ASPECTS OF THE CONTROL OF ADRENOCORTICAL SECRETION IN MAN

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by

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CONTENTS

	Page
Acknowledgment	4
Summary	5
Nomenclature	7
1. Introduction	8
2. Adrenocortical Secretion and its Measurement	10
2.1. The Nature of the secretion	10
2.2. Estimation of Adrenocortical Activity	16
2.2.1. Secretion and excretion rate methods	16
2.2.2. The determination of steroids in blood and plasma	22
2.2.2.1. Extraction and purification of corticosteroids	22
2.2.2.2. Methods of assaying purified corticosteroids	34
2.3. Development of Multiple Assay Technique	43
2.3.1. Materials	43
2.3.2. Extraction and acetylation of corticosteroids	46
2.3.3. Separation of the major corticosteroids	47
2.3.4. Purification of aldosterone, corticosterone and cortisol acetates	49
2.3.5. Measurement of radioactivity and calculation of plasma steroid concentration	54
2.3.6. Evaluation of the assay of aldosterone, corticosterone and cortisol	56
2.3.7. Purification of cortexone and cortexolone acetates	69
2.3.8. Evaluation of the assay of cortexone and cortexolone	71
2.3.9. Discussion	78

	Page
3. Factors Affecting Adrenocortical Secretion	87
3.1. Introduction	87
3.2. The Effect of Corticotrophin	90
3.3. The Effect of Angiotensin	99
3.4. The Effects of Haemorrhage and Hypotension	106
3.5. The Effect of Sodium Depletion	110
3.6. Experimental	114
3.6.1. The effects of corticotrophin and stress in normal subjects	114
3.6.2. The effect of angiotensin in normal subjects and in patients with Addison's disease and primary aldosteronism	126
3.6.3. The effect of haemorrhage	135
3.6.4. The effect of 'exercise' in patients with hypotension	138
3.6.5. The effect of sodium depletion	140
3.7. Discussion	148
Bibliography	163

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SUMMARY

A double isotope derivative method of measuring the concentrations of five biologically active corticosteroids in a single sample has been developed and evaluated using water, normal human peripheral plasma and also plasma from adrenalectomised patients and a few patients with primary aldosteronism. A number of physiological studies have been carried out with the dual purpose of further validating the method and of studying the acute effects of a number of factors on the peripheral plasma concentrations of the individual corticosteroids and on the relationship of these concentrations to each other.

Investigation of the effect of haemorrhage, undertaken as a control for other studies, revealed little significant effect on peripheral plasma corticosteroid concentrations. The effect of ACTH, either administered or induced by insulin hypoglycaemia was to consistently raise the concentrations of cortisol and corticosterone and, contrary to previous reports, to reduce the ratio between them. Aldosterone concentrations, however, were only raised in response to pharmacological doses of ACTH, or to insulin-induced hypoglycaemia in a subject who had previously been deprived of sodium for several days.

Cortexone and cortexolone concentrations in normal human subjects were also relatively unresponsive to ACTH administration or to hypoglycaemia.

Conversely, the infusion of mildly pressor doses of angiotensin increased peripheral plasma aldosterone levels in normal subjects and in a patient with primary aldosteronism, but no effect on cortisol or corticosterone concentrations could be discerned, even when pituitary secretion of ACTH was suppressed by the infusion of dexamethasone, Sodium depletion, both acute and chronic, resulted in a predictable increase in aldosterone concentration in normal subjects and in a patient with sodium-losing renal disease. Chronic sodium deprivation also caused significant increases in the level of corticosterone but those of cortisol, cortexone and cortexolone were not significantly altered.

It is hoped that these studies may contribute to our understanding of variations in the magnitude and composition of the secretion of the human adrenal cortex in response to changes in the environment of the gland.

NOMENCLATURE

The following trivial names have been used:

Aldosterone : 11β : 21-Dihydroxy-3 : 20-dioxopregn-4-en-18-al.

Aldosterone lactone monoacetate : 3 : 20-Dioxopregn-4-ene 18 $\rightarrow$ 11 lactone-21-acetate.

Corticosterone : 11β : 21-Dihydroxypregn-4-ene-3 : 20 dione.

11-Dehydrocorticosterone : 21-Hydroxypregn-4-ene-3 : 11 : 20-trione.

Cortisol : 11β : 17α : 21-Trihydroxypregn-4-ene-3 : 20-dione.

Cortisone : 17α : 21-Dihydroxypregn-4-ene-3 : 11 : 20-trione.

Cortexolone : 17α : 21-Dihydroxypregn-4-ene-3 : 20-dione.

Cortexone : 21-Hydroxypregn-4-ene-3 : 20-dione.

Dexamethasone : 9α-fluoro-16α-methylcortisol.

Prednisolone : 11β, 17α : 21-Trihydroxypregn-1 : 4-diene-3 : 20-dione.

Metapyrone : 2-methyl-1, 2-bis(3-pyridyl)-propen-1-one.

1. INTRODUCTION

The human adrenal cortex secretes a variety of steroid hormones into the blood-stream via the adrenal vein, and these are involved in the control of many facets of metabolism. The ubiquitous nature of the role played by these compounds has stimulated an intense and prolonged investigation of their chemical nature, metabolic effects and the mechanism by which their secretion is regulated. Our extensive knowledge of the steroidal constituents of the adrenal cortex and its secretion is largely due to the pioneering studies of Kendall, Reichstein and their contemporaries, and through their investigations and those of many other workers, the majority of these steroids can now be synthesised, further facilitating a study of their metabolism.

Advances in all branches of science depend upon the elaboration of new techniques. A study of the adrenal cortex is no exception and the acquisition of relevant physiological and pharmacological data has been conditioned largely by the availability of procedures for accurate estimation of the concentration of steroids in tissue and in biological fluids. Consequently, the main aims of the work described in this thesis were to develop or improve methods for the assay of

steroid hormones in peripheral plasma, and to apply these methods to an investigation of some aspects of the control of their secretion in man. Perhaps the most challenging and interesting topic in the field of adrenocortical physiology is the control of aldosterone secretion, and most emphasis has been placed on the determination and study of this hormone.

2. ADRENOCORTICAL SECRETION AND ITS MEASUREMENT.

2.1. The Nature of the Secretion.

Although detailed study has now succeeded in establishing the nature of the steroids present in adrenal vein blood from animals, much of our knowledge of the composition of human adrenocortical secretion has been assumed indirectly by extrapolation from these animal investigations. Only on a limited number of occasions has direct analysis of adrenal vein blood been achieved with satisfactory identification of the steroids present. A number of relevant publications are listed in Table I.

In addition, there is evidence for the production of oestrogens (Bulbrook & Greenwood, 1957) and progesterone (Short, 1960) by the adrenal cortex, and it is possible that many other steroids, possessing unidentified hormonal activity, are also secreted. Adrenocortical secretion is frequently altered by disease, either in magnitude or in composition, and much further investigation is needed before a full understanding of these changes is to be obtained.

While at the present time the significance of the production of sex hormones by the adrenal cortex is unknown, those steroids

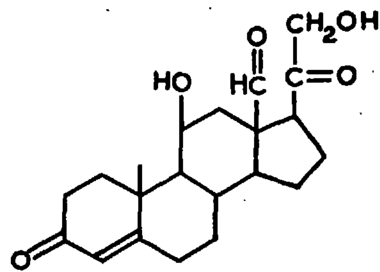
TABLE I. STEROIDS ISOLATED FROM HUMAN ADRENAL VEIN BLOOD.

Authors	Steroids
Sweat, Abbot, Jefferies & Bliss (1953).	Cortisol, corticosterone.
Romanoff, Hudson & Pincus (1953).	Cortisol, corticosterone, 11β-hydroxyandrost-4-ene-3 : 17-dione.
Bush, Swale & Patterson (1956).	Cortisol, corticosterone, 11β-hydroxyandrost-4-ene-3 : 17-dione, 3β-hydroxyandrost-5-en-17-one, 3α-hydroxy-5α-androstan-17-one.
Lombardo, McMorris & Hudson (1959).	Cortisol, corticosterone, cortexolone, 11β-hydroxy-androst-4-ene-3 : 17-dione, 17α-hydroxyprogesterone, 3β-hydroxyandrost-5-en-17-one.
Wieland, de Courcy, Levy, Zala & Hirschmann (1965).	11β-hydroxyandrost-4-ene-3 : 17-dione sulphate, 11β-hydroxyandrost-4-ene-3 : 17-dione, androst-4-ene-3 : 17-dione, androst-5-ene-3β : 17β-diol.

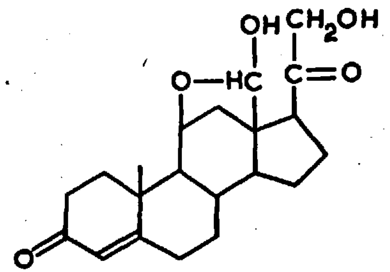
which influence carbohydrate metabolism, probably by changing the rate of protein catabolism (Long, Katzin & Fry, 1940), and those which control electrolyte metabolism profoundly affect the homeostasis of the body. From extensive pharmacological studies of these substances it is apparent that, while each compound may affect both spheres of metabolism, one or other of these effects usually predominates. For this reason they may be classified as glucocorticoids or mineralocorticoids.

Although the presence of other biologically active substances in the adrenal cortex cannot be excluded, the known influence of the gland in metabolism can largely be accounted for by cortisol, cortexolone, aldosterone, corticosterone and cortexone (Bush, 1954) (Fig. 1). Interest has therefore centred around the measurement of the concentrations of these compounds in biological fluids, and in fluctuations in the rate of their production caused by alterations in the internal and external environment of the body.

Assays of steroid hormones and their metabolites in urine have provided valuable information on the secretory activity of the adrenal cortex. Secretion rate methods are a more recent development (see section 2.2.1) and are based on the

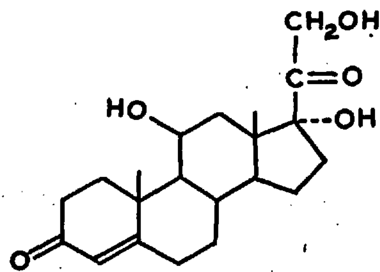


(Free aldehyde.)

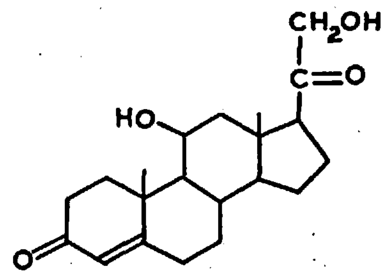


(Hemi-acetal.)

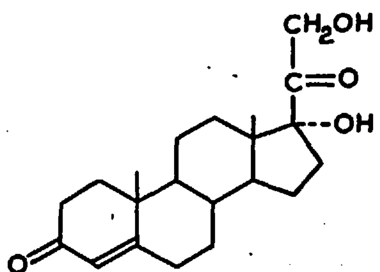
ALDOSTERONE



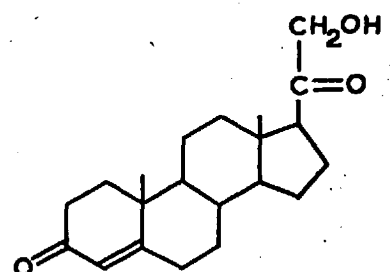
CORTISOL



CORTICOSTERONE



CORTEXOLONE



CORTEXONE

Fig. 1: Structural formulae of adrenocortical steroids.

oral or intravenous administration of radioactive steroid, followed by collection of urine over a period of at least 6 hours and more commonly 24 or 48 hours. The secretion rates can be calculated from the specific activity of the hormone or of a metabolite (see section 2.2.1) and are easier to interpret than excretion rates. While secretion rate measurements provide an integrated picture of the activity of the gland during the period of urine collection, little information about acute changes can be obtained in this way.

The estimation of steroid concentrations in blood or plasma is technically more exacting since frequently only very small quantities are present. In the human, where only peripheral blood is usually available, the concentrations are much lower than in adrenal vein blood, and the technical problems are consequently amplified. However, the advantages of such assays in tracing the minute-to-minute changes in adrenocortical activity are considerable and were the raison d'être of this study.

It has previously been stated that the adrenal cortex exerts its influence on metabolism by means of groups of hormones, rather than by a single steroid, and also that these substances may differ from each other in their ability to alter

carbohydrate or mineral metabolism. It seems possible, even likely, that the ratios of the concentrations of these compounds in blood and plasma may change as the adrenal gland responds to stimuli of different kinds. Those techniques, therefore, which measure the concentration of only one steroid, or alternatively those which estimate the total concentration of several, will ignore these changes and consequently will be open to misinterpretation.

Therefore, in a study of the factors affecting adrenocortical secretion in man, it is necessary to be able to follow short-term changes in the concentrations of as many as possible of the individual biologically active steroid hormones simultaneously, in order that both absolute and relative effects of stimuli to the adrenal cortex can be assessed.

In the following sections, the literature on which a choice of technique was based has been reviewed and a description of the development and validation of a multiple assay method for the five steroids listed above has been given. Physiological studies, described in section 3, were undertaken with the dual purpose of further demonstrating the validity of the method and also to compare the effects of alterations in the adrenal environment on the secretion of its constituent hormones.

2.2. Estimation of Adrenocortical Activity.

2.2.1. Secretion and excretion rate methods.

The activity of the adrenal cortex may be assessed by analysis of the steroid content of adrenal or peripheral vein blood and by measurement of the concentrations of the individual steroids or their metabolites in urine.

(a) Adrenal vein blood analysis.

Collection and analysis of adrenal vein blood provides in theory the most accurate estimate of adrenocortical activity. The concentrations of corticosteroids are relatively high so that assay is simplified, and secretion rates can be calculated for the short period of sample collection. However, the relative inaccessibility of the adrenal glands and their susceptibility to surgical stress (Sayers, 1950) renders such a procedure of questionable value, even in experimental animals. In addition, secretion by other means, such as into the lymphatic system, will not be measured by this technique, and insertion of a cannula into the adrenal vein may both decrease the adrenal perfusion rate by obstruction and increase the loss of steroid by alternative routes. Although controls can be compared with animals subjected to various treatments, it is doubtful whether true basal or resting secretion rates can be obtained in either

group by this method.

The problem has to an extent been overcome in the merino sheep (Blair-West, Coghlan, Denton, Goding, Wintour & Wright, 1963) by transplanting the adrenal gland to the voluminous skin folds of the neck. After a recovery period, the adrenal vein effluent can be repeatedly sampled with little apparent discomfort to the animal.

While much valuable information can be obtained in this way, the dangers of extrapolating data from one species to another are apparent from a consideration of the differences of aldosterone metabolism between man and the rat (Eilers & Peterson, 1964; Bojesen, 1966). Adrenal vein techniques are rarely applicable to man.

(b) Urinary excretion rates.

A valuable alternative measure of adrenal activity is the analysis of urine for steroid hormones and their metabolites. Urine collected over a period of 24 hours contains such compounds in quantities far greater than in the volume of adrenal vein blood normally available from an animal. However, the interpretation of assays of steroid hormones in urine is complicated by the fact that these may represent only a small proportion of the adrenocortical

secretion. Moreover, the ratio of the unchanged hormone to its metabolites may very from person to person and from day to day. This is certainly true of cortisol (Cope & Black, 1958; James & Caie, 1964) and may also be the case with aldosterone (see discussion by Tait & Tait, 1962). The excretion rate of a urinary metabolite, such as tetrahydrocortisol or the 3-oxo-conjugate of aldosterone may thus bear a variable relationship to the secretion rate of the parent compound.

Providing that, during the period of study, all or most of the metabolites of a given hormone are excreted via the kidney, methods which measure all, or a major fraction, of the urinary metabolites will give values which approximate to the secretion rate of the hormone. Such methods, based on the reducing properties of the α-ketol side chain of many metabolites, have been described by Talbot, Saltzman, Wixom & Wolfe (1945), Heard, Sobel & Venning (1946), and Chen, Wheeler & Tewell (1953), and another method, relying on the reaction of phenylhydrazine in sulphuric acid with the group of metabolites possessing the 17α, 21-dihydroxy-20-ketone group, has been used to obtain an estimate of cortisol secretion rate (Porter & Silber, 1950). The methods

of Norymberski (1952) and Appleby, Gibson, Norymberski & Stubbs (1955) measure the quantities of 17-oxosteroids and 17-hydroxy corticosteroids in urine.

(c) Estimation of secretion rates by isotope methods.

Information which is far easier to interpret can be obtained by indirect methods of measuring secretion rates. Two basic techniques have been developed for this purpose, both of which rely on the use of pure steroids labelled with [^{3}H] or [^{14}C], which if administered to an experimental subject, are assumed to mix rapidly with the endogenous steroid and to be metabolised with it by identical pathways and at an identical rate.

(i) Isotope dilution methods.

In one technique, the isotope content (i.e., specific activity) of a urinary metabolite known to be derived solely from the steroid under investigation (i.e., a 'unique metabolite') is compared with the specific activity of the administered dose, and the secretion rate is calculated from the degree of dilution of the urinary isotope. Thus, after a period of high secretion rate, the specific activity of the unique metabolite will be much reduced by dilution with unlabelled endogenously synthesised steroid. Such methods

have been developed for cortisol (Cope & Black, 1958), corticosterone (Peterson, 1957), aldosterone (Johnson, Lieberman & Mulrow, 1957 ; Cope, Nicolis & Fraser, 1961) and cortexone (Crane & Harris, 1966; Biglieri, Herron & Brust, 1966).

(ii) <u>Measurement of secretion rates using blood</u>.

An alternative method of measuring the secretion rate of a steroid hormone relies on the rate of disappearance of a single injected dose of radioactive steroid from the peripheral circulation. During steady-state conditions in which the rate of steroid secretion into the bloodstream is equal to the rate of removal, the specific activity of the steroid in the peripheral circulation will fall logarithmically, and from this fall the secretion rate can be calculated (Peterson, 1959). This relatively simple technique is applicable to the measurement of the secretion rates of those steroids which are bound to plasma proteins, since their distribution in the body approximates to a single compartment, but the disappearance curves of the less firmly bound steroids are composed of more than one exponential, representing a distribution in more than one compartment, and calculation of secretion rates from these curves leads to considerable inaccuracy. For those steroids,

the secretion rate is more accurately measured by the continuous, constant rate infusion of radioactive steroid until equilibrium with the endogenous circulating steroid is reached. The secretion rate is then calculated from the infusion rate, the final radioactive concentration of the hormone and the non-isotopic steroid plasma concentration (Tait, Little, Tait & Flood, 1962).

