cirrhosis with signs of Cushing's syndrome.

The nature of the acid-hydrolysable steroid conjugates persisting after serial enzymic hydrolysis is obscure. If experimental error is excluded, CS must combine to a small extent with substances other than glucuronic and sulphuric acids. This is believed to be the case with urinary steroids. The point is probably of academic importance only, for acid hydrolysis is known to destroy some 50% of 17-OHCS (Klein, Papadatos, Fortunato and Byers, 1955) in plasma and is accordingly unlikely to be adopted

as a routine procedure in determining adrenal function.

SUMMARY.

Simultaneous determinations have been made of plasma cortisol and 17-hydroxycorticosteroids, the latter including the cortisol degradation product tetrahydrocortisone. Figures are given for free steroids, glucuronosides, sulphates and residual acid-hydrolysable conjugates which are mentioned in decreasing order of abundance (under basal conditions).

About half the plasma cortisol is free under basal conditions; ACTH stimulation or cortisol administration elevates the free cortisol markedly and rapidly. Subsequently the cortisol conjugates, notably the glucuronoside, increase.

The 17-OHCS are largely present as conjugates, and the proportion of conjugated to free 17-OHCS rises after cortisol or ACTH. Free 17-OHCS show a fairly considerable rise, though slower than that of free cortisol; but a prominent late rise in tetrahydrocortisone glucuronoside is evident.

The order in which ACTH and cortisol affect these measurements suggest that cortisol is metabolised to tetrahydrocortisone before conjugation takes place.

SECTION V

VARIATIONS IN PLASMA CORTISOL

In this section observations are reported on some variations in plasma cortisol levels which may occur under physiological and pathological conditions. The extent to which the level rises during maximal adrenocortical stimulation, and after administration of a single large dose of cortisol, were first determined. By comparison, the fluctuations in health have been observed in the following experiments to be rather small (except in the late stages of pregnancy); it appears that the secretion of cortisol is less labile than has been suggested.

Until recently, physiologists were compelled to infer the degree of adrenal activity from indices several degrees removed from the actual rate of CS release from the gland. For example, protein catabolism and negative potassium balance are common consequences of injury, and have been ascribed to adrenal hypersecretion (Selye, 1946). Histological studies on adrenal glands have supported this concept. However, Ingle (1951) and Engel (1951) have observed these responses in adrenalectomised animals maintained on constant doses of cortisone; Ingle has coined the term "permissive action" of cortisone to describe, but not explain this phenomenon. Similar observations have been made in humans, (Rosenbaum, Popper and Ashley, 1955, Mason, 1955, Wilkinson, 1956). Eosinopenic responses have

been noted even in adrenalectomised animals maintained on sodium chloride.

There is no doubt that a variety of factors may temporarily alter adrenal activity, but it appears that this has on the whole been over-emphasized, and that in health the plasma cortisol at least is relatively stable. This greatly increases the clinical value of this determination.

A. CORTISOL ADMINISTRATION.

Following an oral dose of 120 mg of cortisol "free alcohol" to a normal subject 4 hours after a meal, the plasma level of this steroid rises within 1 - 2 hours, reaches a maximum in 6 hours and declines to a near-normal value at 9 hours. (Figure 15). The rise is delayed if the administered

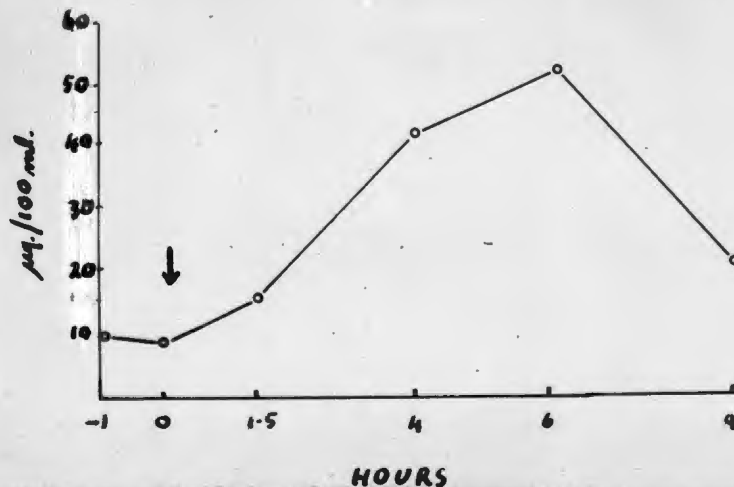


Figure 15. Rapid increase in plasma cortisol after oral administration of that steroid.

steroid is esterified; cortisol acetate produces a detectable rise at 2½ hours, but the peak is still at 6 hours. If the free steroid is administered after a moderately heavy meal, the rise is first noted at 2½ hours, but the maximum is lower (27 μg as compared to 52 and 45 μg/100 ml in the preceding two experiments).

The observation (described in Section IV) that the 17-OHCS maximum occurs later than the cortisol peak, suggests that the method for cortisol determination is specific in that it is unaffected by cortisol degradation products such as tetrahydrocortisone.

Prolonged oral administration of cortisol acetate in the management of moderately severe ulcerative colitis and acute rheumatic fever (20 mg, 6-hourly, commencing 10 and 6 days respectively, prior to investigation) did not result in remarkably high cortisol levels in plasma. Samples drawn 4 hours after doses of the steroid had been given contained 32 and 24 μg/100 ml; at the time of administration the figures were 35 and 30 μg/100 ml respectively. No doubt the suppression of endogenous cortisol secretion partly accounted for the lower values as compared with the response to a single dose; this suggestion is supported by the following observations:

B. EFFECT OF LONG-CONTINUED ADMINISTRATION OF PREDNISONE AND CORTISONE.

A chronic asthmatic was treated with prednisone (Δ1:4 pregnadiene-3:20 dione-11:17:21 triol), 10 mg three

times daily by mouth. A month after treatment commenced, the plasma cortisol level was 5.4 μg /100 ml. Prednisone is more potent than cortisol in suppressing ACTH secretion; the subnormal cortisol levels illustrate the danger of sudden withdrawal of "steroid therapy", adrenal insufficiency being a serious hazard. A single observation on a rheumatoid arthritic who had received an average of 75 mg cortisone acetate daily for a year showed 3.6 μg cortisol/100 ml plasma.

This determination indicates that presumed high blood levels of cortisone do not affect the determination of cortisol by the present method.

A further implication is that the degree of adrenal atrophy during cortisone therapy can be assessed by cortisol determination. In addition, it appears that although cortisone may be converted to cortisol in the tissues, this cortisol does not enter the blood stream to a significant extent.

C. ACTH STIMULATION.

Continuous intravenous infusion is the most efficient route for ACTH administration; when the polypeptide is injected intramuscularly, there is reason to believe that it is in part destroyed at the injection site. The response to an ACTH infusion has been assessed by eosinophil counts (Thorn, 1953), by urinary 17-ketosteroid excretion and 17-OHCS excretion, and plasma 17-OHCS level. (Eik-Nes, Sandberg, Migeon, Tyler and Samuels, 1955).

In a limited clinical experience with plasma

cortisol determinations by the present method, single measurements have on every occasion clinched the diagnosis of Addison's disease, levels being unequivocally low; but Mason (1955) has referred to discrepancies between clinical data and plasma CS (presumably Porter-Silber chromogens). It is conceivable that partial destruction of adrenal tissue, associated with compensatory pituitary-induced hypertrophy of the remainder of the gland, would be associated with normal basal plasma cortisol levels; but an inadequate response to ACTH would reveal the true situation, surviving adrenal tissue being already under maximal or near-maximal stimulation.