The general problems of interpretation of secretion rate measurements are discussed by Tait (1963). However, whatever the validity of these methods, their use is confined to circumstances where changes in steroid secretion occur gradually over the course of days or weeks, whereas many of the determinants of adrenocortical secretion rates exert their effects extremely rapidly. Corticotrophin, for example, causes an increase in plasma cortisol concentration within minutes of administration to human subjects (Landon, James, Cryer, Wynn & Frankland, 1964). It is not possible, using the techniques described in (i) and (ii), to discern such acute changes.

On the other hand, changes in the concentrations of steroids in peripheral blood or plasma can be studied at much shorter intervals and acute alterations can therefore be demonstrated. However, such methods are not without disadvantages. Changes in steroid concentration in the peripheral circulation depend,

not only on the rate of secretion of the substance, but also on the rate at which it is removed from the blood (i.e., the metabolic clearance rate). Corticosteroids are present in low concentrations in peripheral blood and withdrawal of volumes sufficient for accurate measurement of concentration may itself provoke adrenocortical secretion. With these reservations, the blood concentration of a hormone represents most closely that to which the tissues are subjected and, as such, may have more relevance to studies of the importance of steroids in metabolism. In a study of the control of adrenocortical secretion, as in eighteenth and nineteenth century politics, 'little is accomplished without bloodshed'.

2.2.2. The Determination of Steroids in Blood and Plasma.

2.2.2.1. Extraction and purification of corticosteroids

(a) The blood sample.

The concentrations of corticosteroids in the peripheral circulation can be measured using whole blood, plasma or serum. It is possible that the steroids may not be uniformly distributed between the cells and the plasma, and whole blood assay data would therefore be easier to interpret than those obtained from either plasma or serum. However, some steroids are not readily extracted from blood cells. For example, incubation of red

corpuscles with radioactive cortisol (Bush, 1957) showed that this hormone was rapidly absorbed and adsorbed by the cells and subsequent extraction recovered only about 30%. Evidence also exists that the corticosteroids present in plasma migrate into the erythrocytes when blood is allowed to stand (Migeon, 1957), but this is precisely the procedure required to obtain serum. Bojesen (1964) has reported that aldosterone distributes itself between corpuscles and plasma according to the distribution of water so that date from assays of plasma should correspond to those obtained from whole blood, and since little information is available on the distribution of the remaining corticosteroids in blood, or of the labelled compounds added to blood, plasma would appear to be the most satisfactory medium for assays.

It is recommended (Péron, 1962) that plasma should be separated by centrifugation as soon as possible after withdrawal and stored in a refrigerator or deep freeze cabinet. The effect of refrigeration on corticosteroids in plasma is the subject of conflicting reports (Braunsberg & James, 1960; Guillemin, Clayton, Smith & Lipscombe, 1958; De Moor, Steeno, Raskin & Hendriks, 1960), and should therefore be investigated during studies involving lengthy storage of samples. In

experiments where serial samples over a period of several hours are required, it may not be possible to separate the plasma immediately and in this case, the samples should be quickly cooled to reduce loss of steroid to the erythrocytes (Neher, 1958).

Care must also be taken that the use of anticoagulants does not interfere with the method of assay to be employed. For example, heparin does not affect the results of colorimetric or fluorimetric assay (Nelson & Samuels, 1952; Guillemin et al., 1958), but citrate and oxalate may both interfere with the former method (Nelson & Samuels, 1952). It is unlikely that methods involving extensive purification will be affected by these compounds.

Standardisation of sampling time and also of the posture of the experimental subject during sampling may also be critical. It is well known, for example, that corticotrophin secretion is subject to a nycthemeral rhythm and plasma concentrations of the corticosteroids which it controls will consequently also vary with time. More recently, nycthemeral rhythms in the production and excretion of aldosterone, attributable at least partly to postural changes, have been described in man (Gordon, Wolfe, Island & Liddle, 1966). Unless these conditions of

sampling are standardised, such changes will be superimposed on the results of experimental treatment of the subject.

(b) Direct estimation of corticosteroids in plasma

Only rarely can corticosteroid concentrations be estimated without some prior purification. Cortisol in plasma is bound specifically to a protein fraction - 'transcortin' (Daughaday, 1956) - and competitive binding to this protein has been made the basis of a rapid and sensitive assay of cortisol (Murphy & Pattee, 1964). A similar method has been described by Nugent and Mayes (1966). The extent and specificity of binding to plasma proteins by other corticosteroids is variable and, as yet, no similar methods of assay for these compounds have been developed. The majority of assay methods have relied on extraction of the steroids from plasma, usually followed by some degree of purification.

(c) Extraction with organic solvents.

Steroid hormones are readily extracted from aqueous media by partitioning with organic solvents. In addition to providing an advantageous partition coefficient for corticosteroids, suitable solvents should be easily purifiable, stable after purification and should not present a hazard to the health of laboratory personnel or to the structural integrity of the

substances to be extracted. The properties of the solvents commonly used for extractions from urine and plasma have been reviewed by McLaughlin, Kamieki and Gray (1958) and Braunsberg and James (1961) who concluded that there was little to choose between ethyl acetate and methylene chloride for corticosteroids. However, Burstein and Kimball (1963) have shown that ethyl acetate is unstable and must be repurified at frequent intervals. Failure to do so may result in destruction of corticosteroids in the extract due to the formation of peroxides in the solvent.

Methylene chloride, on the other hand, is stable for long periods after purification (Peterson, 1957) and its low boiling point facilitates evaporation from plasma extracts at temperatures low enough to avoid thermal damage to the corticosteroids. Unfortunately, it is toxic and must be handled with circumspection. Another disadvantage of this solvent is its tendency to form emulsions with plasma, but this can be overcome by using large volumes of organic phase compared with aqueous phase. Recoveries of cortisol and corticosterone from plasma by a single extraction with methylene chloride (ratio - plasma : solvent, 1 : 5 v/v.) are estimated to be in excess of 90% (Braunsberg & James, 1961). This procedure rapidly denatures plasma proteins, breaking steroid-protein bonds, and good recoveries of all the biologically

active corticosteroids are obtained (Bush, 1957).

(d) Assay of crude extracts for corticosteroids

Simple fluorimetric techniques (see below), such as that of Mattingly (1962), are based on the development of fluorescence in the crude methylene chloride extract. Although such methods are purported to measure only 11-hydroxycorticosteroids, the most important of which are cortisol and corticosterone, a direct comparison of this technique with the more specific double isotope derivative assay (Fraser & James, 1967) has shown that the fluorescence cannot be accounted for entirely by these two compounds (James, Townsend & Fraser, 1967). In some samples, as much as 50% of the fluorescence was non-specific and little improvement resulted from the preliminary treatment of the crude extract by the benzene partition recommended by Gornall and McDonald (1953). Interference from plasma cholesterol (McLaughlin et al., 1958) or 20β-hydroxysteroids (De Moor et al., 1960) may be partly responsible for these discrepancies.

(e) Preliminary purification of the extract

Purification procedures vary considerably from method to method. A large measure of the specificity of the longer procedures is conferred upon them by rigorous purification.

Where the concentrations of more than one corticosteroid are to be measured, preliminary separation must be carried out.

The removal of acidic compounds such as oestrogens, fatty acids and numerous coloured pigments by means of an alkaline wash is standard practice. Dilute sodium hydroxide is most commonly used, but minor destructive losses of some steroids may occur (Venning, 1954), although this does not seem to be the case with cortisone (Romanoff, Rodriguez, Seelye & Pincus, 1957). The extract is then washed with dilute acid and finally with water. Volumes of aqueous solutions should be minimised to prevent loss by partition, and back-extraction of the pooled washes with organic solvent may improve recoveries (Péron, 1962).

(f) Chromatography

Corticosteroids have no remarkable chemical properties to distinguish them from each other, so that small differences in their physical properties must be employed to obtain further purification. Chromatographic techniques have been widely used for this purpose. A number of methods of measuring the concentration of more than one corticosteroid in a single biological sample have

been published and these rely for purification on one or more chromatographic steps. Some of these publications are listed in Table 2. The methods described in the publications are further discussed in later sections.

Chromatographic analysis has been adapted to the separation of steroid alcohols (e.g. Braunsberg & James, 1960) acetates (e.g. Kliman & Peterson, 1960) or other derivatives (e.g. Bojesen, 1966). Steroids from dog adrenal vein blood and from adrenal tissue have also been separated and purified by countercurrent distribution (Cartensen, 1962) but this procedure has been described by its author as long and tedious and would be difficult to adapt to the analysis of large numbers of plasma samples.

There is a limit to the degree of purity which can be obtained by repeated chromatography and the most successful procedures include, during purification, a chemical step which alters the polarity of the steroid. For example,Ködding et al. (1962) begin purification with the corticosteroids in the form of free alcohols, converting these to acetates at a later stage, while Peterson, Kliman and Bollier (1957) convert aldosterone diacetate to aldosterone lactone monoacetate during purification. Bojesen (1966) synthesises p-toluene sulphonate derivatives of aldosterone and cortico-

TABLE 2. MULTIPLE STEROID ASSAY TECHNIQUES

STEROIDS	AUTHORS
Cortisol, corticosterone.	McLaughlin et al., 1958. Ely, Hughes & Kelly, 1958. Braunsberg & James, 1960. Eechaute, 1966.
Cortisol, cortexolone, corticosterone.	Hoet, Materazze & Ekka, 1964.
Aldosterone, corticosterone, cortisol.	Ködding, Wolff, Karl & Torbica, 1962 Stachenko, Laplante & Giroud, 1964.
Cortisol, cortexolone.	Kliman, 1966.
Aldosterone, cortexone.	Rapp & EikNes, 1966.
Aldosterone, corticosterone.	Bojesen, 1966.

sterone, which are then oxidised and finally converted to the corresponding hydrazones. To obtain the maximum benefit from these chemical changes, the reactions should be as specific as possible to the compound being purified. Enzyme-catalysed reactions have a high degree of specificity, a fact which has been employed in the purification of the acetates of cortexone and cortexolone described in section 2.3.7. Where radioactive derivatives, such as acetates, are synthesised, derivatisation of the steroids in the crude extract produces the best results in terms of purity and recovery.

Chromatography on columns, thin layers and paper have each been used in purification schemes. Thin layers of silica gel have the advantage of rapid development, and incorporation of fluorescent compounds makes possible the location, visually in ultra-violet light, of microgram quantities of steroids possessing the 4-en-3 -one group characteristic of the biologically active corticosteroids. However, difficulties have been experienced in eluting the more polar corticosteroids such as cortisol from thin layers of silica gel (James, 1965). Mattox and Mason (1956) also report losses of cortisol on thin layers which they ascribe to the conversion of the steroid to 11β -hydroxyandrostenedione,

and Neher (1964) states that aldosterone may decompose during application to thin layer plates.

A more detailed study of cortisol losses during thin layer chromatography has been made by Idler, Kimball and Truscott (1966). Their recoveries varied with the type of silica gel used and were particularly reduced when two thin layer chromatography systems were used consecutively. Losses of cortisol acetate were less serious, but the authors suggest that steroid diacetates may be variably hydrolysed to monoacetates. It is not possible to ascertain from the results whether the losses of cortisol which they describe were due to degradation or incomplete elution of the compound.

The use of thin layer systems is particularly valuable for initial purification of bulky extracts of biological material since they have a high capacity, and also when radioactive derivatives have been synthesised. In the latter case, the excess reagent and the radioactive derivatives of contaminants remain on the thin layer plate and do not contaminate the chromatography equipment as they might in an over-run paper chromatography system (Burger, Kent & Kellie, 1964).

Further purification has most frequently been accomplished

by paper chromatography using the systems described by Bush (1952) or modifications thereof. Reverse phase systems, in which the polar aqueous methanol phase is mobile, have also been used to advantage (Burger et al., 1964). In general terms, efficient purification is only to be achieved by judicious variation of the type of chromatography system used and by transformation of the polarity of the compound involved by derivative synthesis.

As the number of purification steps increases, total losses of corticosteroids will obviously become greater, and it therefore becomes necessary to correct for these losses. Fortunately, many of the corticosteroids are now commercially available as pure substances labelled with [^{14}C] or [^{3}H], and addition of small quantities of these early in the procedure, allows an accurate correction to be made by assaying an aliquot of the final residue for radioactivity.

2.2.2.2. Methods of assaying purified corticosteroids

Many current methods were originally developed for the measurement of urinary steroid concentrations and have since been adapted to plasma analysis. However, while the quantities of steroid present in extracts of urine are comparatively large, if the results reported by Tait et al. (1962) and Kono & Mikaye (1964) are correct, concentrations of aldosterone in normal human peripheral plasma may only be in the range 5-15 mμg./100 ml. That is, in a 25 ml. sample of plasma a maximum of 4 mμg. may be present, 70-90% of which may be lost during preliminary purification. Thus, the analytical problem presented is to measure specifically and precisely 1 mμg. or less of steroid. While concentrations of cortisol in these plasma samples may be a thousand times greater than this, those of cortexolone (Kliman, 1966) and corticosterone (Braunsberg & James, 1961) are probably also very low. The concentration of cortexone in human adrenal vein blood (Biglieri, 1965) suggests that unless the metabolic clearance rate of this steroid is markedly less than for other corticosteroids, its concentration in normal peripheral plasma is little higher than that of aldosterone. Thus, choice of an assay method will be largely governed by its sensitivity.

(a) Colorimetric methods.

It is doubtful whether the colorimetric techniques at present available could be modified to provide the necessary sensitivity. Those which rely on the reducing properties of corticosteroids are particularly lacking in specificity and sensitivity. Chen, Wheeler and Tewell (1953), for example, were able to measure a minimum of 5 μg. of reducing steroid with tetrazolium blue (3 : 3' -dianisole-bis-4 : 4' -3 : 5-diphenyl-tetrazolium chloride) in alkaline solution. Development of the colour is difficult to standardise and background colour is high, although improvements have resulted from the use of the organic base tetramethylammonium hydroxide (Weichselbaum & Margraf, 1957). A greater sensitivity (2 μg.) has been achieved in the measurement of urinary aldosterone concentration (Neher & Wettstein, 1956; Nowaczynski, Koiw & Genest, 1957; Dyrenfurth & Venning, 1959), but this is never-the-less inadequate for peripheral plasma steroid analysis.

The method of Porter and Silber (1950) (see section 2.2.1) has also found wide use in the estimation of plasma steroid concentrations. It is more specific than the reduction of tetrazolium blue and may also be more sensitive (Peterson,

Karrer & Guerra, 1957) although Weichselbaum and Margraf (1957) found little difference in sensitivity between the two techniques. Whilst the phenylhydrazine reaction is not applicable to the measurement of corticosterone and cortexone concentrations, it has been successfully adapted to the determination of aldosterone in urine (Lewbart & Mattox, 1962) where, however, the sensitivity limit (c. 1 μg.) falls far short of the requirements of a plasma method.

(b) <u>Ultra-violet absorption and fluorescence measurement.</u>

Absorption of ultra-violet light at 240 mμ. by the 4-en-3-one group of the corticosteroids has also been adapted to their determination (Weichselbaum & Margraf, 1957; Nowaczynski et al., 1959) but again high background absorption, even following extensive chromatographic purification and acetylation (Gerst, Schamway, Schwarz & Farrell, 1960) prohibits the measurement of quantities of less than 3 μg. of steroid.

When treated with ethanolic sulphuric acid and then activated with light at 465 mμ., cortisol and corticosterone produce an intense fluorescence which is readily measured by a fluorimeter. While this reaction is by no means specific (see section 2.2.2.1), it has never-the-less been the basis of a number of simple but valuable techniques such as have

previously been discussed. Assessed by the determination of pure steroids fluorimetric techniques are potentially of a much higher order of sensitivity than colorimetry, but when used with extracts of biological fluids containing a multiplicity of fluorogenic substances, the sensitivity in practice is considerably less than theoretical. However, sensitivity of 0.5 mμg. (Glick, von Redlich & Levine, 1964) is certainly adequate for the determination of cortisol and corticosterone in normal human peripheral plasma. Purification of steroids from plasma extracts by means of chromatography on silica gel columns (Sweat, 1955; Braunsberg & James, 1960) or on paper (Peterson, 1957) both increases specificity and allows individual measurements of cortisol and corticosterone concentrations to be made in the same plasma samples. Unfortunately, neither cortexone nor cortexolone fluoresce when treated in this way.

When heated with sodium hydroxide and activated with ultra-violet light aldosterone also produces a characteristic fluorescence which can be developed directly on a paper chromatogram, and fluorescence intensity compared with standard quantities of aldosterone, either visually (Flood, Layne, Ramcharan, Rossipal, Tait & Tait, 1961) or by means

of a fluorimeter (Ayres, Simpson & Tait, 1957; Tait & Tait, 1960). However, the sensitivity of this technique (0.1 µg.) is not adequate to measure the quantities of aldosterone normally present in plasma.

(c) Enzyme methods and gas-liquid chromatography.

The method of Margraf, Margraf & Weichselbaum (1963) is based on the reduction of the α ketol side chain, present in all biologically active corticosteroids, by $NADH_2$, a reaction catalysed by the enzyme 20β-hydroxysteroid dehydrogenase. The oxidation of $NADH_2$ can be measured by spectophotometry at 340 mµ. and is proportional to the quantity of steroid in the reaction mixture. While this technique has the advantage that it can be applied to each of the corticosteroids to be studied, the sensitivity (0.2 µg.) is again too low.

Little success has been obtained in the determination of corticosteroids by gas-liquid chromatography because these compounds are particularly prone to thermal destruction. In theory, the combination of halogenated steroid derivatives and electron capture detection could be developed into methods of very high sensitivity if the problem of degradation on the column can be overcome.

(d) Double isotope derivative assay techniques.

None of the techniques so far described can be applied to all the corticosteroids under review while at the same time being sufficiently sensitive to measure their concentrations in normal human plasma. The methods of choice, possessing both these qualities, are those which employ the formation of radioactive derivatives by esterification of one or more alcohol groups, notably the 21-hydroxyl group, present in all the biologically active corticosteroids. The ester is carefully purified and the quantity determined from a knowledge of the specific activity of the esterifying reagent. Pure corticosteroids labelled with an isotope different from that in the reagent are mixed with the plasma to determine recoveries during purification, and non-radioactive 'carrier' ester, added immediately after derivatisation of the steroids in the extract, does not affect the final assay but allows ease of location of the compound during purification.

In theory, the sensitivity of these methods depends solely on the specific activity of the esterifying reagent while specificity relies, perhaps even more so than in colorimetric and fluorimetric techniques, on the efficacy

of the purification procedures.

Acetic anhydride, p-iodosulphonylic anhydride and p-toluene sulphonic anhydride have been used to form their respective esters. Kliman and Peterson (1960) introduced the use of [^{3}H]-acetic anhydride for the assay of aldosterone in urine and adrenal vein blood, correcting for recoveries by adding aldosterone-18 : 21-[^{14}C] diacetate immediately after acetylation of the steroid in the crude neutral extract, since [^{14}C]-aldosterone was not available at that time. Unfortunately, losses during the preliminary extraction and acetylation are not corrected for in this method. Similarly, in the method of Ködding et al. (1962), the residue from extraction of the plasma passes through two chromatographic purification stages before acetylation and subsequent addition of the recovery standard, although [^{14}C]-cortisol and [^{14}C]-corticosterone are added to the plasma before extraction. A similar scheme has been used to measure aldosterone, cortisol and corticosterone concentrations in rat adrenal vein blood and in <u>in vitro</u> media containing slices of rat adrenal cortex (Stachenko, Laplante & Giroud, 1964).

Coghlan, Hudson, Wintour and Dulmanis (1965) and Coghlan, Cain, Dulmanis, Hudson and Scoggins (1966) used [^{14}C]-aldosterone,

added to plasma, to monitor recoveries in their method of determination of peripheral plasma aldosterone with $[^{3}H]$-acetic anhydride in sheep and man, and an analogous method for cortexolone and cortisol in man has also been developed (Kliman, 1966). The use of $[^{14}C]$-acetic anhydride and $[^{3}H]$-corticosteroids (Peterson, 1964), while having some advantages (see later), is less acceptable because the reagent is prohibitively expensive and available at a lower specific activity than its $[^{14}C]$-labelled equivalent.

The synthesis of esters with $[^{35}S]$-p-iodosulphonylic anhydride, and the use of corticosteroid $[^{131}I]$-p-iodosulphonates as recovery standards (Bojesen & Degn, 1961) has been adapted to the measurement of steroids in dog peripheral plasma. The recovery standard can only be added after esterification and the method has other serious disadvantages. For example, these derivatives are less stable than acetates and therefore more difficult to purify, and $[^{35}S]$ and $[^{131}I]$ are isotopes with relatively short half-lives, a fact which may assume considerable importance in lengthy purification procedures. In addition, the reagent cannot be stored before use for more than a short time. A method using $[^{35}S]$-p-toluene sulphonic anhydride with

$[^{3}H]$-corticosteroids has proved to be of extreme sensitivity in the hands of Bojesen (1966).

Ketonic reagents, such as thiosemicarbazide, form thiosemicarbazones with ketone groups such as are present in positions 3 and 20 of all biologically active corticosteroids. $[^{35}S]$-Thiosemicarbazide forms the basis of a sensitive method for the measurement of plasma concentrations of testosterone (Riondel, Tait, Gut, Tait, Joachim & Little, 1963; Lim & Brooks, 1966) and progesterone (Riondel, Tait, Tait, Gut & Little, 1965), but it has not been used to determine corticosteroids.

A combination of $[^{3}H]$-acetic anhydride with the use of $[^{14}C]$-corticosteroids was chosen for the study described in this thesis because of the chemical simplicity of acetylation and the stability of steroid acetates. The anhydride is relatively inexpensive and is available at very high specific activity. The following sections describe the development of a multiple assay technique and the evaluation of this technique for the determination of corticosteroids in human peripheral plasma.

2.3. Development of Multiple Assay Technique.

2.3.1. Materials.

All solvents except methanol and ethanol were obtained from Hopkins & Williams, Ltd. Methanol was purchased from Imperial Chemical Industries, and ethanol was ER quality from James Burroughs. Dioxane, carbon tetrachloride, cyclohexane and toluene were used without further purification; benzene and ethanol were purified as described by Bush (1961), and methylene chloride was redistilled. Acetic acid was refluxed over chromium trioxide (Hopkins & Williams, Ltd.) (20g./litre) for four hours. This procedure was repeated twice more, decanting the acid on to fresh chromium trioxide each time. Finally it was redistilled and stored at 4°C. The oxidation reagent - 0.5% chromium trioxide (A.R. Hopkins & Williams, Ltd.) in acetic acid - was prepared immediately before use. Pyridine was refluxed over potassium hydroxide pellets, and then distilled from fresh pellets; acetic anhydride was refluxed over calcium carbide and then redistilled. Both pyridine and acetic anhydride were stored at 4°C. [^{3}H]-Acetic anhydride (100mC/mM, 15% in benzene, Radiochemical Centre, Amersham) was twice distilled <u>in vacuo</u> immediately before use (Henderson, Crowley & Gaudette, 1964). The specific

activity of the anhydride was measured as described by Kliman and Peterson (1960) using acid-induced fluorescence to measure the concentration of cortisol acetate. Aldosterone-18 : 21-diacetate was prepared by acetylating d-aldosterone, and purified by chromatography in systems 1 and 2 (see below). A solution of approximately 1 mg./1 ml. was prepared in methanol : ethyl acetate (1:1, v/v). Solutions of the 21-acetates of cortisol, cortexone, cortexolone (Steraloids, Ltd.) and corticosterone (Sigma, Ltd.) were prepared at similar concentrations. [^{14}C]-labelled aldosterone (46 mC/mM), corticosterone (8 mC/mM) and cortisol (30 mC/mM) were obtained from Tracerlab Inc., Waltham. Mass., U.S.A. as a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda. U.S.A. [^{14}C]-Cortexone and [^{14}C]-cortexolone (35 mC/mM) were purchased from The New England Nuclear Corp., Boston. Mass., U.S.A. The [^{14}C]-steroids were purified before use by chromatography in system C (Bush, 1952) and were stored at 4°C in 10% methanol in benzene. Purification was repeated at intervals of approximately 8 months during the study. 20β-Hydroxysteroid dehydrogenase (Boehringer Corp.) was stored at -20°C., and a solution of 5mg./ml. of $NADH_2$ in tris-buffer (0.015 M, 0.2% EDTA, pH 8.0) was prepared as required.

Whatman chromatography paper No. 2 was used as received except for final stage chromatograms, for which it was washed chromatographically with ethyl acetate : methanol (1 : 1 v/v; 100ml./40 cm. width) shortly before use. The scintillator solution used was 0.3% terphenyl and 0.004% dimethyl POPOP (Nuclear Enterprises, Ltd.) in toluene. Silica gel GF_{254} was obtained from Merck, Ltd.

All glassware was rinsed in unpurified methanol and water immediately after use. Apparatus used for acetylation or for thin layer chromatography was steeped in chromic acid or strong detergent (Decon 75; Gallenkamp) overnight and rinsed thoroughly in tap water. All apparatus was cleansed in warm, strong detergent (Pyroneg; Diversey, Ltd.) solution (c. 45°C., for 45 mins.) in an ultrasonic agitator (Ultrasonics Industries, Inc.). Finally, it was rinsed several times in warm tap water, then in glass-distilled water, and dried in an oven.

Heparinised blood samples were centrifuged as soon as possible after collection. Where some delay was inevitable, blood samples were cooled in a refrigerator until separation was possible. The plasma was divided into aliquots, usually 25 ml., and stored at -20°C until required for assay.

2.3.2. Extraction and acetylation of corticosteroids

Known quantities of [^{14}C]-steroid (750 dpm. aldosterone, 1125 dpm. corticosterone, 5000 dpm. cortisol, 750 dpm. cortexolone, 750 dpm. cortexone), in 25-30 µL were added to plasma before extraction. The amounts of [^{14}C]-cortisol and [^{14}C]-corticosterone added were reduced to 900 dpm. and 725 dpm. respectively during the latter part of the study. The plasma was then extracted (1 x 200 ml. of methylene chloride) and the extract washed (1 x 20 ml. N/10 sodium hydroxide, 1 x 20 ml. N/10 acetic acid, 1 x 20 ml. distilled water) and evaporated to dryness as described by Kliman and Peterson (1960). The residue was transferred to a stoppered centrifuge tube in methylene chloride (3 x 3 ml.) which was evaporated at 35-40°C under a stream of nitrogen. The residue was carefully washed down to the bottom of the tube and placed in a vacuum desiccator overnight. Apparatus required for purifying and dispensing the radioactive acetic anhydride was also placed in the desiccator.

After redistilling the acetic anhydride the dry residues were dissolved in 0.025 ml. of pyridine and 5 mC. (0.025 ml.) of anhydride solution. The tubes were tightly stoppered. To avoid radioactive contamination of the laboratory the tubes were then placed in a small desiccator containing potassium

hydroxide pellets, which in turn was placed in an incubator at 37°C for 24 - 48 hours.

Excess anhydride was hydrolysed with 0.5 ml. of $^{N}/10$ hydrochloric acid (3 hours at 37°C) (Burger, Kent & Kellie, 1964) and the [^{3}H]-acetates extracted (1 x 5 ml. of carbon tetrachloride) and washed (1 x 1 ml. water) as described by Kliman and Peterson (1960). Approximately 200 μg. of each of the appropriate non-radioactive carrier acetates was added to the carbon tetrachloride extract, which was then evaporated to dryness in a 40°C water bath under a stream of nitrogen. All subsequent evaporations were carried out in this way.

2.3.3. Separation of the major corticosteroids.

Crude fractions containing individual corticosteroids were obtained by means of chromatography on thin layers (250 μ.) of silica gel. Residues from acetylation were applied to the origin in ethyl acetate : methanol 1:1 v/v (1 x 0.05 ml., 2 x 0.025 ml.) as a series of three or four spots using a fine Pasteur pipette constructed of carefully washed glass tubing which was discarded after use. The plates were developed in a methanol : benzene (1:9 v/v) system. The required steroid acetate zones (Fig. 2) were located in ultra-violet light (220-230 mμ., Chromatolite, Hanovia, Ltd.) and removed carefully,

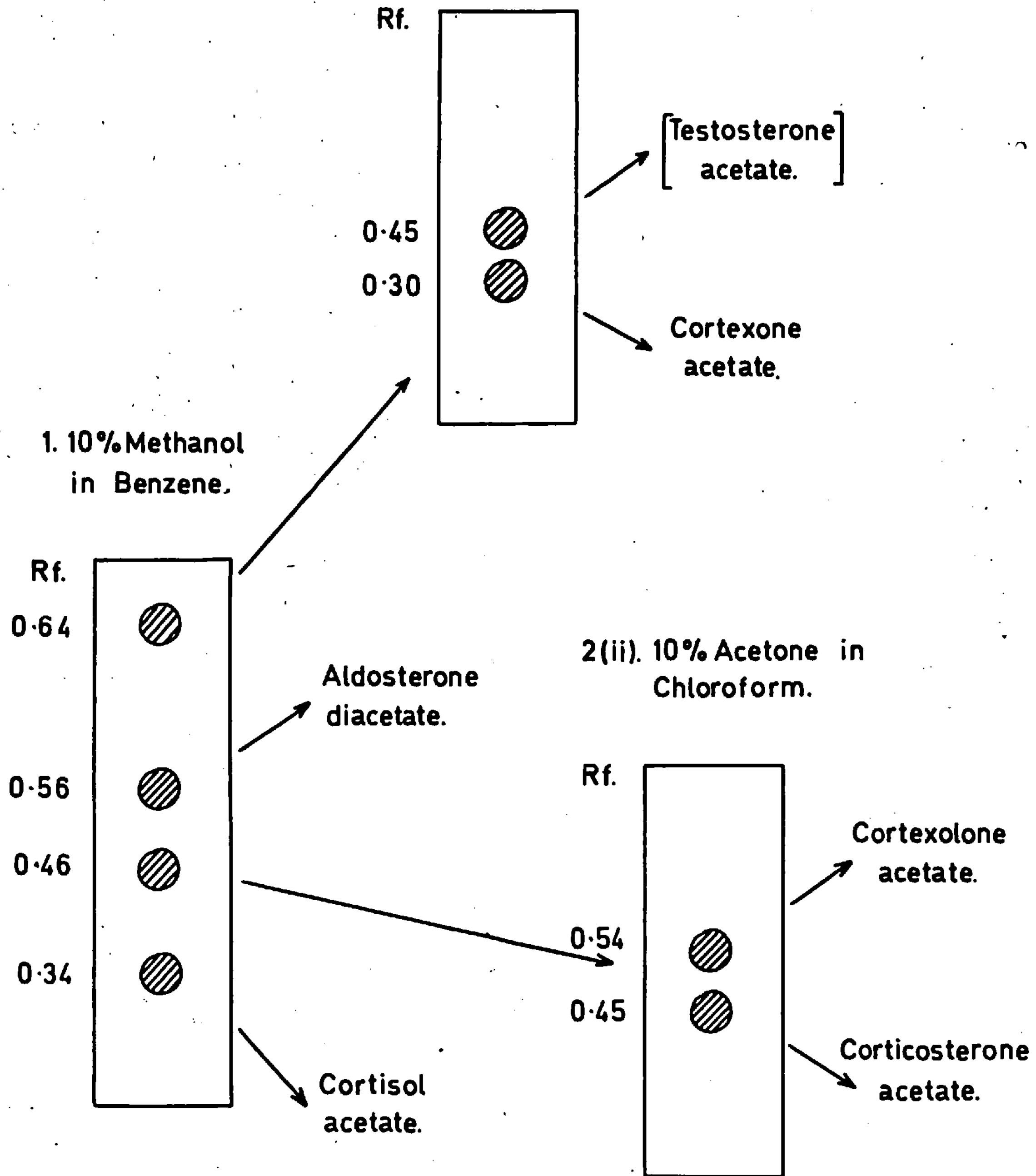


Fig.2: The separation of major corticosteroids by thin layer chromatography.

using a spatula, to a test tube where the substance was eluted (3 x 3 ml. of ethyl acetate : methanol, 1:1 v/v, containing 1% of water). The eluates were evaporated to dryness.

This chromatography system was inadequate for the separation of the acetates of corticosterone and cortexolone. Cortexone and testosterone acetates also migrated at similar rates. In order to separate these compounds, further thin layer systems were used. The residue containing corticosterone and cortexolone acetates was rechromatographed in a system containing 10% acetone in chloroform v/v; 25% Acetone in hexane was used for the mixture of cortexone and testosterone acetates. Application and elution were carried out as described above. Migration rates are also shown in Fig. 2. The following sections describe the further purification of the individual steroid acetates and their final estimation.

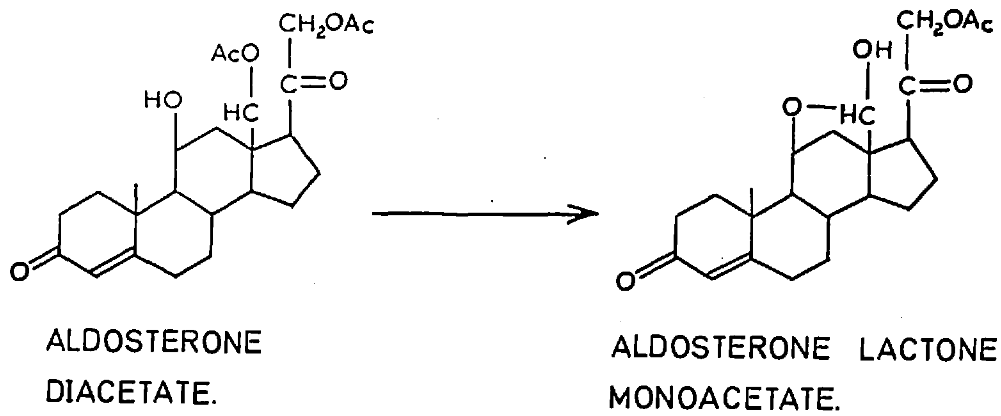
2.3.4. Purification of aldosterone, corticosterone and cortisol acetates

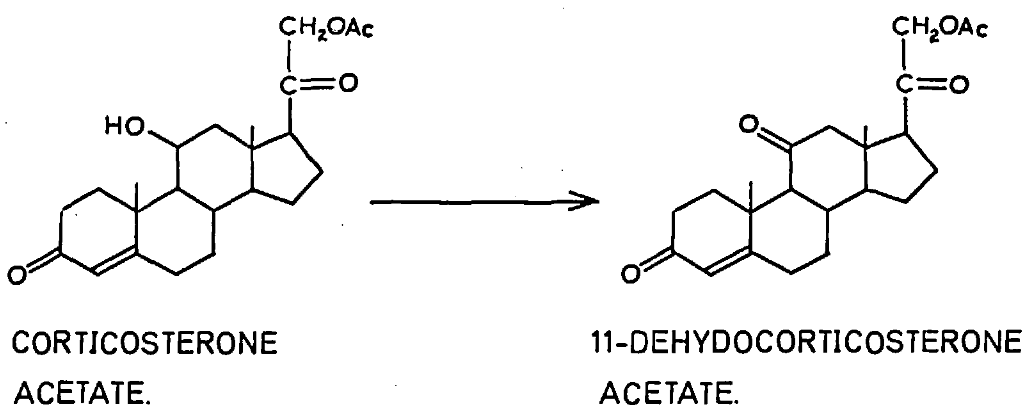
The acetates of aldosterone, corticosterone and cortisol were purified separately using successively the paper chromatography systems 1 and 2 of Kliman and Peterson (1960) (see Table 3). The approximate development times of these systems, maintained at a constant temperature of approximately 24°C, are shown in Table 4. Steroid acetate zones were again

located in ultra-violet light, eluted (3 x 3 ml. of ethyl acetate : methanol, 1:1 v/v), and the eluates reduced to dryness.