In view of this possibility, it seemed worthwhile to determine the extent of the normal rise in plasma cortisol after ACTH. This was done in six subjects, data being presented in Figure 16.

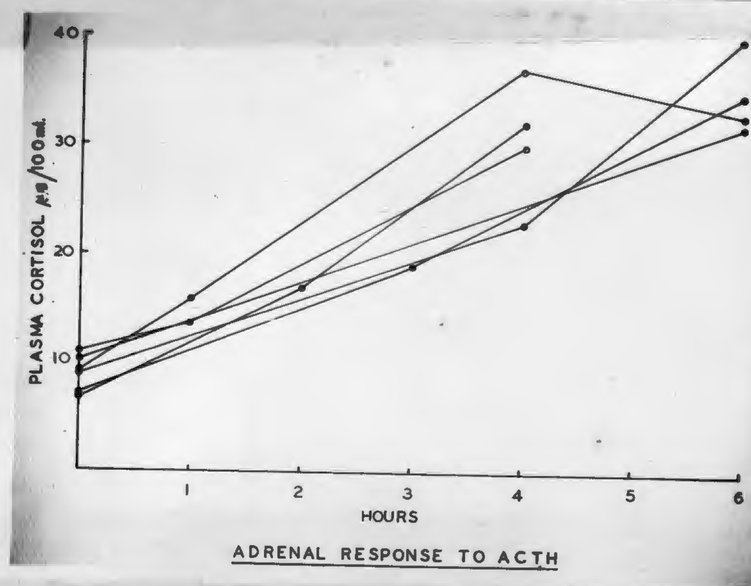


Figure 16.

The 6-hour infusion (of 40 units ACTH (Organon) in 500 ml of 5 % glucose), produced a maximum level of not less than 30 μg/100 ml. Four subjects were male; the ages ranged from 13 - 63.

D. EXERCISE.

Adrenalectomy impairs the work capacity of the skeletal musculature. This has formed the basis of Ingle's bio-assay procedure (1944). While work capacity may be restored to normal with cortisone, no publication has been found by the writer in which supernormal work output has been induced by CS.

It is widely held that severe exertion induces increased adrenocortical secretion. This belief stems largely from fallacious indices such as the eosinophil count. Thorn (1953) could not show an increase in Porter-Silber chromogens in the urine following exercise, using the method of Reddy, Jenkins and Thorn (1952).

Some observations on subjects undergoing moderate to severe exercise are shown in Table 15. The experiment, carried out early in this study, includes data on corticosterone levels.

There is no clear evidence for adrenocortical hyperfunction under these experimental conditions, except in the second subject, whose cortisol level rose by 4 μg/100 ml, which is significant at $p = 0.02$. It has been argued that increased "utilisation" might mask a rise in CS secretion.

TABLE 15

EFFECT OF EXERCISE ON PLASMA CS LEVELS

	CS μg/100 ml		
	One hour before	Immediately after	Two hours after
2-hour walk	11(0)	9(0.5)	10.5(1)
Competitive squash	7(0)	11(0)	11(0)
Competitive squash	10(1)	10(2)	8(2)
6-hour route march	8.9	-	7.9

If the liver were responsible for increased clearance of CS from the blood stream, a rise in tetrahydrocortisone glucuronoside might be detectable.

Part of the samples drawn from a subject before and after a route march (included in Table 15), were hydrolysed with β-glucuronidase and estimated by the Bayliss and Steinbeck procedure. "Total" (free plus glucuronoside-conjugated) 17-OHCS levels were 20 and 24 μg/100 ml respectively.

Lastly, an attempt was made to demonstrate an increase in the arteriovenous cortisol difference of an exercised limb. The lower limb was used in preference to the arm, as antecubital vein blood drains skin capillaries to a large extent, while the femoral vein, though slightly less accessible, mainly contains the venous effluent from muscle. An athletic

subject exercised his right leg to the point of exhaustion by raising himself repeatedly on to an 18" stool. Blood was immediately drawn from left and right femoral veins, and from the right femoral artery. The cortisol levels were:

Arterial blood	9.1 μg/100 ml plasma.
Venous blood from resting limb	8.7 μg/100 ml plasma.
Venous blood from exercised limb	9.8 μg/100 ml plasma.

No significant cortisol uptake in either limb was demonstrable with the method of determination used. A slightly better experiment would have been the comparison of arterial and venous levels before and after exercise, but was precluded by fear of haematoma formation.

E. WATER LOADING.

The deficient water diuresis in adrenal insufficiency is corrected only by 11, 17-oxygenated CS; as cortisol is the major "glucocorticoid" in peripheral and adrenal venous blood, it has been of interest to follow its blood level during a heavy water load. 20ml tap water per kilogram body weight was ingested by two healthy subjects over periods of 15 minutes. (Soffer and Gabrilove, 1952). Basal and five subsequent cortisol levels were determined; in one subject, freezing point depression was measured on each plasma sample; considerable haemodilution was evident. Satisfactory water diuresis occurred in both subjects, more than 80 % of the water load being excreted in 4 hours. Results are shown in Table 16. No remarkable change in plasma cortisol took place. The transitory rise from 6.7 to 8.9μg/100ml

in one subject, and 10.3 to 12.1 in the other, were significant at $p = 0.05$.

TABLE 16.

	SUBJECT (i)		SUBJECT (ii)
	Cortisol μg/100ml	Plasma osmolarity mOsmols/L	Cortisol μg/100ml
Basal	7.5	413	10.3
30 minutes	6.7	352	11.1
1 hour	8.9	331	12.1
2 hours	7.5	312	11.5
3 hours	7.9	340	11.7
4 hours	7.8	320	10.6

F. COLD EXPOSURE.

Two healthy young males, weighing 132 and 157 lb were immersed to the neck for 30 minutes in water at 54° F. Minimal activity was permitted. In one the cortisol level fell from 8.1 to 7.2 μg/100 ml plasma; in the other the figures were 6.3 and 6.4 μg/100 ml.

G. "PSYCHIC STRESS".

This unhappy term has been used to denote situations such as anger, operation of a "pursuitmeter", and being cox in a boat race. The affective state in students immediately before their final Medicine orals could presumably be described as "stressful"; two such individuals were prevailed

upon to submit to cortisol determinations ten minutes before and 24 hours after their ordeal. The initial values were 10.9 and 7.9 µg/100 ml, the subsequent ones 7.7 and 9.8 µg/100 ml. Unless the subsequent celebration was more 'stressful' than the pre-examination state, there was no indication of an adrenocortical response to fear in the second subject, while the higher pre-examination figure in the first subject, though significantly different, is still within the normal range.

It is indeed probable that individual variation is marked in this, a psychosomatic field. Hetzel, Schottstaedt, Grace and Wolff (1955) have reported transitory elevation of the urinary 17-OHCS after 'stressful life experiences' in some subjects.

H. DIURNAL VARIATION.

Variations in adrenal activity during the course of the day have been reported. Flustuations in the circulating eosinophil count and in sodium and potassium excretions take place, and Porter- Silber chromogens in blood and urine are highest at 6 a.m. and lowest at 10 p.m. (Tyler, Migeon, Florentin and Samuels, 1954; Doe, Flink and Flint, 1954; Forsham, 1955; Doe, Flink and Goodsell, 1956). The pattern of variation is agreed upon by these groups, but Tyler et al report far more extensive fluctuations in plasma 17-OHCS than do Doe and co-workers.

The cause of this diurnal variation is by no means

clear; night workers have maximal levels in the early morning, disposing of activity, posture and sleep as determining factors. The fluctuation is not due to changes in light intensity, for it is shown by totally blind subjects. Environmental temperature may be responsible; the negative results of the experiments described in Section V(F) do not favour this, but the period of exposure to cold may have been too short.

Further speculation suggested that the food intake was implicated. Plasma and urine 17-OHCS levels fall throughout the day, and rise fairly steeply during the latter part of the night. The postabsorptive state is known to be associated with hepatic glycogenolysis, while prolonged fasting results in gluconeogenesis. Starvation is known to cause histological changes in the adrenals of rats (Lewis, 1949) and humans (Uehlinger, 1955) compatible with increased cortical activity. Was it possible, then, that "glucocorticoid" secretion rises during the postabsorptive state, varying reciprocally with insulin release?

The normal diurnal variation in night workers, reported by Tyler et al, does not invalidate this hypothesis, for such individuals commonly prefer to avoid a heavy midnight meal, having the main meal before retiring. Tyler does not indicate the number of subjects studied.

Another possibility linking diet with the diurnal variation was that hepatic degradation of CS became reduced

during the latter part of the night, when, among other changes, the liver glycogen is lowered. This is unlikely in view of the similarity of the urine and plasma 17-OHCS patterns.

In testing the dietary hypothesis, the normal diurnal pattern was determined in four subjects; they were then given additional balanced meals of about 800 C. at 10.30 p.m; observations were continued for a further 24 hours. The experiments varied in duration from 2 to 6 days. In two subjects, urinary ketogenic steroid determinations were carried out on urine samples secreted in the 60 minute periods preceding each venepuncture.

Examples of the results obtained are shown in Figure 17. In the control periods, the patterns conform to published

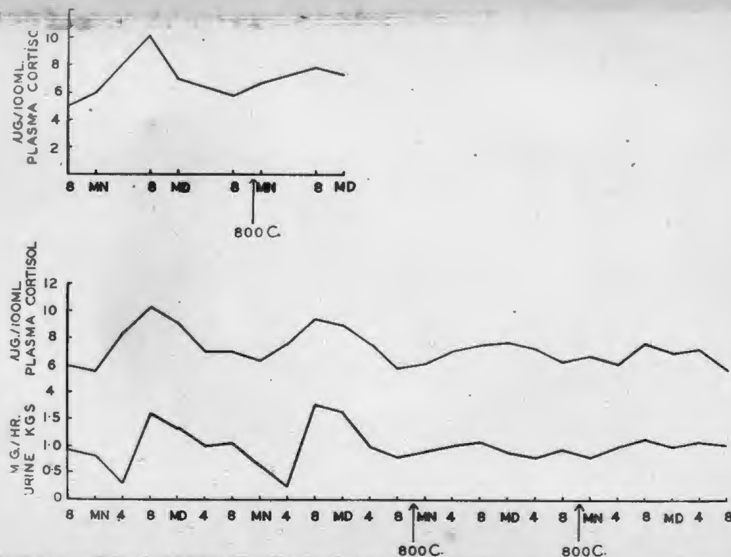


Figure 17. Diminution of diurnal variation by additional food intake at night.

data for 17-OHCS; the plasma cortisol maximum was as much as twice the minimum value. The KGS excretion rate was minimal at 4 a.m. in both cases, while the plasma cortisol was invariably at its lowest at midnight.

In every subject investigated, the diurnal variation was greatly diminished, though never abolished, by introducing an additional late meal.

I. EFFECT OF CALORIE INTAKE.

Variations in food intake were studied in three healthy adult males. The normal 10 a.m. values for plasma cortisol were estimated. The levels after fasting for 24 hours in two subjects, and 48 hours in a third, were obtained. (They were permitted to drink water). After a four day control period, basal values were again determined, and the effect of 72 hours of a 4000 - 4500 C. per day diet noted. Results are shown in Table 17.

TABLE 17.

EFFECT OF CALORIE INTAKE ON PLASMA CORTISOL (in µg /100 ml)

Basal	Fasting	Basal	High intake
7.3	11.1	8.6	9.0
11.0	11.8	9.1	10.5
10.1	13.4	9.6	10.9

The effect of fasting in elevating the plasma cortisol was significant in two subjects. The high-calorie

diet used had no effect.

The dietary constituents were not separately investigated in detail; after a control period, three subjects were given isocaloric diets in which 80 - 90 % of the calories were provided as carbohydrate; no significant alteration in plasma cortisol was observed.

In summarising the data from sections D to I it may be stated that the plasma cortisol is somewhat less labile than might have been anticipated, a point of singular importance in interpreting clinical and experimental data.

The diurnal variation is, however, a source of error; as the pattern is reasonably consistent from individual to individual, both in this and other studies, it appears that values obtained at the same time of the day are sufficiently comparable.

J. PREGNANCY.

The plasma level and urinary excretion of 17-OHCS are known to show a pronounced rise during pregnancy (Gemmell, 1955; Bayliss and Steinbeck, 1955; Robinson et al, 1955); re-investigation with the plasma cortisol technique was carried out mainly to establish the norms with this method prior to studies on disordered carbohydrate metabolism in pregnancy.

With the exception of the survey by Bayliss and Steinbeck (1955), studies have consisted of disconnected observations on women in various stages of pregnancy; the

alternative plan is to follow the same individuals throughout and after their pregnancy. The latter scheme is preferable, each subject acting as her own control so that the pattern of adrenocortical function would become apparent with a smaller, if more time-consuming study. This scheme has been adopted in the present survey, which is as yet incomplete.

Another criticism may be directed at some published work in this field. In pregnancy a considerable hydraemia occurs; CS are transported almost entirely in the plasma, the red cells containing a negligible quantity (in fresh blood). Determinations on whole blood may therefore give falsely high values.

Some results of the pregnancy series are shown in Figure 18. During the second trimester most figures lie