To enhance chromatographic purification, the residues from system 2 were subjected to oxidation. Chromium trioxide reagent (0.2 ml.) was added to each dry residue in a stoppered tube which was gently rotated at intervals over a period of 10 minutes at room temperature. The reaction was stopped by the addition of 1 ml. of 20% ethanol and the oxidation products extracted (1 x 10 ml. of methylene chloride) and washed (1 x 1 ml. of water) as described by Kliman and Peterson (1960). Under these conditions aldosterone diacetate, corticosterone acetate and cortisol acetate are converted to aldosterone lactone monoacetate (Peterson, Kliman & Bollier, 1957; 11-dehydrocorticosterone acetate (Kendall, Mason & Myers, 1936) and cortisone acetate (Sarrett, 1946) respectively (Fig. 3).

Further purification of these products was then carried out using systems 3 and 2 of Kliman and Peterson (1960) as shown in Tables 3 and 4. During the development of the aldosterone assay method, the residue from system 2 was rechromatographed on system A (Bush, 1952), but this was not found to be routinely necessary. Duplicate assays of [^{14}C]-





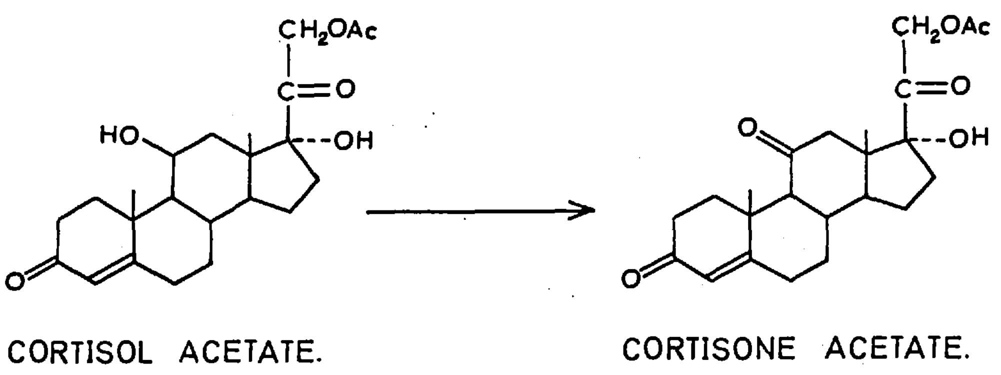


Fig.3: The effect on some corticosteroid acetates of oxidation with chromium trioxide in glacial acetic acid.

steroids added to water were carried out with each batch of plasma samples, the results of which were used as blanks.

TABLE 3 : COMPOSITION OF PAPER CHROMATOGRAPHY SYSTEMS.

System	Composition (by volume)		Reference
1	Cyclohexane	- 4	Kliman & Peterson (1960)
	Benzene	- 2	
	Methanol	- 4	
	Water	- 1	
2	Cyclohexane	- 4	" "
	Dioxane	- 4	
	Methanol	- 2	
	Water	- 1	
3	Cyclohexane	- 4	" "
	Benzene	- 3	
	Methanol	- 4	
	Water	- 1	
A	Petrol	- 5	Bush (1952)
	Methanol	- 4	
	Water	- 1	
Modified A	Petrol	- 5	Burger et al. (1964)
	Acetic Acid	- 4	
	Water	- 1	

TABLE 4 : APPROXIMATE DEVELOPMENT TIMES FOR PAPER CHROMATOGRAPHY SYSTEMS. (HOURS)

System	Aldosterone Diacetate	Corticosterone Acetate.	Cortisol Acetate.
1	12	8	24
2	14	9	12
	Aldosterone Lactone Monoacetate	11-Dehydro-Corticosterone Acetate	Cortisone Acetate
3	16	4	8
2	16	8	8
A	6	-	-

2.3.5. Measurement of radioactivity and calculation of plasma steroid concentration

Measurements of radioactivity were made in a liquid scintillation spectrometer (Nuclear Chicago, 720 series). The final residues from paper chromatography were transferred in toluene (1 x 0.2 ml., 2 x 0.1 ml.) to vials containing 10 ml. of scintillator solution the background radioactivity of which had previously been checked, and counted 5 times for 100 minutes (aldosterone) or 3 times for 100 minutes (cortexolone, cortexone, corticosterone, cortisol). Counting vials (Johnson & Jorgensen, Ltd.) were discarded after use. Efficiencies for $[^{14}C]$ and $[^{3}H]$ under dual label conditions were 39% and 10% respectively and the corresponding backgrounds were approximately 7 and 16 cpm. Determination of $[^{3}H]$ and $[^{14}C]$ was carried out as described by Okita, Kabara, Richardson and Leroy (1957). No correction for quenching was necessary since routine addition of internal standards over a period of several months showed that samples were not significantly more quenched than pure standards.

Under the counter conditions employed $[^{3}H]$ counts appearing in the $[^{14}C]$ channel were negligible (0.01%). $[^{14}C]$- Counts entering the $[^{3}H]$ channel amounted to

approximately 12% of those in the $[^{14}C]$ channel and were corrected for by counting a pure $[^{14}C]$ standard with each batch of assays. Gross plasma concentrations were calculated from these corrected $[^{3}H]$ count rates by the formula:-

Plasma concentration =

$$\frac{[^{3}H]\ \text{cpm} \times \dfrac{100}{\%\ \text{recovery}} \times \dfrac{100}{\text{sample volume}} \times \dfrac{100}{[^{3}H]\ \text{efficiency in } [^{3}H]\ \text{channel}}}{\text{specific activity (dpm/unit wt. of steroid)}}$$

Specific activity values for the appropriate steroid alcohols were calculated from that of cortisol acetate (Section 2.3.1). A net plasma concentration was then obtained by subtracting a mean blank value (see section 2.3.4), which includes the mass of $[^{14}C]$ steroid added.

2.3.6. Evaluation of the assay of aldosterone corticosterone and cortisol

(a) Recovery of [^{14}C]-steroid from plasma.

To determine losses during the purification stages, aliquots (5 or 10%) were removed and their [^{14}C] content measured. The [^{14}C] recovery in final stage residues were, of course, measured for the purposes of the calculation. The results are shown in Table 5. The yield of [^{14}C]-aldosterone during oxidation from diacetate to lactone monoacetate (see 2.3.4) was further studied. Known quantities of [^{14}C]-aldosterone diacetate and 40 μg. of unlabelled carrier, were oxidised as previously described. After extraction, a further 40 μg. of carrier diacetate was added, the extract evaporated, and the residue applied to a thin layer silica gel chromatogram. This was developed in ethyl acetate and the regions corresponding to diacetate (R f : 0.65) and lactone monoacetate (R f : 0.50) eluted and assayed for radioactivity. The remainder of the chromatogram was also eluted and the residue from the eluate counted (Table 6).

TABLE 5. % RECOVERIES OF [^{14}C]-STEROIDS ADDED TO PLASMA DURING PURIFICATION STAGES

STAGE OF	ALDOSTERONE			CORTICOSTERONE			CORTISOL		
PURIFICATION	Range	Mean	N	Range	Mean	N	Range	Mean	N
EXTRACTION	84.0 -104.2	96.33	10	86.1 - 99.6	95.42	4	87.6 -105.5	97.01	4
ACETYLATION	73.8 - 79.3	77.94	10	-	-	-	-	-	-
THIN LAYER SYSTEM	56.1 - 63.8	60.88	10	50.6 - 59.0	55.12	4	47.1 - 51.3	49.99	4
SYSTEM 1	49.6 - 55.0	52.26	10	40.9 - 51.2	44.08	4	38.7 - 43.7	40.13	4
SYSTEM 2	42.1 - 49.9	47.59	10	33.3 - 45.2	40.31	4	36.3 - 39.8	38.60	4
OXIDATION	10.8 - 46.1	26.22	24	18.2 - 34.5	25.86	10	23.1 - 35.0	27.94	8
SYSTEM 3	9.97 - 29.2	17.91	24	14.1 - 27.7	20.32	10	17.2 - 24.6	18.31	8
SYSTEM 4	7.0 - 25.7	12.30	315	7.2 - 34.1	16.57	181	8.1 - 26.5	13.84	115
SYSTEM A	5.2 - 18.6	9.23	6	-	-	-	-	-	-

TABLE 6. % RECOVERIES OF [^{14}C] AFTER OXIDATION OF ALDOSTERONE DIACETATE

Zone	Sample 1.	Sample 2.	Mean
Lactone monoacetate	55.2 %	63.0 %	59.1 %
diacetate	9.1 %	4.4 %	6.8 %
remainder	14.9 %	13.8 %	14.4 %
Total	79.2 %	81.2 %	80.3 %

During the early stages of development of the assay, it was frequently necessary to carry out small numbers of acetylations with a given sample of [^{3}H]-acetic anhydride over a period of several weeks. Although the reagent was stored at -20°C in a dry atmosphere the efficiency of acetylation decreased with time, and recoveries of [^{14}C]-aldosterone through the complete purification were consequently reduced, presumably due to decomposition of the reagent (Table 7).

TABLE 7. % RECOVERIES OF ^{14}C-ALDOSTERONE USING A SINGLE BATCH OF ANHYDRIDE

Weeks after Delivery.	% Recovery.	N
0	23.8	2
2	12.5	2
5	5.6	2

All values reported in this thesis were obtained using fresh reagent, a sufficient number of plasma samples being analysed to use a complete 100 mC. ampoule of anhydride.

(b) Purification of the [^{14}C]-steroid [^{3}H] acetates.

The effectiveness of the various purification stages in removing [^{3}H] contamination from the steroid acetate fractions was followed by measuring the [^{3}H] : [^{14}C] ratio after oxidation and after each of the subsequent chromatographic stages. The results are shown in Table 8. A marked fall in the ratio was found after chromatography in system 3, but the remaining stages had little effect. Chromatography in system A was, therefore, omitted from routine aldosterone assays.

The effect of redistilling the [^{3}H]-acetate anhydride on the blank value and plasma concentration of aldosterone were examined in water and a normal plasma pool (Table 9).

TABLE 9. EFFECT OF DISTILLATION OF [^{3}H]-ACETIC ANHYDRIDE

No. of Distillations.	Water (μug.)	N	Plasma(μug/100ml)	N
0	2.5, 2.7	2	35.8, 34.4	2
1	2.0, 1.9	2	17.0, 16.4	2
2	1.9 ± 0.5(SD)	18	10.5 ± 1.8(SD)	18
6	1.2, 1.9	2	——	-

TABLE 8. $[^3H] / [^{14}C]$ OF STEROID ACETATES DURING THE LATER STAGES OF PURIFICATION

Sample	Stage of Purification	Aldosterone Lactone Monoacetate	11-Dehydro-Corticosterone Acetate	Cortisone Acetate
Water	a	0.86	3.0	1.9
	b	0.45	1.4	0.8
	c	0.42	1.3	0.8
	d	0.43	-	-
Plasma from an adrenalectomised subject.	a	0.90	3.2	3.2
	b	0.46	1.6	1.2
	c	0.44	1.6	1.2
	d	0.45	-	-
Normal plasma	a	1.09	16.2	26.0
	b	0.54	8.8	16.1
	c	0.51	8.8	16.0
	d	0.51	-	-

(a - after oxidation b - after system 3
c - after system 2 d - after system A)

(c) Reliability

A number of studies were carried out to evaluate the reliability of the method for aldosterone, corticosterone and cortisol. The results obtained are summarised in Table 10.

(i) Precision:

The precision (repeatability) of the method was investigated by carrying out duplicate assays on a series of duplicate plasma samples obtained from control subjects. The standard deviation was calculated from the formula

$$S.D. = \sqrt{\frac{\Sigma d^2}{2N}} \qquad \text{(Youden, 1951)}$$

(d = difference between duplicates; N = number of duplicates)

(ii) Recovery of added steroid:

Plasma (50ml) from a normal subject was divided into two equal parts, and known weights of steroid (aldosterone, 2.5 $\mu\mu$g.; corticosterone, 2.4 $\mu\mu$g.; cortisol, 1.0 μg.) were added to one of the plasma samples. Satisfactory recoveries of all three steroids were obtained (see Table 10).

(iii) Sensitivity:

The sensitivity of the method was calculated from the precision data obtained above (i). The values (P = 0.05) for aldosterone, corticosterone and cortisol were found to be 0.005 μg., 0.16 μg., and 1.6 μg. per 100 ml. of plasma respectively when a single plasma sample of 25 ml. is analysed.

(iv) Specificity:

Blank samples of 25 ml. of water and samples of plasma obtained from three patients who had been submitted to total adrenalectomy, and who were subsequently maintained on prednisolone and 9α-fluorocortisol were assayed for aldosterone, corticosterone and cortisol. The blank value for cortisol (48.8 mμg.) was subsequently reduced to 4 mμg./sample following the addition of smaller quantities of [^{14}C]-cortisol (1020 dpm.). The plasma samples from adrenalectomised patients gave values for all three steroids which were below the sensitivity of the method, and were thus not significantly different from zero.

TABLE 10. EVALUATION OF THE RELIABILITY OF THE METHOD

	Aldosterone.	Corticosterone.	Cortisol.
Water blanks	1.9 ± 0.5mµg.	7.9 ± 13.0mµg.	48.8 ± 25.0mµg.
	N = 18	N = 16	N = 14
Adrenalectomy plasma 1)	2.1mµg/100ml.	–	–
2)	1.2 " "	0.02µg/100ml.	0.00µg/100 ml.
3)	4.1 " "	0.09 " "	0.01 " "
Normal plasma pool	10.5 ± 1.8 mµg/100 ml.	0.72 ± 0.08 µg./100 ml.	11.98 ± 0.8 µg./100 ml.
	N = 18	N = 16	N = 14
S.D. (duplicate determinations)	± 1.5mµg/100ml.	± 0.03µg/100ml.	± 0.19µg/100ml.
	N = 12	N = 11	N = 10
Sensitivity (per 100ml)	5 mµg.	0.16 µg.	1.6 µg.
Recovery of added steroid.	118.0%, 102.0%.	95.2%, 98.1%.	100.3%, 99.5%.

Specificity for aldosterone was further examined by following its disappearance rate from plasma. A single intravenous injection of 100 μg. of d-aldosterone (Aldocorten, Ciba, Ltd.) was given to normal male volunteers. Samples of blood (50 ml.) were withdrawn at intervals for plasma aldosterone determination. From the results obtained (Fig. 4) a half-life of 29 minutes was calculated for aldosterone.

(v) Effect of storage of plasma samples:

Since it was necessary to store plasma samples for various lengths of time before assay, the effect of storage at -20°C was investigated. A large pool of plasma from a number of control subjects was divided into 25 ml. aliquots, and at approximately monthly intervals, duplicate samples were withdrawn for assay. Over a period of twelve months no systematic change in the concentration of any of the three steroids estimated could be detected.

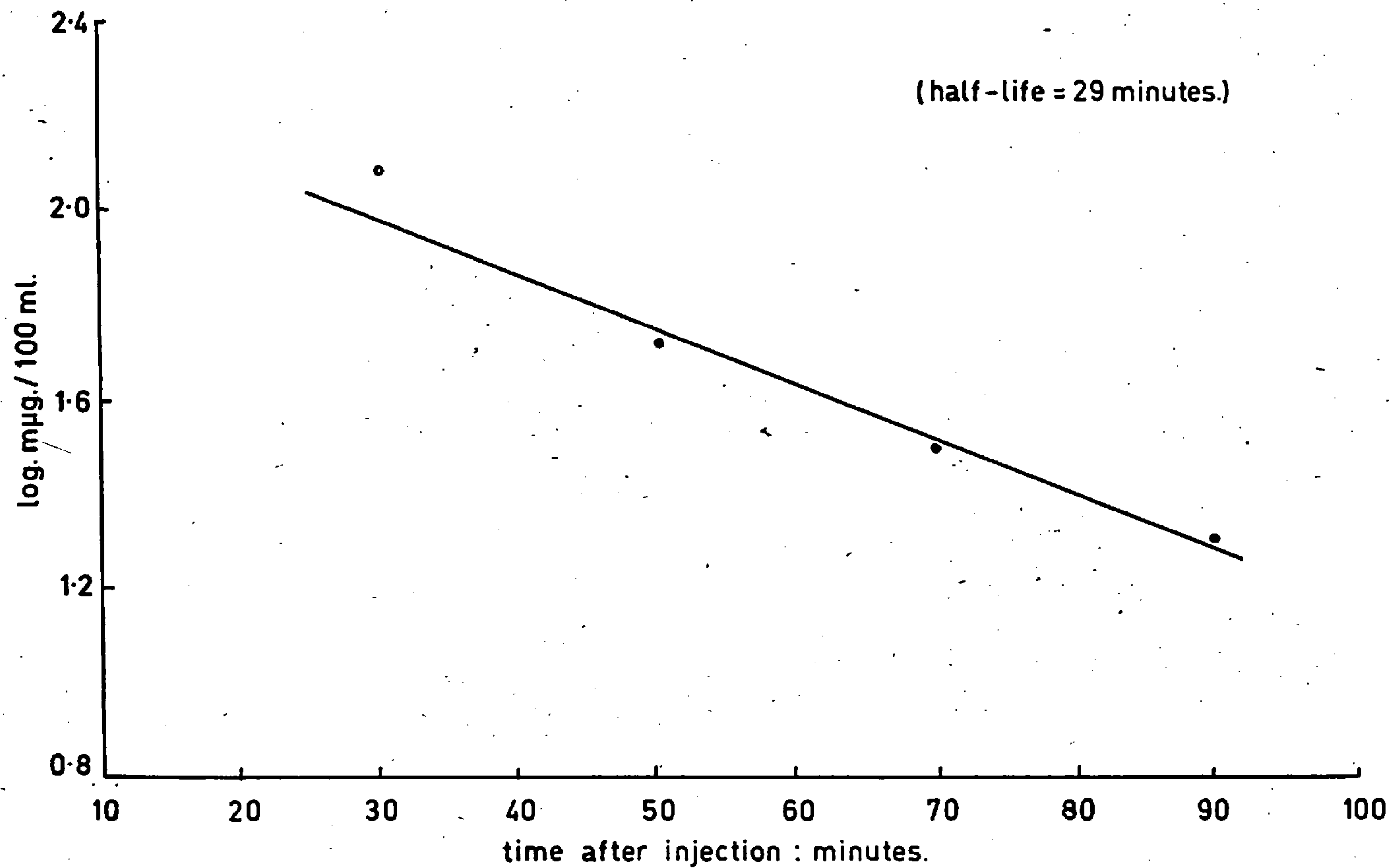


Fig. 4: The disappearance of aldosterone from normal plasma.

(d) Normal Range.

Plasma samples were obtained from a series of healthy control subjects to establish a normal range for each steroid. The subjects of these studies were medical students and laboratory staff under no dietary restrictions. Samples were withdrawn at random times throughout the day, and no night samples were used. The mean values and ranges are shown below (Table 11).

TABLE 11. RANGE OF PERIPHERAL STEROID HORMONE CONCENTRATION IN NORMAL HUMANS

	Aldosterone ($\mu\mu$g./100ml.)	Corticosterone (μg./100ml.)	Cortisol (μg./100ml.)
Range	5 - 17	0.13 - 2.30	3.1 - 20.2
Mean	8.0	0.66	9.8
N	34	29	17

(e) Plasma aldosterone in primary aldosteronism (Conn's Syndrome)

Plasma from 14 patients with untreated primary aldosteronism was assayed for aldosterone. The diagnosis of primary aldosteronism was based on the demonstration of hypokalaemia in the presence of high urinary potassium excretion, and all patients were hypertensive with plasma

sodium levels in the upper normal range. Plasma renin concentrations (Brown, Davies, Lever, Robertson & Tree, 1964) were measured in the Medical Unit, St. Mary's Hospital and were in the low normal range or below. In 9 cases the diagnosis was confirmed by surgery; the remainder have not yet been submitted to surgery. Plasma samples were obtained from 6 cases after removal of the tumour. The results are shown in Fig. 4(a), together with the range of values obtained for the control group. With two exceptions (discussed below), concentrations of plasma aldosterone were above the upper limit of the normal range and varied between 11 and 145 mμg./100 ml. Following the removal of the tumour, levels in the 6 patients studied returned to normal. In 2 of these, two different post-operative assays showed normal concentrations. One patient was studied on three separate occasions prior to surgery. On the first occasion an elevated concentration was observed (68 mμg./100 ml.). At a later date, two samples obtained within an hour of each other showed normal values of 11 and 16 mμg./100 ml.

Another patient was also shown to have a plasma aldosterone level within the normal range, but this was accompanied by a markedly elevated plasma corticosterone concentration(15.2μg/100ml.)

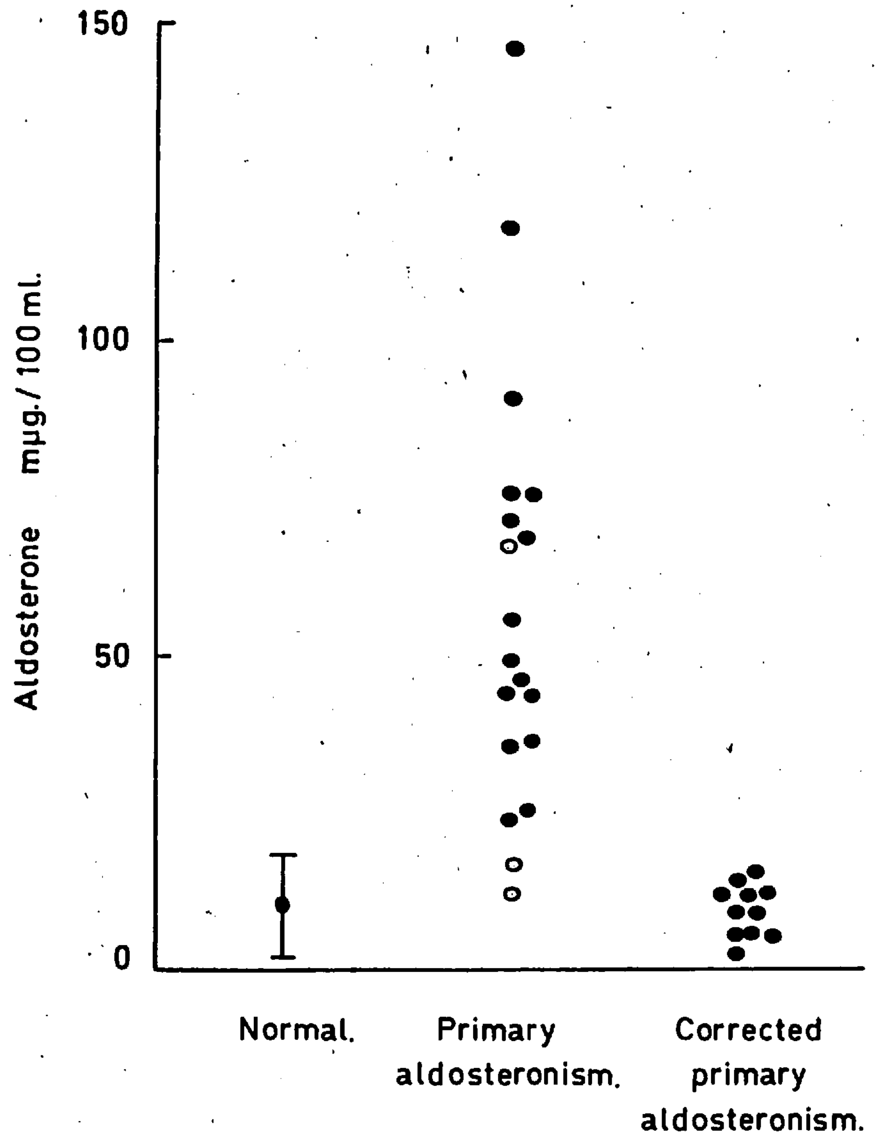


Fig. 4(a): Concentrations of aldosterone in the plasma of normal subjects and in patients with primary aldosteronism before and after surgery. Values represented by o are from a single patient.

Cortisol concentration (6.6 μg./100 ml.) was within the normal range. Examination of the adrenals revealed an adrenal carcinoma.

2.3.7. Purification of cortexone and cortexolone acetates

Crude cortexone acetate and cortexolone acetate fractions from silica gel chromatograms (2.3.3), like the acetates of aldosterone, corticosterone and cortisol, were purified by paper chromatography and chemical transformation. Both compounds were first chromatographed in system A (Bush, 1952) modified by Burger et al. (1964) (see Table 3), and eluted as previously described (2.3.4).

The residues were dissolved in a drop of ethanol in a stoppered tube, to which 1.8 ml. of tris buffer solution (0.05 M, 0.01% EDTA, pH 7.3), 0.025 ml. of $NADH_2$ solution and 0.02 mg. of 20β-hydroxysteroid dehydrogenase protein were added. The reagents were mixed and placed in an incubator (37°C, 20 hours). The solution was then diluted to 3 ml. with water, and extracted with ethyl acetate (3 x 3 ml.). The pooled extracts were evaporated to dryness and rechromatographed in system 2 (Kliman & Peterson, 1960). Cortexolone and cortexone acetates both yielded two products visible in ultra-violet light, the least polar of which was eluted in

each case and the residue acetylated (0.1 ml. acetic anhydride, 0.1 ml. pyridine, 16 hours at 37°C) in a stoppered tube.

The reagents were evaporated under a stream of nitrogen. The product of cortexone acetate was then chromatographed in the modified system A, while that of cortexolone acetate was purified in system 2. The approximate times of development are shown in Table 12. The residues obtained by eluting the final chromatograms were assayed for radioactivity as described in section 2.3.5.

TABLE 12. APPROXIMATE DEVELOPMENT TIMES FOR PAPER CHROMATOGRAPHY SYSTEMS (Hours)

System	Cortexone Acetate	Cortexolone Acetate
A	10 '20-dihydrocortexone acetate'.	30 '20-dihydrocortexolone acetate'.
2	8 'Cortexone 20 : 21 diacetate'	8 'Cortexolone 20 : 21 diacetate'
A	6	-
2	-	6

2.3.8. Evaluation of the assay of cortexone and Recovery of [^{14}C]-steroids.

Aliquots were removed from samples at stages during the purification and their [^{14}C] content measured. The results are shown in Table 13. Recovery from the enzymic reduction stage was further examined using known quantities of [^{14}C]-steroid acetate, prepared with unlabelled acetic anhydride. After reduction for 3 or 20 hours, the products were extracted (section 2.3.7), and separated by thin layer chromatography (10% methanol in benzene). Regions corresponding to the two products and also to the unchanged acetate and its corresponding alcohol were eluted and assayed for [^{14}C]. (Table 14).

Purification of steroid acetates.

The effectiveness of the various purification procedures was assessed as described in section 2.3., using water and normal plasma. The data are shown in Table 15. A marked fall in the ratio of [^{3}H] to [^{14}C] occurred in both cortexone and cortexolone acetates after enzymic reduction and chromatography on the modified system A. A further decrease in this ratio in the plasma extracts resulted from subsequent acetylation followed by chromatography, but this procedure had little effect on the blank sample. Rechromatographing in system 3 had no effect on the purity of the water or plasma samples and was not routinely used.

TABLE 13. RECOVERY OF [^{14}C]-CORTEXONE AND [^{14}C]-CORTEXOLONE DURING PURIFICATION

	CORTEXONE			CORTEXOLONE		
STAGE OF PURIFICATION	Range	Mean	N	Range	Mean	N
EXTRACTION	92.6, 102.4	97.50	2	94.2, 91.5	92.85	2
SECOND THIN LAYER SYSTEM	62.5, 63.3	62.90	2	47.6, 53.8	50.65	2
MODIFIED SYSTEM A	55.1, 58.3	56.70	2	38.9, 46.3	42.60	2
ENZYME REDUCTION AND SYSTEM 2	20.3, 21.6	20.95	2	16.0, 16.9	16.45	2
ACETYLATION AND MODIFIED SYSTEM A	6.7, - 25.3	15.08	32			
ACETYLATION AND SYSTEM 2				5.9 - 23.1	11.17	32

TABLE 14. RECOVERY OF [^{14}C]-CORTEXONE AND [^{14}C]-CORTEXOLONE FOLLOWING ENZYMIC REDUCTION

Substrate	Product	R f	% [^{14}C] Recovery 3 hours	20 hours
Cortexone Acetate	Cortexone Acetate	0.62	10.7	6.8
	Cortexone	0.38	1.1	1.8
	Least polar product	0.32	43.1	48.1
	More " "	0.24	15.7	20.2
Cortexolone Acetate	Cortexolone Acetate	0.40	9.6	2.7
	Least polar product	0.27	48.0	57.1
	More " "	0.20	6.1	10.3
	Cortexolone	0.10	0.7	0.9

TABLE 15. $[^{3}H]/[^{14}C]$ OF STEROID ACETATES DURING THE LATER STAGES OF PURIFICATION

Sample	Stage of Purification	Cortexone Acetate	Cortexolone Acetate
Water	a	5.3	9.3
	b	2.8	3.1
	c	2.7	-
	d	-	2.6
	e	2.7	2.7
Normal Plasma	a	194.0	170.3
	b	40.3	55.8
	c	20.7	-
	d	-	29.0
	e	20.3	29.2

(a : after modified system A, b : after reduction and system 2,
c : after acetylation and modified system A,
d : after acetylation and system 2, e : after system 3)

Reliability.

Precision was estimated by carrying out duplicate assays of cortexone and cortexolone in plasma from control subjects. The standard deviation was calculated as previously described (section 2.3.6(c)) and used to obtain the sensitivity of the method. For a probability of $p = 0.05$, the sensitivity was 0.033 µg. and 0.11 µg./100 ml. for cortexone and cortexolone in plasma respectively (section 2.3.6 iii). Specificity was evaluated by assaying 25 ml. samples of water, and the recovery of known weights (0.05 µg. cortexone, 0.05 µg. cortexolone) of steroid added to plasma was also measured. The results are presented in Table 16.

Normal Range.

Plasma samples were obtained from normal healthy control subjects (section 2.3.6(d)) to establish a normal range.

The plasma concentrations of cortexone and cortexolone in these samples are summarised in Table 17.

TABLE 16. EVALUATION OF THE RELIABILITY OF THE METHOD

	CORTEXONE	CORTEXOLONE
Water blanks	2.9 ± 1.2 mμg. N = 8	3.5 ± 0.63 mμg. N = 7
Standard deviation (duplicate estimations)	± 0.017 μg./100ml. N = 5	0.052 μg./100 ml. N = 5
Sensitivity (p = 0.05) (per 100 ml)	0.033 μg.	0.11 μg.
Recovery of added steroids	104 %	93 %

TABLE 17. RANGE OF NORMAL PLASMA CONCENTRATIONS OF CORTEXONE AND CORTEXOLONE

	Cortexone (μg./100ml)	Cortexolone (μg./100ml)
Range	0.10 - 0.52	0.27 - 0.86
Mean	0.22	0.36
N	13	13

2.3.9. DISCUSSION

The merits of double isotope derivative assays have already been enumerated. In the present study, five plasma corticosteroids have been simultaneously acetylated and subsequently estimated individually, but there is in theory no reason why the procedure should not be extended to include the assay of several additional steroids in a single plasma sample. Although this may reduce the ease of manipulation, the relative cost per assay is lessened. Reagent for the acetylation of a single plasma extract costs £1, and the cost of reagent per assay will obviously decrease as the number of steroid assays increases, and will thus represent a relatively small proportion of the total cost.

The use of [^{3}H]-acetic anhydride, which is relatively cheap and of high specific activity, has as an attendant disadvantage the need to employ [^{14}C]-corticosteroids. While this allows compensation for losses throughout the entire purification procedure, it involves adding to the plasma a significant quantity of steroid (e.g. 3 mμg. of aldosterone to a sample of 25 ml. of plasma often containing less than this) the mass of which must obviously be allowed for in the final calculation. Peterson and Eilers (1964) have clearly

shown that the theoretical error of isotope dilution assay techniques depends on the ratio of unlabelled steroid present in the sample to labelled steroid added for recovery purposes, and that below a ratio of 10, a situation which prevails in the majority of peripheral plasma aldosterone determinations, the error increases rapidly. At present it is the precision with which the added steroid is measured which imposes a sensitivity limit on the method. For aldosterone, this limit is approximately 5 $\mu\mu$g./100 ml. On this basis, samples obtained from patients with Addison's disease (see later) or those who had been adrenalectomised, and also from a proportion of normal subjects, showed values which were below the sensitivity limit of the method. No measurements of cortexone and cortexolone concentrations in plasma from adrenalectomised subjects or patients with Addison's disease were made.

It is desirable, although at present not possible, to employ [^{14}C]-steroids of high specific activity, particularly aldosterone, as tracers, so that a smaller mass could be added to the plasma. Even so, improvement in this direction is limited by the maximum specific activity which can be achieved. Alternatively, an increase in the overall recovery of [^{14}C] during purification and an increase in the efficiency of counting

[^{14}C] would allow a reduction in the quantity added to plasma. Improvements in the precision of the assay and in its sensitivity may result from the use of even higher specific activity [^{3}H]-acetic anhydride, available at specific activities up to 1000 mC/mM., and from an increase in the efficiency for [^{3}H] of the liquid scintillation equipment. Such improvements would allow a reduction in the volume of plasma required for a single assay.

The other factor which limits the sensitivity of the procedure is the difficulty which has been encountered by all investigators in this field, of purifying the isotopic derivative from extraneous radioactivity. In agreement with others (Coghlan et al. 1965) redistillation of the acetic anhydride proved to be the most important single step towards improvement in this direction, although no further decrease in blank values resulted after distillation had been carried out twice (c.f. Coghlan et al. 1966). The blank values obtained for aldosterone compare favourably with those reported by other groups (Peterson, 1964; Coghlan, 1965). That the problem is not insuperable, however, is indicated by the studies of Coghlan et al. (1966) who, by extensive chromatography succeeded in obtaining blank values

as low as 0.13 mμg./sample. The purity of extracts at intermediate stages of purification was assessed by the [^{3}H] / [^{14}C] ratio in an aliquot of the total eluate. In the light of recent reports of isotope fractionation (Benraad, 1966; Cezka & Venneman, 1966) variation of the ratio across a steroid acetate zone on a paper or thin layer chromatogram may not necessarily indicate impurity, and scanning the final residue for uniformity of isotope ratio was not therefore used as a criterion of purity.

A study of aldosterone concentration in a series of 34 normal subjects on an unrestricted diet showed levels of up to 17 mμg./100 ml., with a mean concentration of 8 mμg./100 ml. There are few published figures with which these can be compared. They agree with the normal range reported by Peterson (1964) and with the normal range of mean plasma concentrations of 2-15 mμg./100 ml. (mean 6.6 $\pm$ 1.2, n = 10), and 6-9 mμg./100 ml., calculated respectively by Tait et al. (1961) and by Kono & Mikaye (1965) from secretion rates and metabolic clearance rates. The range of 6-59 mμg./100 ml., obtained by Wolff, Lommer & Torbica (1965) is wider than that reported here. A few data are available from the analysis of peripheral plasma from animals. For example, Bojesen &

Degn (1961) found concentrations between 20 and 30 mμg./100ml. in the dog. More recently, Bojesen (1966) showed that concentrations of aldosterone between 2 and 8 mμg./100 ml., in salt replete rats increased to a mean of 300 mμg./100 ml., following three days of salt deprivation. The biological half-life of 29 minutes obtained for aldosterone is in good agreement with those obtained by other methods (e.g. Tait et al. 1961). The lack of sensitivity of the aldosterone method described here precludes the determination of low concentrations of aldosterone; indeed, some normal values were below the limit of sensitivity. Improvement in this direction must necessarily precede a study of situations in which the secretion of aldosterone is reduced or inhibited.

Valuable, although circumstantial, evidence of the specificity of the determination of aldosterone can be obtained by measuring the concentration in situations in which the general level is predictable, or by examining anticipated changes in plasma level caused by procedures which are known to influence the secretion rate of the hormone. The failure of a technique to reveal such changes would indicate lack of specificity.