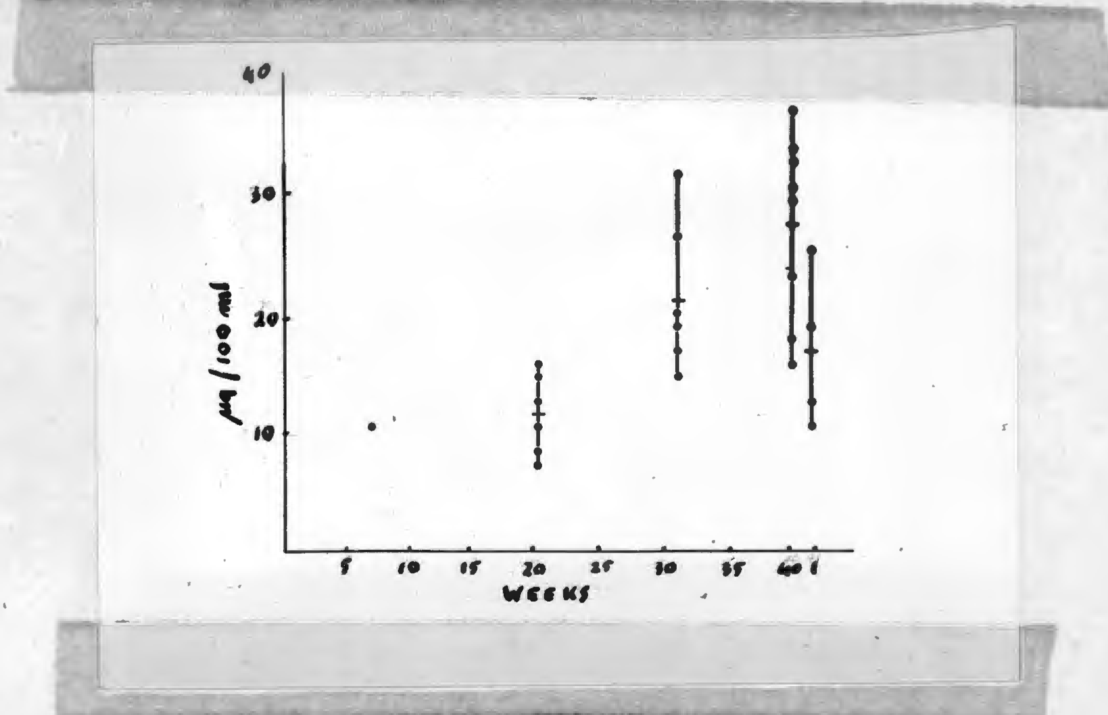


Figure 18. Rise in plasma cortisol during pregnancy.

within the normal range, but the mean is 11 μg cortisol/100ml plasma (normal mean 9.2). In the last trimester a considerable rise occurs, but the values at 40 weeks must be viewed with suspicion, as some of the subjects were in early labour.

In three women at term but not in labour, cortisol levels were 17, 22 and 28 μg/100 ml; late in the first stage of labour, values rose to 30, 29 and 33 μg/100 ml respectively. In the first subject the level fell on the second day of the puerperium to 10 μg/100 ml, but the mean figure of 18 μg/100ml from four subjects on the 7th day, was significantly above normal.

Several women at various stages of pregnancy are at present under investigation, but have been excluded from the series now under discussion.

K. MENSTRUATION.

Four subjects between the ages of 17 and 28 were investigated weekly during the menstrual cycle. The absence of significant variation is evident from Table 18, and contrasts with the minor increase in urinary 17-OHCS in the second and third weeks reported by Maengwyn-Davies and Weiner (1955).

TABLE 18.

PLASMA CORTISOL DURING MENSTRUAL CYCLE

	1st week	2nd week	3rd week	4th week
	9.6	10.6	9.9	10.9
	6.8	6.0	8.0	8.4
	7.5	7.9	8.8	7.4
	10.1	9.1	8.8	10.5
Mean	8.5	8.4	8.9	9.3

L. PLASMA CORTISOL IN DISEASE.

The acid test of a new technique in clinical biochemistry is its degree of correlation with the clinical assessment of the patient, as reinforced by proved pathological investigations. In a year's experience with this method, results have been consistent with clinical data; one is indebted to several physicians at Groote Schuur Hospital for supplying a wealth of specimens from their endocrine and other cases.

The majority of cases were under investigation for Cushing's syndrome; with one exception the diagnosis was not supported; these cases will be referred to later.

Some data are listed in Table 19, most of the cases being selected because they illustrate a feature or application of the technique of cortisol estimation.

The first three cases, of severe adrenal insufficiency, had levels of 0.5, 0.0 and 0.2 μg/100 ml. This offers useful support for the contention that the method is a specific measure of adrenal activity. Two of these patients were maintained on deoxycorticosterone acetate, which in the doses used does not affect the results of the test.

A patient with bilateral phaeochromocytomata (in whom the diagnosis was established preoperatively in this laboratory by noradrenaline bioassay), provided some informative data (4 and 5). His hypertension was persistent, with exacerbations. Plasma cortisol was normal, suggesting that noradrenaline did

not stimulate the pituitary-adrenal axis in the considerable amounts released (diastolic pressure averaged 160 mm Hg, urinary noradrenaline between 1 and 2 μg/ml). Removal of the tumours with almost all adrenal tissue corrected the blood pressure and noradrenaline excretion. Symptoms of adrenal insufficiency were held in check by sodium chloride supplements; his postoperative plasma cortisol was 2 μg/100 ml. His fasting blood sugar was normal; increasing pigmentation was not observed.

TABLE 19

EXAMPLES OF PLASMA CORTISOL LEVELS IN DISEASE

		μg/100ml
1.	Addisonian crisis	0.5
2.	Addison's disease, on deoxycorticosterone	0.0
3.	Total adrenalectomy, on deoxycorticosterone	0.2
4.	Subtotal adrenalectomy, maintained on sodium chloride	2.0
5.	Phaeochromocytomata	8.0
6.	Essential hypertension	7.0
7.	Gonadal agenesis (age 15)	4.5
8.	Gonadal agenesis (age 11)	10.8
9.	Ovarian hyperthecosis (Stein-Leventhal syndrome)	4.4
10.	Ovarian hyperthecosis (Stein-Leventhal syndrome)	6.0
11.	Cushing's syndrome (adrenal hyperplasia)	39
12.	Diabetic coma	26
13.	Surgical shock (compound fracture of femur)	18
14.	Exploratory laparotomy, 3 hours post-operatively	15
15.	Partial gastrectomy for carcinoma, 1 hour post-operatively	27

A normal cortisol level was found in patient 6, with hypertension and grade IV retinopathy but normal urea clearance. The important role of the adrenal cortex in essential hypertension has been reviewed by Sapeika (1948, 1955), but in agreement with published data, this patient and three others did not have excessive adrenal cortical function as assessed by plasma cortisol levels.

In gonadal agenesis, described inter alia by Turner, shortness of stature has been ascribed by Albright, Smith and Fraser (1942) to deficiency of adrenal androgen. Plasma cortisol was slightly low in one case, normal in another; in the latter the response to ACTH was normal (38 μg/100 ml at 6 hours). Stunting of growth may simply be one of the many developmental abnormalities in this condition.

If Turner's syndrome is an important cause of primary amenorrhoea, the ovarian hyperthecosis (Stein-Leventhal) syndrome is attaining even greater prominence in the differential diagnosis of secondary amenorrhoea and oligomenorrhoea. The hirsutes in this condition has been ascribed to excess adrenal androgen, (Muller, 1955, personal communication). High pregnanediol excretion by the Sommerville and Marrian method was not easily explicable in patients with polycystic ovaries. With the discovery of pregnanetriol (Cox and Marrian, 1953) and its likely origin from 17-hydroxyprogesterone, adrenal hormones may well be implicated in the pathogenesis; the high "pregnanediol" figures may be ascribed to

excessive pregnanetriol excretion, as both yield chromogens with sulphuric acid. Following this train of speculation, an analogy may be drawn with virilising adrenal hyperplasia, where high pregnanetriol excretion marks a block in cortisol synthesis, leading to increased ACTH secretion and consequent rise in adrenal androgen output. In this light the low or low normal cortisol levels in the two patients studied are of great interest; further investigations are proceeding.

In only one of the eleven patients investigated for Cushing's syndrome was the diagnosis established on full investigation. The pathology was bilateral adrenal hyperplasia and the cortisol level of 30 μg/100 ml was the highest observed in the clinical series. Of ten subjects admitted for investigation for Cushing's syndrome, in whom the diagnosis was discarded, six had cortisol levels of 9 - 13.8 μg/100 ml with a mean of 11.6. These high normal figures, in patients who were in essence obese diabetics, are notable in view of current research into the etiology of this type of diabetes, in which plasma insulin levels are often normal, (Vallance-Owen, Hurlock and Please, 1955).