Examination of plasma samples from patients with a

clinical diagnosis of primary aldosteronism, which in the majority of cases was later confirmed surgically revealed with two exceptions, that aldosterone concentrations were above the limit of the control group. In one case, a grossly elevated concentration was found initially, but two more samples obtained at a later date gave values which were within the normal range. It is possible that the adrenocortical adenoma, subsequently revealed by surgical exploration, was secreting aldosterone intermittently. In the second exception, it appears that the clinical manifestations, although similar to Conn's syndrome, were in fact due to the excessive secretion of corticosterone.

Previous estimates of peripheral plasma corticosterone concentration have been reviewed by Braunsberg & James (1961). The values reported here are higher than those reported by Braunsberg & James (1960), and are closer to those of 0.5 - 2.0 µg./100 ml., found by Peterson (1957).

11-Hydroxycorticoid concentrations in plasma have been published for a wide variety of conditions in the human. This has been made possible by the simplicity of the method of Mattingly (1962). Such values, however, are aggregates of cortisol and corticosterone at least, and probably also

contain other steroid and non-steroid substances (James et al. 1966; 1967) (see also section 2.2.2.1(d)). However, the cortisol values reported here are in close agreement with those obtained by the best alternative techniques which have been reviewed by Braunsberg and James (1961).

The importance of altering the polarity of a steroid or steroid derivative during purification has previously been discussed. Neither cortexone nor cortexolone possess an 11-hydroxyl group, and a different method from oxidation with chromic oxide in acetic acid was therefore necessary in order to alter the polarity of these compounds. Enzymes have considerable substrate specificity and are in theory ideal for this purpose. An enzyme preparation from Streptomyces hydrogenans described by Hübener and Lehmann (1958), and now commercially available, specifically reduces the 20-ketone group present in all the glucocorticoids and mineralocorticoids to a 20β-hydroxyl group, in the presence of the coenzyme $NADH_2$. The reaction has been extensively used in the identification of adrenocortical steroids in plasma (Hübener & Sahrholz, 1959) and in a system of micro-analysis of steroid mixtures (Henning & Zander, 1962). Unfortunately, the preparation used in this study was impure

and catalysed the formation of two distinct products, neither of which on the basis of thin layer chromatography, was the unchanged substrate, the corresponding alcohol released by hydrolysis, or the enzyme-reduced product of this alcohol. While no further attempt was made to identify the products, it seems likely that the increase in their polarity compared with the substrate was due to the presence of an extra hydroxyl group, and it is possible that they differed in the position, number or orientation of the hydroxyl groups. Acetylation of the major product in each case resulted in a decrease in polarity so that the final derivative migrated at a faster rate on the paper chromatogram than the original unreduced steroid acetate, indicating the presence of an additional acetate group.

Estimates of the plasma concentration of cortexone have not previously been published. Ayers, Barlow, Garrod, Tait, Tait and Walker (1958) calculated that the secretion rate of this hormone in normal subjects is less than 500 μg./24 hours and, more recently, Crane and Harris (1966) measured its urinary secretion rate in ten normal subjects, finding rates of 585 $\pm$ 274 (S.D.) μg./24 hours. Metabolic clearance rates have not been measured and estimates of mean plasma concentration

cannot, therefore, be made. Biglieri (1965) has published measurements of the urinary excretion of tetrahydrocortexone and cortexone in normal subjects, but again predictions of plasma concentration from such data are not possible.

Cortexolone concentrations in peripheral plasma have been measured by Kliman (1966) who reports a concentration range of 0 - 0.5 μg./100 ml. (mean 0.11 μg./100 ml.) which is somewhat lower than that found here.

The multiple assay technique described in the preceding sections was used to study the effects of different treatments on the individual steroids. The effect of some of these treatments on one or more steroids is predictable from previous studies of secretion rates and will facilitate further evaluation of the technique.

3. FACTORS AFFECTING ADRENOCORTICAL SECRETION

3.1. Introduction.

In response to stresses such as pain or hypoglycaemia, the anterior pituitary gland secretes a trophic hormone - corticotrophin or ACTH - which causes the adrenal cortex to accelerate the secretion rate of one or more of its steroid hormones into the blood stream. Conversely, a rise in the concentration of these steroid hormones in the peripheral circulation, whether due to increased endogenous secretion or to exogenous administration, may result in suppression of ACTH secretion followed by a fall in the rate of adrenocortical secretion by a 'negative feedback' mechanism. While the secretion of cortisol, and possibly also corticosterone, is clearly dependent on ACTH, the effect of this trophic hormone on aldosterone production is less well defined.

Excessive natriuresis and the administration of large quantities of potassium are factors which markedly increase the secretion rate of aldosterone in man and some animals while, in man at least, they do not affect the secretion rate of cortisol. The effect of these stimuli on aldosterone secretion appears to be mediated by the kidney.

From recent studies of the mechanism by which the rates of secretion of the constituent steroid hormones of adrenal vein blood are controlled, it has become increasingly obvious that the concept of such a dichotomy is too facile and that the apparent selectivity of the kidney and the pituitary trophic hormones is not absolute but is a matter of degree. That is, when present at low concentrations in the circulation, a trophic hormone may increase selectively the secretion rate of one or a small group of corticosteroids, but at higher concentrations it may have a more general effect on the adrenocortical secretion. In a study of the control of adrenocortical secretion it is therefore of paramount importance that experimental manipulation of external and internal environmental factors should be as far as possible within the range likely to be normally experienced by the subject. Administration of massive pharmacological doses of a trophic hormone, for example, may induce grossly abnormal patterns of secretion.

The statement that 'the proper study for man is man' may be particularly true of adrenocortical physiology: the extent to which knowledge acquired by a study of animals can be applied to man is a matter of debate. The rat, for example,

secretes large quantities of corticosterone but little cortisol, the reverse being true of the human (Bush, 1953). In addition, it now seems reasonably certain that the renin-angiotensin system (see section 3.3) has less importance in the rat than in man in controlling the secretion of aldosterone (Eilers & Peterson, 1964; Bojesen, 1966), while in the dog this same system may influence the secretion rate of the 17-hydroxycorticosteroids as well as that of aldosterone.

In the following sections, a more detailed review of a small number of factors which influence adrenocortical secretion has been given and this is followed by a description of experiments designed to ascertain the specificity of these factors with respect to man.

3.2. The Effect of Corticotrophin.

ACTH is released from the anterior pituitary in response to a wide variety of stimuli, loosely classified as stressors (Sayers, 1950; Moore, 1957), and including surgical trauma, infection, particularly that which results in pyrexia (Bliss, Migeon, Eik-Nes, Sandberg & Samuels, 1954), pain such as that caused by burning (Egdahl, 1967), and insulin-induced hypoglycaemia (Bliss et al. 1954). Exposure to cold elicits a similar response in the rat (Boulouard, 1960) and guinea pig (D'Angelo, 1960) but although the dog responds normally to surgical stress, this animal shows no response to cold (Hume & Egdahl, 1959). Even relatively mild emotional stimuli such as the movement of rats from one room to another or handling of experimental animals prior to experimental treatment has been shown to cause the release of ACTH (Guillemin, 1967).

The control of ACTH release is a subject of some complexity, but it can be subdivided into the stimulation and the inhibition of secretion. Lesions in the hypothalamus, particularly in the region of the median eminence, depress or inhibit the release of ACTH (de Wied, 1961), indicating that this region may receive afferent stress stimuli from the central nervous system. It

is postulated that a corticotrophin releasing factor - CRF - is secreted by the nerve endings and enters the minute vessels of the hypothalamic-hypophyseal portal system, stimulating an increase in the secretion of ACTH.

It has been pointed out (McCann, 1957) that the action of vasopressin, a pressor hormone from the posterior pituitary gland, on the release of ACTH is very similar to that expected of a CRF, and a number of tests of anterior pituitary function have been developed using vasopressin analogues (e.g. Gwinup, 1965; Landon, James & Stoker, 1965). However, it is now clear that these substances may not be specific for this purpose but may act also at other points in the pituitary-adrenal system (Hedge, Yates, Marcus & Yates, 1966).

The inhibitive effect of natural and synthetic glucocorticoids on adrenocortical secretion is well documented in man, and is explained by the postulate of a 'negative feedback' mechanism whereby a rise in the circulating concentration of glucocorticoids results in a decrease or inhibition of ACTH secretion. However, since high levels of blood glucocorticoid are readily produced by stress in the normal intact animal, it is obvious that some further control mechanism allows continued ACTH secretion in these circumstances, even in the presence of glucocorticoid. Yates and Urquhart (1962) suggest that the

concentration of such steroids in blood required to inhibit ACTH secretion during stress is 'reset' at a higher level, but more recently it has been demonstrated that, even in the presence of massive, pharmacological quantities of infused glucocorticoid, stresses such as surgery, pyrexia and hypoglycaemia may still elicit an anterior pituitary response (Estep, Island, Ney & Liddle, 1963; James, Landon & Fraser, 1967). Motta, Mangili & Martini (1965) state that the circulating level of ACTH itself may be of importance in controlling further ACTH release. Obviously, further investigation is necessary to clarify the situation.

ACTH has a profound effect on the size of the adrenal cortex, and on the proportions of its constituent tissues. Prolonged infusion of the trophic hormone causes hypertrophy, while inhibition of secretion by the infusion of steroids, or hypophysectomy lead to atrophy. Evidence exists that the adrenal cortex is subject to a functional zonation, the outer zone or zona glomerulosa being responsible, in rat and beef adrenals at least, for the synthesis of aldosterone, while the zonae fasciculata and reticularis synthesise cortisol and much of the corticosterone. The outer zone may also contribute to corticosterone production (Stachenko & Giroud, 1962). Atrophy of the zona glomerulosa after

hypophysectomy or cortisone administration (Deane & Greep, 1946; Farrell, Banks & Koletsky, 1956; Miller, 1965) in animals occurs at a slower rate than the remaining zones indicating that the zone, and consequently its hormone product aldosterone, may be relatively free of pituitary control.

Much information has been published concerning the effect of pituitary extracts or ACTH preparations on excretion, secretion and plasma concentrations of adrenocortical steroid hormones. Of particular interest are the studies of individual steroids using specific methods of estimation and these are relatively few.

The secretion rate of cortisol in normal humans (Cope & Black, 1958) was increased nine or ten-fold by the administration of ACTH, while low ACTH secretion due to pituitary insufficiency or to prednisolone, prednisone or 9α fluorocortisol therapy resulted in very low secretion rates. Similarly, Braunsberg and James (1960) found marked increases in plasma cortisol concentration in subjects receiving an intramuscular injection of ACTH, but the rise in plasma corticosterone concentration was small and difficult to interpret, bearing in mind the low sensitivity of the method. Cameron and Kilborn (1964) used a fluorimetric technique to demonstrate that both cortisol

and corticosterone concentrations rise sharply during the infusion of ACTH, and administration of synthetic β^{1-24} ACTH (Kappeler & Schwyzer, 1961) or insulin-induced hypoglycaemia leads to elevated 11-hydroxycorticosteroid concentrations in human plasma (Landon, Wynn & James, 1963; Landon et al. 1964).

According to Bush (1953) the ratio of cortisol to corticosterone is genetically controlled and is typical of a species. He states that the effect of ACTH is to increase the concentrations of both hormones while leaving their ratio unchanged. The method used in this study, however, was at best only semi-quantitative and the process by which blood samples were obtained from the adrenal vein of the experimental animals would almost certainly have caused the release of ACTH in large quantities. It is doubtful, therefore, whether true basal conditions could have been obtained with which to compare ACTH-infused animals. The results reported more recently by Cameron and Kilborn (1964), however, also show little change in the ratio of these two hormones in the human after ACTH infusion. In contrast, Krum and Glenn (1965) found that, in the rabbit, which secretes corticosterone predominantly, prolonged ACTH infusion eventually reduced the corticosterone to cortisol ratio from about 20 : 1 to 1 : 1. Further

investigations, using sensitive methods of steroid assay and less traumatic blood sampling techniques, are obviously necessary to clarify the situation.

Little is known of the effect of ACTH on cortexolone and cortexone secretion in the intact normal human. Inhibition of 11-hydroxylation in metapyrone (2-methyl-1, 2-bis(3-pyridyl)-propan-1-one) results in a rapid fall in the circulating concentration of glucocorticoid, an increase in the rate of secretion of ACTH and consequently a marked increase in the secretion rate of the 11-deoxy-compounds and excretion rate of their products (Jenkins, Pothier, Reddy, Nelson & Thorn, 1959). Similarly, a congenital deficiency in the 11-hydroxylation system is accompanied by elevated excretion of cortexone and cortexolone metabolites which can be further increased by ACTH administration (Crane & Harris, 1966). While Biglieri, Herron and Brust (1966) report increased cortexone secretion with ACTH treatment in man, Bledsloe, Island and Liddle (1966) could not demonstrate such an effect unless 11-hydroxylation was inhibited. An increase in plasma cortexolone concentration in man in response to ACTH infusion has been reported (Hoet, Matterazzi & Ekka, 1964) but the quantities of the trophic hormone used in the study were

very large and almost certainly outside the normal physiological range.

The effect of ACTH on aldosterone secretion has been studied by many groups of research workers by as many different methods. For several years it was accepted that the pituitary gland exerted little or no influence on this steroid hormone, and Farrell (1958), in favour of this view, cites the lack of effect of hypophysectomy on the zona glomerulosa (see above), and the absence of impaired aldosterone production in hypophysectomised dogs or in humans with panhypopituitarism or undergoing cortisone therapy. However, later studies have shown that some reduction in aldosterone secretion does result from pituitary insufficiency or hypophysectomy (Eilers & Peterson, 1964 - rat; Ganong, Lieberman, Daily, Yven, Mulrow, Leutscher & Bailey, 1959 - dog; Ross, van't Hoff, Crabbe & Thorn, 1960 - man), and that the response of the adrenal gland to salt deprivation is impaired in the dog (Davis, Bahn, Yankopoulos, Kliman & Peterson, 1959; Binion, Davis, Brown & Olichney, 1965) and the human (Ross et al. 1960), but not in the rat (Eilers & Peterson, 1964).
That treatment of an animal with large quantities of cortisol reduces the capacity of the gland to produce aldosterone <u>in vitro</u> (Vecsei, Farkas, Kemeny & Harangozo, 1965 - rat) and <u>in vivo</u>

(Farrell, 1963 - dog) suggests that inhibition of ACTH secretion by the negative feedback mechanism may reduce secretion of this steroid hormone, but neither Bledsloe et al. (1966) nor Liddle, Duncan and Bartter (1956) could confirm this effect in man.

Histological examination of rat adrenal glands from hypophysectomised animals (Stack-Dunne, 1960) reveals, in addition to reduced volume and rate of cell division of the zona glomerulosa tissue, occlusion of blood vessels between this zone and the adjacent zona fasciculata, probably resulting in reduced perfusion and impaired metabolism. Such degeneration did not occur when the rats were maintained on ACTH.

A rise in the secretion rate of aldosterone can be induced by ACTH administration in a variety of animals, examples of which are the rat (Singer & Stack-Dunne, 1955) sheep (Blair-West et al. 1963) dog (Mulrow & Ganong, 1961) and man (Venning, Dyrenfurth, Giroud & Beck, 1957). The effect, however, may be short-lived. For example, Liddle et al. (1956) found that, while urinary excretion of aldosterone increased during the first few days of treatment with ACTH, further treatment did not prevent levels returning to normal, but abrupt withdrawal of ACTH was followed by a fall in the excretion rate to below

normal levels.

The quantity of ACTH required to produce an increase in aldosterone production depends on the electrolyte status of the animal. Dogs on diets containing unrestricted quantities of salt did not respond to ACTH unless quantities far in excess of those required to produce maximal increases in the secretion rates of the 17-hydroxycorticosteroids were administered, but those on low sodium diets responded at lower levels of treatment (Ganong, Biglieri & Mulrow, 1966). Binion et al. (1965) report that the increase in aldosterone caused by sodium depletion in dogs is greater in the presence than in the absence of the pituitary. A similar increase in the sensitivity of aldosterone secretion in the salt-depleted sheep has been noted (Blair-West et al. 1963), but in man the increase in aldosterone excretion rate following ACTH therapy was not enhanced by salt deprivation (Liddle et al. 1956).

The experiments described in section 3.6.1 represent an attempt to distinguish between the pharmacological and physiological effects of ACTH on aldosterone secretion, and to discover the effect of dose level and sodium status in the human on the response of the secretion of the individual corticosteroids.

3.3. The Effect of Angiotensin.

The fact that, in man, dog and sheep at least, the secretion of aldosterone is relatively unresponsive to ACTH has stimulated the search for an alternative control mechanism, specific to this hormone. That this mechanism is of a humoral nature is suggested by the studies of Carpenter, Davis, Ayers and Bahn (1961) and of Blair-West et al. (1962) which showed that the transplanted, denervated adrenal gland did not lose the ability to respond , by increasing the rate of production of aldosterone, to such stimuli as haemorrhage and sodium depletion (see also sections 3.4 and 3.5). Davis, Carpenter, Ayers, Holman and Bahn (1961), having first demonstrated that hypophysectomy of the dog failed to inhibit aldosterone secretion, by a series of ablation studies also eliminated both the possibility of a specific neurohormone or pineal secretion by decapitating the animal, and the participation of the liver by removing this organ, since none of these treatments could prevent a rise in aldosterone secretion in response to the above stimuli. However, the hypophysectomised, nephrectomised dog failed to respond, and a similar sheep preparation, although it could still respond to severe sodium depletion, failed to respond to haemorrhage

(Blair-West et al. 1962.).

These experiments suggest that the kidney may have an important role to play in the control of adrenocortical secretion with respect of aldosterone. Such a view is further enhanced by the results of cross-circulation experiments (Yenkopoulos, Davis, Kliman & Peterson, 1959) where plasma from dogs in which increased aldosterone secretion had been induced by constriction of the thoracic vena cava caused a similar rise in secretion when infused into untreated animals.

The nature of the trophic hormone has been further investigated. Saline extracts of kidney, administered to dogs, caused increases in aldosterone secretion rate which were directly proportional to the dose given (Mulrow & Ganong, 1962) and further fractionation of such an extract (Davis, Higgins & Urquhart, 1964) provided strong evidence that the biologically active principle was the kidney enzyme renin. Several animals have since been shown to respond to renin by increasing their aldosterone secretion rate.