A patient in diabetic coma, in whom the precipitating cause was obscure, had a cortisol level of 26 μg/100 ml. It is difficult to distinguish cause and effect in this case. "Steroid diabetes" is insulin resistant, but ketosis is rare. Hepatic insufficiency may contribute to the high cortisol level.

Gemmell has used the Nelson and Samuels procedure to

demonstrate high 17-OHCS levels postoperatively, particularly in patients with shock, (Gemzell, Frankson and von Euler, 1954). Subjects subsequently dying from shock display CS levels at least as high as those surviving; there is no "adrenal exhaustion". An unshocked patient, after an exploratory laparotomy under general anaesthesia, had an unremarkable rise in plasma cortisol from 9.4 μg/100 ml 3 hours preoperatively to 15 μg/100 ml, 3 hours after leaving the table. Severe surgical shock due to a compound fracture of the femur in a 40-year old male was associated with a plasma cortisol of 18 μg/100 ml. A figure of 27 μg/100 ml, was obtained in an elderly man who had had a partial gastrectomy for carcinoma an hour previously; shock was not evident.

SUMMARY.

That the method of cortisol determination is a sensitive index of adrenocortical function is shown by pronounced rises in plasma level after administering cortisol or ACTH. Despite this, plasma levels are little affected by exercise, water loading, cold and "psychic stress". The diurnal variation is provisionally ascribed to lack of food intake during the night; to be comparable, blood samples should be drawn at a fixed time of the day. Plasma cortisol rises considerably during late pregnancy and labour.

Clinically, the plasma cortisol determination appears to be of most use in diagnosing adrenal insufficiency (coupled if necessary with ACTH infusion). Studies in other endocrine conditions have indicated some future lines of research.

SECTION VI

COMPARISON OF PLASMA CORTISOL WITH URINARY KETOGENIC STEROIDS

Reference has been made in the introduction to the relative merits of plasma and urine studies in measuring adrenocortical activity. It is facile to assume (as is so often done) that blood levels are of greater value than excretion rates, and such attention as has been paid to the problem has in the main been speculative.

A direct approach to the subject was attempted by making simultaneous plasma and urine determinations of CS and their metabolites under varying conditions of adrenal activity.

In selecting a technique for urine studies it was clear that the estimation of cortisol itself was of no value, for a negligible fraction of this steroid is excreted unchanged, or even in conjugated form. One of the procedures which detect CS metabolites was required. The Porter-Silber reaction for dihydroxyacetone steroids is employed in the method of Reddy, Jenkins and Thorn (1952), and is theoretically unsuitable as a significant part of the CS output is reduced at C20 producing a 20-hydroxy derivative; one is not alone in having experienced unworkably high blank values, and it appears a common finding that butanol of sufficient purity is extremely difficult to prepare.

The more involved technique of Norymberski, Stubbs and West (1953) is based on the ability of sodium bismuthate to oxidise steroids with α-ketol, dihydroxyacetone, glycerol and glycol side chains to 17-ketosteroids. These, the ketogenic steroids (KGS) are measured by the difference between the 17-ketosteroid content of normal and oxidised urine. One might anticipate difficulties with a method in which the results of two relatively complicated determinations have to be subtracted, and some workers have in fact experienced trouble; in this laboratory, fortunately, the published method has given reproducible results without major modification. It has the additional advantage that 17-ketosteroids are simultaneously determined.

Urine samples were obtained during the ACTH infusions described in Section V(C). A basal sample was collected at the end of the 60 minute period preceding the infusion; the second sample was that secreted between 3½ and 4½ hours after commencing ACTH administration, and was compared with the 4-hour plasma level. The third represented the period 5½ - 6½ hours.

Urine and blood samples were obtained from a patient who had undergone subtotal adrenalectomy and was being maintained on sodium chloride supplements without CS therapy. His blood urea was normal. The patient with Addison's disease was studied before commencing therapy, and was in a mild crisis.

Results are shown in Table 20 and Figure 19.

TABLE 20

COMPARISON OF PLASMA CORTISOL AND URINARY KGS IN NORMAL SUBJECTS AND AFTER ACTH STIMULATION, AND IN PATIENTS WITH ADRENAL INSUFFICIENCY

	BASAL		4 HOURS		6 HOURS	
	Plasma μg/100 ml	Urine mg/hr.	Plasma μg/100 ml	Urine mg/hr.	Plasma μg/100 ml	Urine mg/hr
Normal 1	8.8	1.3	23	1.7	38	3.2
Normal 2	9.2	0.8	34	1.5	34	2.2
Normal 3	10.8	1.1	30	2.1	-	2.5
Normal 4	6.7	0.9	32	1.9	-	-
Subtotal adrenalectomy	2.0	0.17	-	-	-	-
Addison's disease	0.5	0.12	-	-	-	-

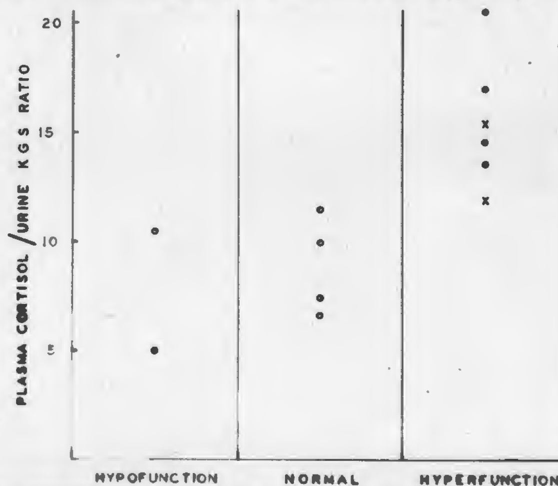


Figure 19. Variation in ratio of plasma cortisol to urinary KGS at different levels of adrenal activity. (In hyperfunction, circles denote ratios after 4 hours ACTH infusion, while crosses indicate the ratios after 6 hours).

While a rough correlation between plasma and urine levels is evident, the urinary excretion does not follow plasma cortisol at all closely with the methods employed. Only tentative conclusions can be drawn from this limited number of observations. The poor correlation could be ascribed to inaccuracy of the methods of determination; plasma cortisol data are regarded with some confidence; the urine estimations show higher excretions than might be expected in Addison's disease; it is conceivable that bismuthate oxidation of substances from extra-adrenal sources may contribute to the colour with the Zimmerman reagent; this would account for the low plasma/urine ratios in the two cases of adrenal insufficiency.

It is more plausible, however, to interpret data in terms of physiology than chemistry. Roughly half the plasma C3 and their metabolites appear to be conjugated, contrasting with the proportion in urine, where conjugates greatly predominate over free steroids. It is clear that the kidney excretes the water-soluble conjugates far more readily than the uncombined steroids. After administering ACTH or cortisol (Section IV), the rise of plasma steroid conjugates occurs some hours after the rise in free plasma cortisol. In consequence, the rise of urinary conjugated KGS appears less steep than that of plasma cortisol so that the plasma/urine ratio increases during the first 4 hours of the experimental period (Figure 19).

Table 21 shows that the rise in plasma cortisol after ACTH is more pronounced than that of urinary KGS. This is not surprising, for the urine is only one of the routes for elimination of CS and their metabolites; bile and sweat contain these substances too, and complete degradation of part of the adrenal output is possible.

TABLE 21

MEAN VALUES DURING ACTH INFUSIONS INTO FOUR NORMAL SUBJECTS

	Basal	4 hours	6 hours
Mean plasma cortisol, μg/100 ml	9.0	30	36
Mean urine KGS, mg/hour	1.0	1.8	2.6

Urinary corticoid studies in general have been credited with certain advantages over plasma determinations. Methods are undoubtedly less complex; this may be attributed to the greater quantities of steroid available in urine samples, and to the relatively simple composition of urine, reducing the need for purification of extracts. In addition, a 24-hour urine specimen will "smooth out" diurnal and other physiological fluctuations in adrenal activity though data in Section V suggest that the plasma cortisol is less labile than has been supposed. It is conceivable, too, that maintained minor variations in adrenal activity would be better reflected by changes in excretion than plasma concentration; an analogy

has been drawn (Jackson, 1954) with some decalcifying diseases, in which a high urinary excretion of calcium may co-exist with a serum level in the normal range.

The plasma level, it is true, represents the balance at a single point in time between adrenocortical secretion and the elimination of CS from the blood stream. How variable is the latter factor? The renal contribution may alter at pathological extremes of adrenocortical activity and chronic hepatic disease has been shown to reduce the proportion of conjugated 17-OHCS in blood, (Klein, Papadatos, Fortunato, Byers and Puntereri, 1955); this group of workers has also described a Cushing's syndrome-like picture in hepatic insufficiency.

The problem of hydrolysis of steroid conjugates has perplexed endocrinologists for some years. Acid hydrolysis undoubtedly reduces yields of less stable CS, notably those with an oxygen function at 17, and artefact formation is possible. Enzymic hydrolysis with β-glucuronidase is a notable advance, and ester sulphatase preparations are now available; these methods are time-consuming however, and sulphatase is inhibited by phosphate and sulphate in concentrations prevailing in urine. The bismuthate oxidation circumvents the main ill-effect of acid hydrolysis by converting CS to 17-KS before hydrolysis, but even then the yield is uncertain.

This technical stumbling-block does not apply to plasma studies to any serious extent, for extensive conjugation of plasma CS has not been shown; in the case of cortisol,

at least half is unconjugated (Section IV).

SUMMARY.

The present investigations have made the following contributions to the "urine versus blood" controversy:

(1) ACTH stimulation produces a more dramatic rise in plasma cortisol than urinary KGS, as determined by the methods described.

(2) Plasma cortisol is not particularly variable in health, except in certain well-defined circumstances such as pregnancy. The diurnal variation must be borne in mind when drawing samples.

(3) Although experience to date is limited, no plasma cortisol level has yet been inconsistent with clinical and other biochemical data. This claim cannot be made for urinary formaldehydogenic steroid determinations, nor for butanol-soluble Porter-Silber chromogens in urine. However, the same criticism may be levelled at plasma 17-OHCS estimation, both in Cushing's syndrome (Mason, 1955) and Addison's disease (Eik-Nes, Sandberg, Nelson, Tyler and Samuels, 1954). Ketogenic steroid excretion in a case of partial adrenal failure (Table 20) was 4 mg/day, while a sample during an Addisonian crisis was 2.9 mg/day. In these cases adrenal function was better indicated by the plasma cortisol.

(4) Hydrolysis of conjugates is unnecessary when determining plasma cortisol, avoiding a difficulty always present in urine studies.

SECTION VII

A CASE OF ALDOSTERONE-SECRETING ADENOMA (CONN'S SYNDROME)

A 33-year old female, N. van R. presented at Groote Schuur Hospital with a 2-year history of severe muscular weakness. Hypertension and a hypokalaemic alkalosis were found, and a presumptive diagnosis by Dr L. Eales of aldosterone-secreting tumour of adrenal origin was supported by radiographic studies using perirenal air insufflation coupled with toxography. Surgical removal of the tumour resulted in dramatic clinical improvement.

Pre-operative studies on urinary and plasma CS were made in this laboratory; the tumour and attached adrenal tissue were investigated, and a post-operative aldosterone determination on urine was carried out. This description of the CS determinations is included, not because of any great originality in the methods used (they lean heavily upon classical and recent techniques in steroid chemistry) but because of the apparent rarity of the syndrome and the striking way in which the secretory activity of the tumour was modified by the potassium intake. This correlation has important implications in diagnosis, for Chalmers, Fitzgerald, James and Scarborough (1956) have reported a proved case in whom urinary and blood aldosterone were usually within the normal range.

URINARY ALDOSTERONE.

The method was essentially a modification of that introduced by Neher and Wettstein (1956). The urine was extracted with freshly distilled chloroform, immediately after acidification to pH1, and again after 24 hours at room temperature. Reversed-phase paper-chromatographic purification of the extract replaced the adsorption method on silica gel columns; chromatography was performed on strips of Whatman no. 4 paper, 1.5 cm wide (previously washed with ethanolic sodium hydroxide). The benzene/50 % methanol system was used first, and was followed by elution of the aldosterone region and resolution of aldosterone and cortisone in toluene-ethyl acetate/methanol-water. These are respectively the B5 and C systems of Bush. The benzene system has certain advantages over the toluene/propyleneglycol method adopted by Neher and Wettstein from Zaffaroni; the latter requires prolonged overrunning; apart from the time required, Rf values are not obtained, so that slight differences in running speed could result in discrepancies between unknown and reference strips.

Determination in the first analysis was by the same semiquantitative procedure as used by Neher and Wettstein, namely, visual assessment of the intensity of the spots shown by the combined blue tetrazolium - 10 % aqueous sodium hydroxide fluorescence reaction. Subsequently this has been coupled with the more precise potassium tert-butoxide fluorescence reaction (Abelson and Bondy, 1955); the eluate from

the first chromatogram is divided into two parts, both being run in the C system. One strip is sprayed with blue tetrazolium-20 % sodium hydroxide and assessed as above; the aldosterone region in the second is thus located, and is eluted with methanol for fluorimetric determination. The use of blue tetrazolium with 20 % sodium hydroxide makes it possible to detect less than 0.1 µg/sq cm of aldosterone (Nowaczynski, Goldner and Genest, 1955); for quantitative work, however, the method of Abelson and Bondy is more sensitive than techniques based on tetrazolium reduction.

Using the quantitative fluorimeter procedure, norms and recoveries were determined on 24-hour urines from apparently healthy subjects on normal diets; three were males, and two females; the ages ranged from 18-27. The specimens were divided into two equal parts; to one, authentic aldosterone was added; recoveries are shown in Table 22.

TABLE 22

NORMAL ALDOSTERONE EXCRETION, AND RECOVERY DATA

	Aldosterone excretion µg [illegible]	Aldosterone added µg	Total µg	Recovery %
	2.7	1.0	3.55	85
	0.5	1.0	1.2	70
	6.4	1.0	7.1	70
	4.95	3.0	7.0	68
	5.4	3.0	7.7	77
Mean	4.0			74

TUMOUR TISSUE, AND ADRENAL GLAND: ALDOSTERONE AND CORTISOL.