It is currently postulated that renin is released from the juxtaglomerular cells of the kidney in response to changes in the perfusion pressure of the organ, perceived by stretch receptors possibly in the juxtaglomerular apparatus (Tobian, 1960).

The enzyme catalyses the conversion of a plasma polypeptide-angiotensinogen - to a decapeptide - angiotensin I - which is then hydrolysed to the biologically active octapeptide - angiotensin II - by a plasma enzyme. Angiotensin II, which will now be called angiotensin, is rapidly destroyed by angiotensinases in plasma.

The role of the kidney may therefore be assessed by following the changes of renin or angiotensin concentration in blood in situations where alterations in corticosteroid content can be predicted, or alternatively, by administering renin or angiotensin to the animal under investigation and following the consequent changes in adrenocortical secretion. Each of these approaches has been attempted in animals but the protein nature of renin and the difficulty of obtaining suitably pure preparations makes its use in human subjects inadvisable.

Recent studies have revealed that the renin-angiotensin system may assume more importance in one species than another. In man, infusions of angiotensin at low rates selectively increase the excretion (Genest, Koiw, Nowaczynski & Sandor, 1960) and secretion (Ames, Borkowski, Sicinski & Laragh, 1965) rates of aldosterone, but, at rates above that required to

maximise aldosterone secretion, cortisol production is also increased (Ames et al. 1965). Similarly, Laragh, Cannon and Ames (1964) found that aldosterone secretion rate, but not that of cortisol, was raised by mildly pressor doses of angiotensin, and that this was not solely due to its effect on blood pressure since equipressor doses of norepinephrine exerted no influence on adrenocortical secretion. They further showed that the effect of angiotensin could be sustained by infusion for up to 11 days.

Studying the dog, Slater, Barbour, Henderson, Casper and Bartter (1965) report that, while hypophysectomy did not reduce the rate of aldosterone secretion, both renin and angiotensin in non-pressor doses caused similar rises in the secretion rates of aldosterone, corticosterone and cortisol in the absence of the pituitary. Similar studies are summarised by Ganong et al. (1966) who state that large doses of renin or of angiotensin cause increases in aldosterone secretion in the hypophysectomised, nephrectomised dog and that the secretion of the 17-hydroxycorticosteroids rises also, but to levels which do not approach those attained by ACTH infusion. Changes in corticosterone secretion were not consistent. Neither of the above groups have used intact animals. It is pointed out

that, in the presence of the anterior pituitary gland, increases in the blood cortisol concentration, and possibly also that of corticosterone, would be counteracted by an inhibition of ACTH secretion and that the effect of angiotensin would therefore be specific for aldosterone. Ganong et al. (1966) also explain that the same increase in plasma concentration which, in the case of aldosterone would profoundly affect sodium balance, would in the case of cortisol and corticosterone probably not be physiologically significant.

Both Eilers and Peterson (1964) and Bojesen (1966), investigating the control of aldosterone secretion in the rat, conclude that the renin-angiotensin system may be of little importance in this animal. Although nephrectomy and hypophysectomy, separately and simultaneously, depress aldosterone secretion, normal secretion rates were not restored either by renin or by angiotensin administration. In addition, while sodium restriction caused supranormal secretion rates in both intact and nephrectomised rats, renin and angiotensin infusion could only induce small rises in aldosterone and corticosterone production. In contrast, Glaz & Sugar (196 0 report that aldosterone production _in vitro_ in adrenal glands from rats infused with angiotensin is greater than in those

from untreated control animals, but Muller (1966) could not confirm this.

Alterations in plasma renin concentration have been described for a variety of conditions in which aldosterone secretion is reputedly also changed. For example, Brown, Davies, Lever and Robertson(1964) and Brown, Davies, Doak, Lever and Robertson (1963) have shown convincingly that renin concentration is increased by sodium deprivation in normal humans and also in cirrhosis and pregnancy, while haemorrhage causes similar increases (Brown, Davies, Lever, Robertson & Verniory, 1966) (see also section 3.4). Measurements of angiotensin concentrations in plasma are technically more difficult because of the labile nature of the compound, but this also is reported to rise following haemorrhage (Scornik & Paladini, 1964) and salt deprivation (Mulrow, Lytton & Stansel, 1966).

The effect of angiotensin on the adrenal cortex appears to be a direct one. Infusion into the adrenal vein of the dog (Ganong, Mulrow, Boryczka & Cera, 1962) and the sheep (Blair-West et al. 1962) increases aldosterone secretion to a greater extent than similar systemic infusion, but renin and angiotensin I are ineffective unless infused at this latter

site.

An attempt has been made to investigate the effect of angiotensin, if any, on the human adrenal cortex, and to discover, by suppressing ACTH secretion, to what extent the anterior pituitary modifies such an effect.

3.4. The Effects of Haemorrhage and Hypotension.

The poor sensitivity of the method of estimating plasma aldosterone concentrations in relation to the low concentration of this steroid in plasma necessitates the withdrawal of considerable volumes of blood which, particularly in those experiments requiring repeated sampling, might itself amount to a significant stimulus to the rate of secretion of hormones by the adrenal cortex.

The effect of haemorrhage on the human adrenal gland was noted as early as 1914 by Elliot who reported increased weight and reduced lipid content in subjects who had succumbed to perforated gastric ulcers, compared with those who had suffered instantaneous, accidental death. In 1945, Sayers, Sayers, Liang & Long showed that the cholesterol and ascorbic acid concentration in the rat adrenal gland, which are inversely proportional to its secretory activity, fell markedly after severe, non-fatal haemorrhage and fatal haemorrhage, but that in the former case, the concentrations returned to normal as the animals recovered. A similar adrenal response was elicited by the administration of an ACTH preparation, but no comparable alterations occurred in brain or liver tissue.

The release of ACTH in response to haemorrhage, causing an increase in the secretion rate of adrenocortical steroid hormones is now well-documented (e.g. Carpenter, Davis, Holman, Ayers & Bahn, 1961; Gann & Egdahl, 1965).

However, the effect of haemorrhage on the adrenal cortex does not appear to be mediated entirely by the anterior pituitary gland since the consistent rise in the aldosterone secretion rate following a 250 ml. bleed in the dog is not affected by hypophysectomising the animal but is inhibited by nephrectomy (Davis et al. 1964). Severe fatal haemorrhage results in a progressive increase in the plasma concentration of angiotensin in the dog (Scornik & Paladini, 1964), while in the same species a less severe (25 ml./Kg.) bleed causes consistent increases in plasma renin concentration (Brown et al. 1966). Withdrawal of smaller blood volumes (12 ml./Kg.) has an inconsistent effect on dog plasma renin concentration, and that of the normal human is unaffected by bleeds of between 400 and 500 ml. The same authors report a markedly elevated plasma renin concentration in a patient suffering from intestinal haemorrhage, and that a return to normal resulted from transfusion.

Some mention has already been made of the mechanism by

which the kidney may affect the secretion rate of aldosterone (section 3.3.), but the stimulus to the kidney which induces renin secretion can as yet only be speculated upon. Haemorrhage reduces the splanchnic blood volume and clearance rate of dyes (Price, Deutsch, Marshall, Stephen, Behar & Neufield, 1966), and renal blood flow is reduced to a greater extent than blood flow through other organs such as the liver in the anaesthetised cat (Greenaway & Lawson, 1966). A similar reduction in renal perfusion following haemorrhage in the dog was reported by Muller, Manning, Moret & Mégévand (1964) who also showed simultaneous increases in aldosterone secretion rate. Whether the reduction in blood flow itself or a decrease in the fullness of the renal vasculature was responsible for the effect on aldosterone secretion could not be ascertained.

The study of the effect of haemorrhage in the normal human described in section 3.6. was designed mainly as a control for other studies since it was not possible to subject normal human subjects to haemorrhages as severe as those reported in animals. However, one result of severe blood loss is a fall in blood pressure and the opportunity occurred during the period of this study of measuring plasma cortico-

steroid concentrations in a number of patients suffering from postural hypotension, the results of which are described in section 3.4.3.

Although it is well-known that aldosterone secretion (Bojesen, 1964; Gordon et al. 1966) and plasma renin concentration (Brown, Davies, Lever, McPherson & Robertson, 1966) are higher in humans in an upright position than while recumbent, few similar data are available in patients with postural hypotension. Wolff, Lommer & Torbica (1965) reported an increase of approximately 100 $\mu\mu$g./100 ml. in the plasma aldosterone concentration in a normal human subject following an orthostatic collapse, but Hall & Hökfelt (1966) investigating two patients with postural hypotension noted their low resting aldosterone excretion rates and their inability to respond to sodium restriction by increasing this rate. Similarly, such patients do not appear to respond normally to angiotensin administration (Biglieri & Slaton, 1966), although ACTH administration causes a normal elevation in the production of 17-hydroxycorticosteroids. No reports of plasma corticosteroid levels and their response to postural changes has been discovered in the literature.

3.5. The Effect of Sodium Depletion.

Adrenalectomy causes profound alterations in the distribution and excretion of sodium, as well as chloride and potassium, in all animals so far studied, and it is this disturbance, rather than interference with carbohydrate and protein metabolism, which leads to the eventual death of the animal. The rapid fall in plasma sodium and chloride concentrations is accompanied by haemoconcentration (Baumann & Kurland, 1927) and by a rising serum potassium level (Hastings & Compere, 1931) until shock and death ensue. Human adrenal hypofunction - Addison's disease - is characterised by similar symptoms (Loeb, 1932). The efficacy of extracts of adrenal glands (Rogoff & Stewart, 1927) and also of saline administration (Loeb, 1933) in counteracting these symptoms is further evidence of the importance of the organ in electrolyte metabolism. The mineralocorticoid activity of the adrenal gland was finally explained by the isolation and characterisation of aldosterone (Simpson, Tait, Wettstein, Neher, v. Euw, Schindler & Reichstein, 1954).

The increase in the rate of aldosterone excretion (Leutscher & Axelrad, 1954; Bartter, Mills, Biglieri & Delea, 1954; Johnson, Lieberman & Mulrow, 1957) and secretion (Ulick, Laragh &

Lieberman, 1958; Cope, Nicolis & Fraser, 1961) and also in the concentration of this steroid in peripheral plasma (Peterson, 1964) is now well-documented in man, as is also the fall in some of these parameters during sodium repletion. Similar results have been obtained in several animals. Such knowledge provides an excellent means of testing, physiologically, the specificity of new methods of measuring aldosterone.

The mechanism by which sodium depletion stimulates the secretion rate of aldosterone is not yet clear. Evidence exists that a low plasma sodium concentration may influence the adrenal cortex directly (Blair-West et al. 1963; Davis, Urquhart & Higgins, 1963) but alterations in concentration do not readily occur in normal animals whereas aldosterone secretion responds in the absence of altered plasma electrolytes, to relatively mild sodium deprivation. A more tenable hypothesis (Binion et al. 1965) is that excessive sodium loss, with concomitant loss of water from the extracellular fluid, results in a reduction of blood volume and consequently renal blood flow which is perceived by sensitive volume receptors in the kidney (see section 3.3) and leads to the release of renin. While this theory may hold for man, the dog and the sheep, some other mechanism may prevail in the rat (see section

3.3).

The effect of variations in sodium status on the secretion of other corticosteroids has not been widely studied. No information is available on the alterations, if any, of cortexone and cortoxolone production in response to sodium depletion. Davis, Ayers and Carpenter (1961) and Binion et al. (1965) could find little alteration in corticosterone secretion rate in the sodium depleted dog, neither did Eilers and Peterson (1964) or Bojesen (1966) find that such treatment affected corticosterone secretion in the rat. A small increase in this value in one sheep reported by Blair-West et al. (1963) may have been due, not to sodium depletion, but to the diurnal variation of ACTH secretion.

Jailer (1951) has summarised the evidence for the existence of a salt-excreting hormone. In certain types of congenital adrenal hyperplasia, patients experience difficulty in retaining sodium and the severity of this condition is accentuated by ACTH administration. Also cited is the fact that, while cortexone administration causes sodium retention in patients with Addison's disease, massive doses of the same steroid are needed to produce a similar effect in normal humans, thus indicating the presence of an antagonist, possibly of

adrenal origin. More recently, Erlich (1966) has suggested that cortisol, among its other properties, may act as a salt excreting hormone. He reports that cortisol excretion rates increase in humans subjected to high sodium diets and that, after salt depletion with diuretics, cortisol excretion falls. However, in cases where secretion rates, as opposed to excretion rates, were measured the changes were smaller and less convincing.

The effect of dietary sodium restriction on plasma corticosteroid concentrations has been compared in normal male subjects and in a patient with sodium-losing renal disease. An attempt has also been made to study the effect of acute sodium depletion, achieved by means of the diuretic Frusemide, which causes sodium diuresis while having a minimal effect on potassium excretion (Kleinfelder, 1963). These experiments are described in section 3.6.5.

3.6. Experimental.

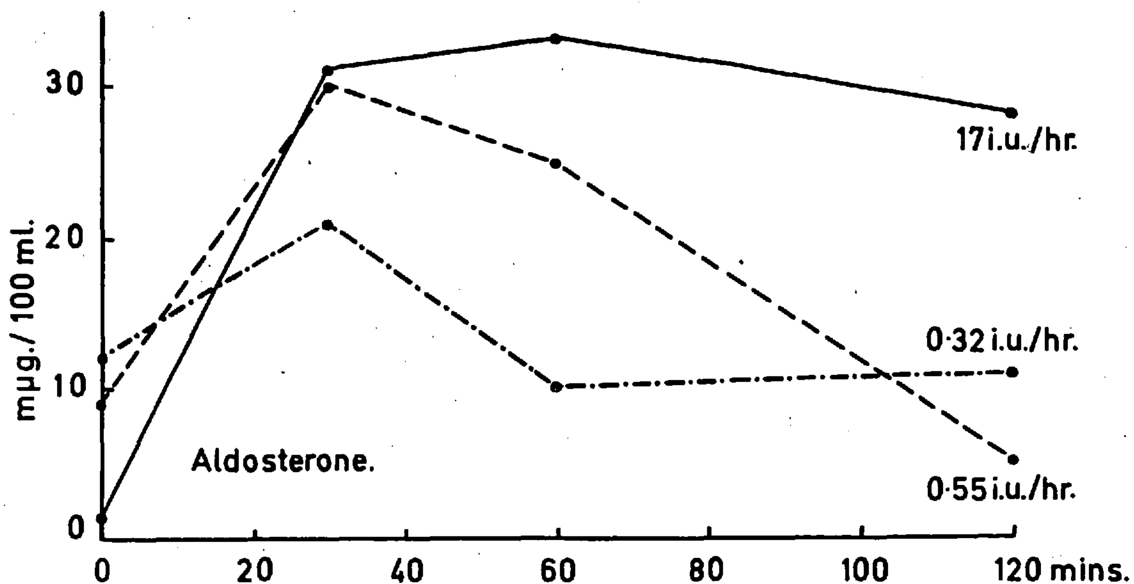
3.6.1. The effects of ACTH and stress in normal subjects.

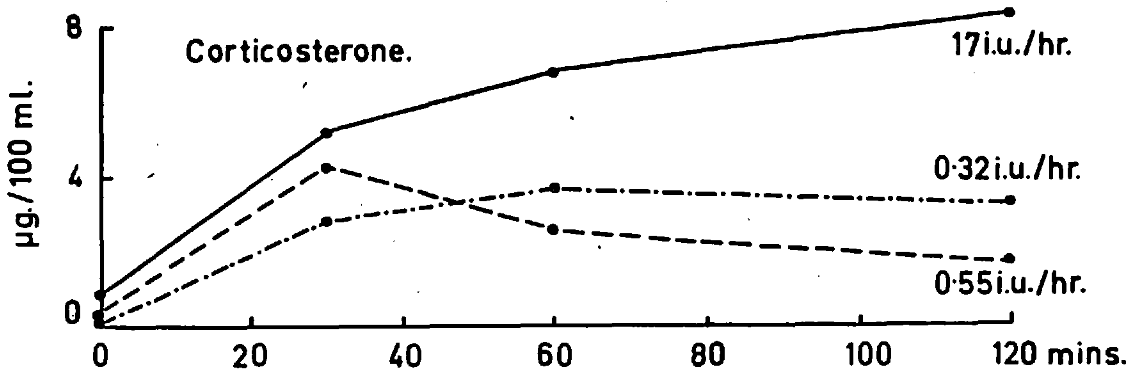
(a) The effect of porcine ACTH.

The effect of infusions of porcine ACTH (Ciba, Ltd.), at three different rates (17 iu/hr., 0.55 iu/hr., 0.32 iu/hr.), on the plasma concentrations of aldosterone, corticosterone and cortisol was examined in a single normal male volunteer. In each of the infusion experiments, which were separated from each other by at least one week and which commenced at approximately 10.0a.m., an indwelling needle was inserted into the antecubital vein at least 30 mins. before withdrawing the resting sample, and the subject remained recumbent throughout. These conditions were observed in all infusion experiments unless otherwise stated. Infusion of ACTH in isotonic fructose was started immediately after the resting sample had been withdrawn, and 50ml. blood samples were taken at intervals over a period of 2 hours. Changes in plasma steroid concentrations are illustrated by Fig. 5, and the effect of the treatment on the ratio of cortisol to corticosterone is shown in Table 18.

TABLE 18. THE EFFECT OF PORCINE ACTH ON THE CORTISOL : CORTICOSTERONE RATIO

	Rate of Infusion. (iu./hr.)		
Time (min.)	0.32	0.55	17.0
0	48	22	22
30	9	7	6
60	8	11	6
120	9	12	5





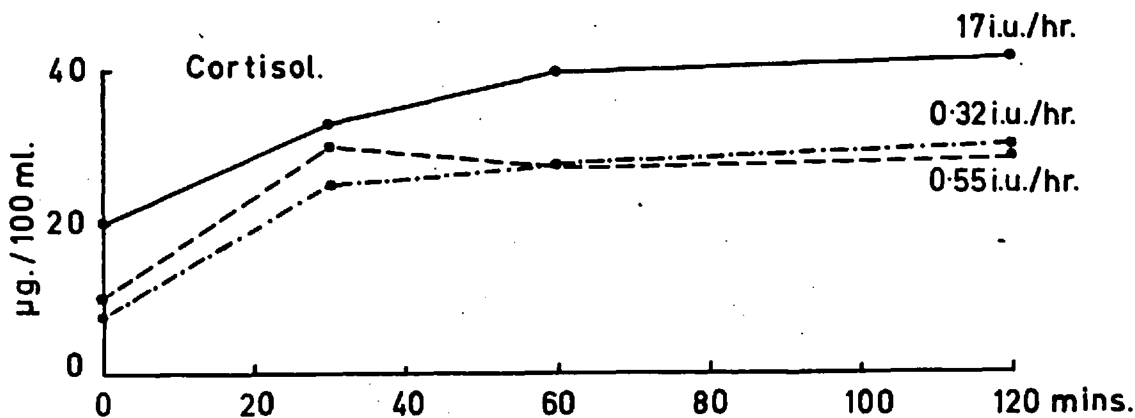


Fig.5: The effect of porcine ACTH. on plasma corticosteroids.