The extraction procedure was similar to that of Cartland and Kuizenga (1936). After extracting with excess of acetone, the extract was concentrated under reduced pressure; the watery residue was extracted with ethylene dichloride; the organic phase was washed with minimal aqueous sodium hydroxide and acetic acid, and taken to dryness. This residue was partitioned between 60 % methanol and hexane, each phase being back-extracted. The combined aqueous phases were concentrated under reduced pressure, the residue being extracted with ethyl acetate; the extract underwent reversed-phase chromatographic "defatting" in 85 % methanol before resolution in benzene/50 % methanol. The cortisol region was eluted, and an aliquot determined by fluorimetry in sulphuric acid. The aldosterone region, as identified by a reference strip, was eluted; the eluate was further handled as follows:

One aliquot was chromatographed in Bush's C system. A large spot was found which reduced blue tetrazolium, showed yellow fluorescence after heating with sodium hydroxide, and had an Rf value identical to the aldosterone reference spot.

A second part was acetylated; chromatography for 3 hours in benzene/formamide followed; a spot with an Rf value of 0.87 was noted. Aldosterone diacetate had an Rf of 0.90.

The remainder of the eluate was chromatographed in

the C system. The section containing aldosterone was eluted and determined by reduction of blue tetrazolium at 90° (Nowaczynski, Goldner and Genest, 1955), the diformazan absorption being measured at 600 mμ.

PLASMA ALDOSTERONE.

Only 50 ml plasma were available; this was worked up precisely as in the plasma cortisol determination, including chromatography in benzene/90 % methanol, with a 36 cm run. The region from the 13th to the 19th centimetre was eluted and re-chromatographed in the C system. The aldosterone area as identified by a reference strip was eluted and determined by fluorimetry in potassium *tert*-butoxide.

RESULTS.

Tumour: Aldosterone 5600 μg/kg. Cortisol 20 μg/kg.

Gland: Aldosterone not detected. Cortisol 260 μg/kg.

Plasma cortisol (11.11.1955): 6.1 μg/100 ml.

Plasma aldosterone: 0.25 μg/100 ml.

TABLE 23.

	Urine aldosterone μg/day	Serum potassium meq/L	Potassium intake g/day	Comments
5.9.1955	0.5	?	± 5	
17.10.1955	5	2.3	4	
7.11.1955	15	3.1	4	
13.11.1955	25	3.7	12	(x)
16.11.1955	19	3.7	12	On prednisone
20.11.1955	24	3.5	12	On prednisone
27.11.1955	15	?	12	On ACTH
10.12.1955	3.6	4.5	4	10 days postoperatively

(x) A course of 20 g potassium daily had ended two days previously; the serum potassium on that day had been 5.2 meq/L.

The aldosterone content of the tumour is about 100 times greater than that of normal beef adrenal; very little cortisol was present. The attached fragment of apparently normal adrenal gland was not, of course, expected to contain detectable amounts of aldosterone; its cortisol content was similar to that of beef adrenal.

The plasma cortisol level was low normal, and the plasma aldosterone slightly elevated by comparison with the figure of 0.16 μg/100 ml in a pooled sample (Section II).

In five subjects on normal balanced diets the urinary aldosterone excretion varied from 0.5 - 6.4 μg/day, mean 4.0. These figures are somewhat higher than the data of Neher and Wettstein (1955), 0.5 - 3 μg/day. However, still higher excretions are accepted as normal by Simpson and Tait, who investigated a case of Conn's syndrome published by Chalmers, Fitzgerald, James and Scarborough (1956). This group now gives the normal range as 8 - 16 μg/day, mean 12, in six male subjects. Their patient had an excessive aldosterone excretion in only one of three pooled urine samples, the values being 8, 11 and 28 μg/day. The high value had not been reported at the time of operation; the diagnosis was established despite normal figures for urinary and blood aldosterone. (The aldosterone excretion fell to one twenty-fifth of the last preoperative value after surgery. Unless this is explained on technical grounds, it must be postulated that the tumour releases aldosterone intermittently; alternatively,

the hormone must be degraded to unknown metabolic products with great rapidity.

A factor determining such intermittent release is suggested by the data obtained on Dr Eales' patient. The aldosterone excretion varied through the range 0.5 - 25 μg/day before the tumour was removed; a single postoperative value was 3.0 μg/day. A rough correlation is seen between aldosterone excretion and potassium intake; the output of this hormone rose to pathological levels (by the provisional standard set by the five normal subjects studied) only during or immediately after the heavy potassium loading which was necessary to elevate the serum potassium to normal or near-normal concentrations.

The single determination of plasma aldosterone was made when the potassium intake was 4 g daily; the level of 0.28 μg/100 ml may be compared with that of 0.16 μg/100 ml in a sample pooled from two normal subjects. Unfortunately, a urine aldosterone determination was not carried out on the patient on the same day.

It has been established experimentally that aldosterone secretion by the normal adrenal responds in a homeostatic manner to a high potassium or low sodium intake (Singer and Stack-Dunne, 1955; Luetscher and Johnson, 1954); an increased aldosterone production promotes sodium retention and potassium loss by the kidney. The present study suggests that a well-differentiated adrenocortical adenoma may behave in the same

manner.

From a table in the case report by Chalmers et al, the patient did not appear to receive potassium supplements during the period in which aldosterone excretion averaged 8 μg/day. Potassium intake is not recorded during later aldosterone determinations.

Conn was unable to correct the hypokalaemia by oral potassium loading in his patient, who differed in this respect from the case under discussion. It is suggested, nevertheless, that hormone studies in a suspected case should be made when the serum potassium has been increased to as near normal as possible.

It was obviously necessary to know the extent to which the aldosterone output would rise (as determined by the method described) during potassium loading and sodium restriction in a normal subject. Urine samples were collected from a healthy male of 26 years, firstly when on a normal diet containing a calculated 5 g sodium and 4 g potassium daily, then on the last day of a three day period during which potassium chloride supplements increased the potassium intake to 12 g daily. Finally, after a three-day period on a normal diet, the sodium intake was reduced to 0.5 g daily, without markedly altering the potassium or caloric intake, for 3 days. A urine sample was collected during the last day of this period.

The effect of potassium loading in increasing aldosterone output is thus confirmed; sodium lack has a probable

slight effect. However, the changes in the patient were much more pronounced.

TABLE 24

Potassium intake g/day	Sodium intake g/day	Aldosterone in urine µg/day
4	5	1.5
12	5	9.4
4	0.5	4.8

SUMMARY.

A case is presented in whom severe hypokalaemia was associated with an adrenocortical adenoma. The tumour was rich in aldosterone, but the urine contained excessive amounts only when the serum potassium had been elevated to near-normal levels.

SECTION VIII

GENERAL DISCUSSION. FUTURE RESEARCH PROJECTS.

GENERAL DISCUSSION.

The nature of the CS in peripheral plasma has been debated with some heat in recent years. In the present study, cortisol, cortisone, corticosterone, aldosterone and probably tetrahydrocortisone were identified; steroids present in concentrations below 0.15 μg/100 ml would not have been detected, however.

Cortisol is generally agreed to be the most plentiful CS in peripheral and adrenal venous plasma (Bush and Sandberg, 1953; Hudson and Lombardo, 1955), and the concentration found is in good agreement with published data (Morris and Williams, 1953; Sweat, 1955; Bush and Sandberg, 1953).

Corticosterone was present at an average level of 1.2 μg/100 ml, giving an F : B ratio of about 8 in normal subjects not receiving ACTH. The mean recovery of this steroid was 87 %. Sweat (1955), who introduced the sulphuric acid fluorescence method for determining cortisol and corticosterone which was employed in the present study, obtained a mean ratio of 2.25 in six subjects. The resolving power of his adsorption chromatogram is questionable, however. Morris and Williams, (1953, 1955) used partition systems, and determined CS by polarography, blue tetrazolium reduction and

spectrophotometry at 235 mμ. With the polarographic method, the mean F : B ratio in 17 subjects was 1.35, while the latter procedures gave a value of 0.9 in a further 6 individuals.

Bush and Sandberg (1953), and Bush (personal communication, 1955) obtained F : B ratios more in keeping with our own, using paper chromatography and sodium hydroxide-induced fluorescence. In the latter series he studied 50 samples of plasma and three large batches, finding corticosterone on only 10 occasions. Bush (1955) quoted unpublished observations of Simpson and Tait, who found a mean ratio of 6 in 11 males, and of Mills, whose 10 subjects had ratios of 5 - 10. Bush attributed the findings of Morris and Williams to "some systematic difference in technique".

In this connection, there arises the possibility of protein binding of corticosterone, disrupted by the ethanol precipitation used by Morris and Williams, but not by the ethyl acetate or ethylene dichloride extraction used by Bush and ourselves. The finding of intermediate amounts of corticosterone by Sweat, using chloroform extraction, does not exclude this suggestion, which requires investigation.

What is unquestioned, however, is that cortisol is a far more potent "glucocorticoid" than corticosterone. This, and the current state of doubt concerning the validity of methods of corticosterone determination, suggest that in the routine measurement of adrenal "glucocorticoid" function it is sufficient to determine cortisol alone. This policy has

been followed in most of the investigation.

The presence of about 8 μg/100 ml of cortisone in our pooled samples is in agreement with the data of Morris and Williams (1953), who found 2.5 - 5 μg/100 ml. Bush and Sandberg found this steroid in small amounts in some samples. In contrast, Hudson and Lombardo (1955) failed to detect the steroid in adrenal venous blood. The inference is that cortisone appears to arise from non-adrenal sources, possibly by oxidation of cortisol.

Aldosterone was found in one pooled sample of plasma at a concentration of 0.16 μg/100 ml, using fluorimetry in potassium _tert_-butoxide; in another sample, semi-quantitative determination suggested a level of 0.15 - 0.3 μg/100 ml. The patient with hyperaldosteronism had a concentration of 0.25 μg/100 ml. Simpson and Tait (1955), using a highly sensitive bio-assay, reported a blood level of 0.08 μg/100 ml. The steroid has been obtained by Lustacher, Neher and Wettstein (1954) in crystalline form from pathological urine. The plasma level of this hormone is too minute to permit determination in plasma samples of reasonable size, with existing methods of estimation. It is possible that a micro-modification of the blue tetrazolium procedure may render this possible; the potassium _tert_-butoxide technique is theoretically of sufficient sensitivity, but high reagent blanks limit its usefulness. A procedure specific for aldehydes would be ideal; this might be complicated, however,

by the behaviour of aldosterone in solution, when ring closure takes place, reversibly forming a hemi-acetal. Certainly the precise measurement of aldosterone secretion is an urgent want both in clinical and experimental fields.

The method of cortisol determination adopted has not, to our knowledge, been employed in other laboratories; it remains to be seen to what extent it will "transplant", for many procedures of this degree of complexity are somewhat personal. All that can be claimed for it is that in our hands it has been sensitive, adequately reproducible, and reasonably easy to perform once a routine has been established. Like all microchemical procedures it does require a scrupulously careful technique; the additional problems of column chromatography are avoided, however, and it is hoped that the introduction of a quantitative paper procedure is a material advance in methodology. The method is not interfered with by cortisone, prednisone or deoxycorticosterone in doses normally employed, while three cases of Addison's disease had cortisol levels near zero. ACTH leads to a greater percentage rise in plasma cortisol than in free or conjugated plasma 17-OHCS and in urinary KGS.

Correlation with clinical data has been consistently good in a year's experience with the method; in this, our procedure compares favourably with 17-OHCS determinations, which may give normal values both in Addison's disease and Cushing's syndrome.

It has been demonstrated that plasma and urine studies do not show a high degree of correlation. The former is certainly a more direct measure of adrenal function, but urine determinations possess the virtue of simplicity; in addition, they provide a yardstick, however crude, of the rate of hormone production; plasma levels indicate adrenal activity at a single point in time. However one may speculate, the direct observations show that the method of plasma cortisol determination used is a more sensitive index of adrenal function than the procedure adopted for urine studies.

The physiological variations in plasma cortisol are of interest in their own right, and their recognition is essential in interpreting clinical data. The diurnal variation in particular must be borne in mind; samples for routine investigations should be drawn at the same time of the day as that for which norms have been established. The diminution of the nocturnal rise by dietary means requires further investigation, both in confirmation and in analysis of the mechanism of this phenomenon.

The plasma cortisol is not greatly affected by other environmental factors in the normal individual. This may mean that CS secretion is fairly constant, or that CS "utilization", a somewhat vague concept, keeps pace with alterations in adrenal activity. Studies of arteriovenous differences in cortisol may throw some light on this, if they are technically feasible.

Some clinical studies have already proved fruitful,

and have been discussed in Section V(L); current lines of investigation have been indicated.

SECTION IX

SUMMARY

A review of existing adrenal function tests suggested the need for a relatively simple procedure for routine determination of the individual corticosteroids in plasma.

Preliminary qualitative studies on the circulating corticosteroids were necessary, in view of certain controversies attending this subject. Cortisol, cortisone, corticosterone and aldosterone were identified with reasonable certainty. Cortisol was far more plentiful than corticosterone.

A quantitative procedure was subsequently developed. Various methods of extraction were compared. Purification of the extract was achieved by reversed-phase paper chromatography. The corticosteroids were resolved by paper partition chromatography in view of the reproducibility and simplicity of this technique in comparison with column methods; this advance depended on the development of a procedure for reducing hitherto prohibitive paper blanks. Cortisol and corticosterone were eluted and determined fluorimetrically. Average recoveries were 92 and 87 % respectively; a difference of 2.7 μg/100 ml between cortisol levels was significant at $p = 0.05$. Normal values were determined. Clinical studies confirmed the specificity of the method.

A comparative study showed that the plasma cortisol was a more sensitive index of adrenal activity than the plasma 17-hydroxycorticosteroids, and that hydrolysis of steroid conjugates by β-glucuronidase or sulphatase did not contribute materially to the value of the methods.

Simultaneous determinations of plasma cortisol and urinary ketogenic steroids were performed at various levels of adrenal activity; only a rough correlation was noted. Urine studies were simpler but less informative than measurements on plasma.

Exercise, chilling, water loading, and fear had slight and inconstant effects on plasma cortisol levels, which were found to be less labile in health than had been surmised. Plasma cortisol determinations in endocrine and other diseases have been consistent with clinical data, and have stimulated some intriguing lines of research.

Studies on a case of aldosterone-secreting adenoma were carried out. Production of this hormone was excessive only when serum potassium levels had been elevated to near normal.

The significance of these investigations is discussed, and some promising fields for future investigation indicated.

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ABBREVIATIONS AND DEFINITIONS

CS: Corticosteroids, substances with adrenocortical activity proven to be released by the adrenal gland.

AC: Adrenal cortex.

17-OHCS: 17-hydroxycorticosteroids.

Cortisol: 17-hydroxycorticosterone: term suggested by Professor C.W. Shoppee to replace "hydrocortisone".

KGS: Ketogenic steroids, as determined by the method of Norymberski, Stubbs and West (1953).

Corticoids: steroids present in adrenal cortex, but not necessarily active.

17-KS: 17-ketosteroids.