At all rates of infusion there was a rapid, sustained rise in cortisol concentration, but concentrations of corticosterone achieved at the two lower infusion rates were only half of that caused by an infusion of 17 iu./hr. During the infusion at 0.55 iu/hr., the plasma corticosterone concentration fell after an initial peak at 30 mins. The ratio of cortisol to corticosterone was markedly reduced at all infusion rates.

ACTH infusion also elicited a rise in the concentration of aldosterone which, however, was only sustained at the highest infusion rate. The concentration showed a smaller increase at an infusion rate of 0.32 iu/hr., and returned more rapidly to normal levels than at an infusion rate of 0.55 iu/hr.

(b) The effect of synthetic β^{1-24} ACTH

In order to study the effects of small quantities of ACTH on plasma corticosteroids, it was necessary to suppress endogenous ACTH secretion. A normal male subject was given 1mg. of dexamethasone (Merck, Sharp & Dohm, Ltd.) orally at 12 hr., and again at 3 hr. before the experiment commenced. A resting sample was withdrawn through an indwelling needle and a series of doses of β^{1-24} ACTH

(Synacthen - Ciba, Ltd.) in normal saline acidified to pH2 with hydrochloric acid (Stouffer & Lipscomb, 1963) were administered intravenously as single injections. Blood samples were withdrawn 15 mins. after each dose and a period of at least 30 mins. elapsed between doses. Dose and sample times are given below:

Time (min.)	Treatment	Sample No.
0	-	1
5	125 $\mu\mu$g. β^{1-24} ACTH	
20	-	2
35	250 $\mu\mu$g. β^{1-24} ACTH	
50	-	3
80	1250 $\mu\mu$g. β^{1-24} ACTH	
95	-	4

The results are shown in Fig. 6 and cortisol and corticosterone ratios are given in Table 19.

Judged by the concentrations of cortisol and corticosterone in the resting plasma sample which were not significantly different from zero, suppression of endogenous ACTH secretion appears to have been complete, but aldosterone, cortexolone

TABLE 19. **THE EFFECT OF β^{1-24} ACTH ON THE CORTISOL : CORTICOSTERONE RATIO**

Dose (μg.)	Cortisol/Corticosterone
0	12*
125	9
250	7
1250	8

* Both cortisol and corticosterone concentrations were below the sensitivity of the method.

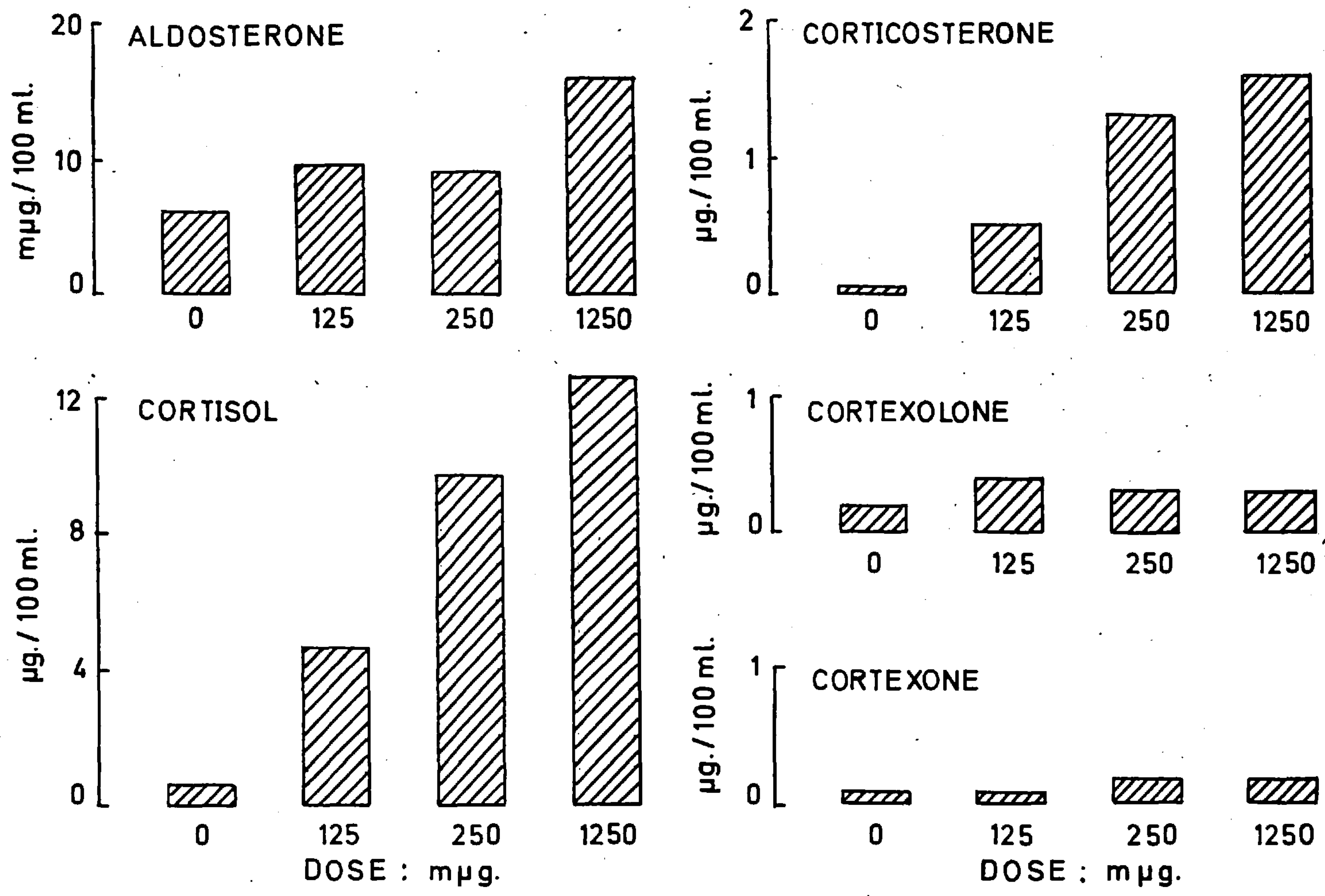


Fig. 6 : The effect of β^{1-24} ACTH on plasma corticosteroids.

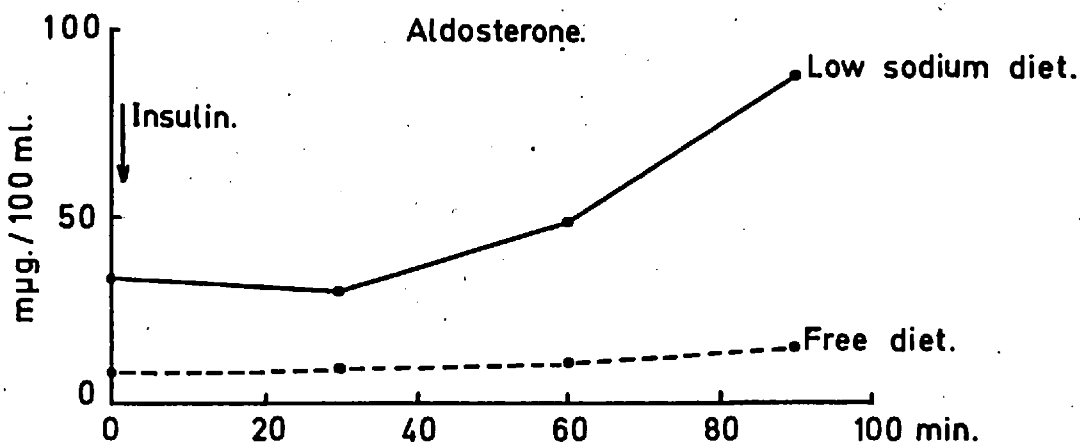
and cortexone concentrations were not reduced below the normal range. From the results, the threshold dose of β^{1-24} ACTH to produce an increase in cortisol and corticosterone levels lies at or below 125 $\mu\mu$g., while the threshold for aldosterone is higher, lying between 250 and 1250 $\mu\mu$g. No significant response in cortexone and cortexolone concentrations was obtained from the range of β^{1-24} ACTH doses used in the experiment; neither was there any significant alteration in the ratio of cortisol to corticosterone.

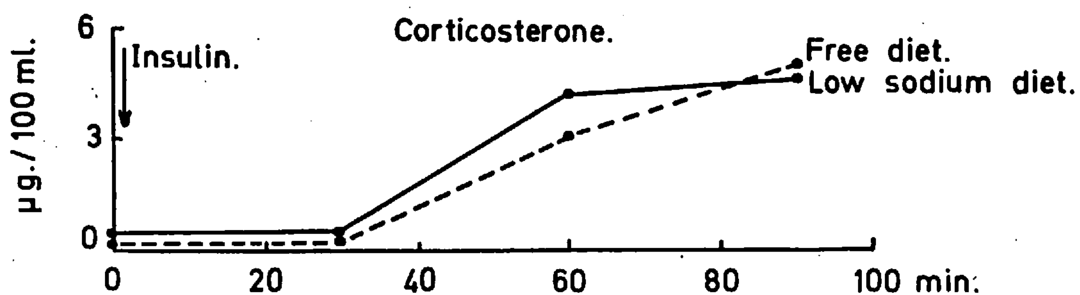
(c) The effect of stress.

The effect of stress, caused by insulin-induced hypoglycaemia, was compared in a single normal male subject on an unrestricted diet and after a period of 6 days on a low sodium diet (see section 3.6.5). Insulin (0.15 iu/Kg., Organon) was administered intravenously immediately after removal of a resting blood sample (50 ml.), and three further samples were taken at 30 min. intervals. Plasma glucose concentrations were measured by the Diagnostic Chemical Pathology Unit, St. Mary's Hospital. The cortisol : corticosterone ratios are again tabulated (Table 20), while changes in plasma steroid concentrations are shown in Figs. 7(a) and (b).

TABLE 20. **THE EFFECT OF STRESS ON THE RATIO OF CORTISOL TO CORTICOSTERONE**

Time (mins.)	Free Diet.	Low Sodium Diet.
0	59	23
30	43	14
60	8	5
90	7	4





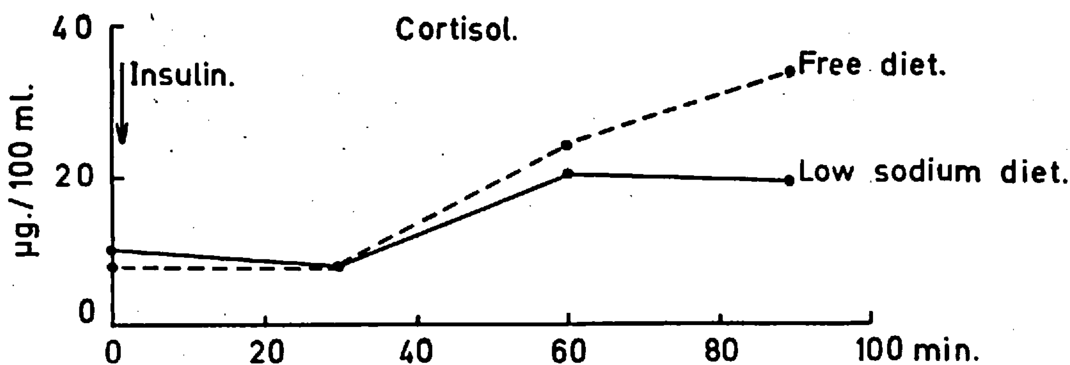
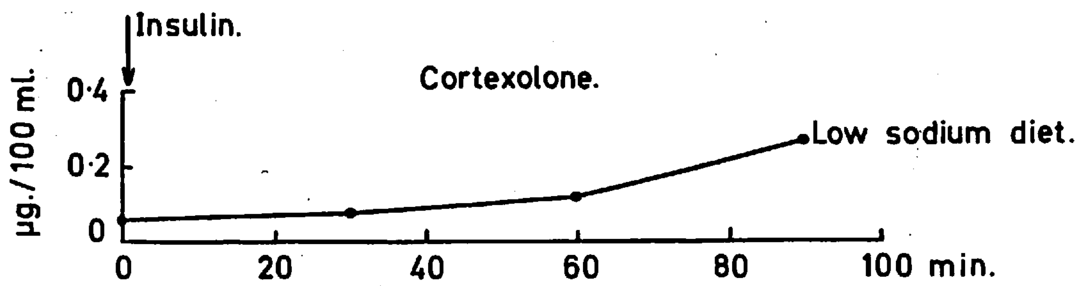
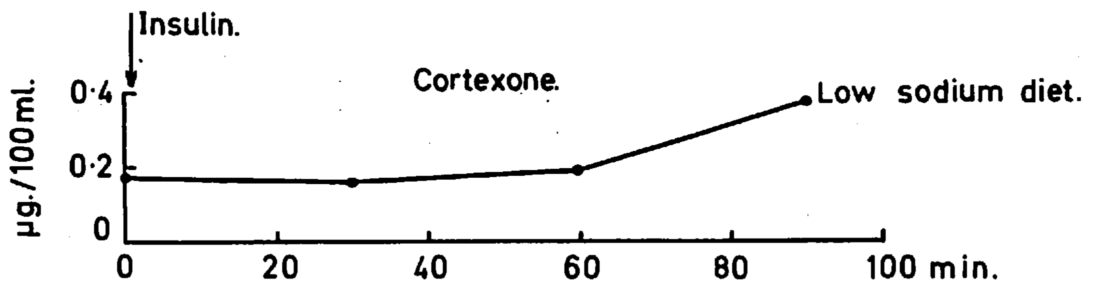


Fig. 7(a): The effect of insulin-induced hypoglycaemia on plasma corticosteroids.





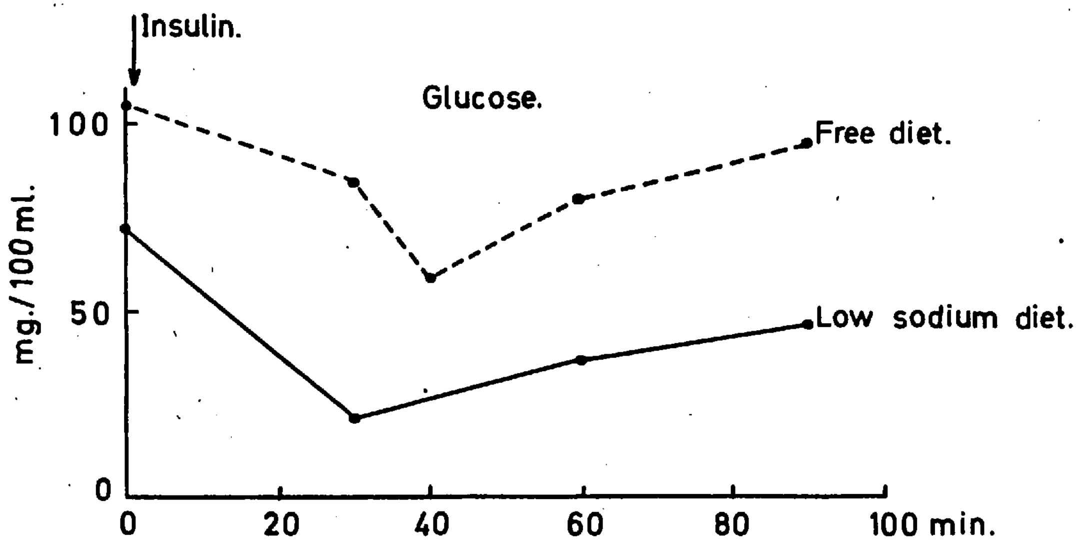


Fig.7(b): The effect of insulin-induced hypoglycaemia on plasma corticosteroids.

A sharp rise in the level of plasma cortisol and corticosterone concentrations occurred after 30 mins. in both experiments. Cortisol concentrations continued to increase throughout the experiment in the subject on a free diet, but in the second experiment, after sodium deprivation, a maximum concentration was reached at one hour. Cortisol : corticosterone ratios again fell markedly during the experiments. Only a small increase in aldosterone concentration occurred in the subject on an unrestricted diet, which contrasted markedly with the rapid, continued rise of this parameter to a very high level in the second experiment. Cortexone and cortexolone concentrations, which were measured only in the second experiment, were unaffected until 90 min. when those of cortisol and corticosterone had already achieved a maximum level.

3.6.2. The effect of angiotensin in normal subjects and in patients with Addison's disease and primary aldosteronism.

(a) The effect of angiotensin in normal subjects

Two normal male volunteers received an infusion of 5% dextrose (0.1 ml. per min.) in the first case, and 0.9% saline solution (0.1 ml. per min.) in the second, through a constant delivery, electrically driven syringe, for a control period, followed by an infusion of angiotensin (Hypertensin, Ciba, Ltd.) at rates of 0.007 µg. per Kg. per min., and 0.0063 µg. per Kg. per min., respectively. The subjects remained recumbent throughout. Blood pressure was recorded at frequent intervals during the experiment and blood samples (50 ml.) were withdrawn during the control period, angiotensin infusion, and also after termination of the infusion. In this experiment, plasma cortisol concentration was determined fluorimetrically (Mattingly, 1962), but aldosterone and corticosterone levels were measured as previously described.

The results are shown in Fig. 8. The greatest increase in blood pressure between control and angiotensin infusions was from 115/70mm. Hg., to 122/84mm. Hg. Neither corticosterone nor 'cortisol' concentrations were significantly affected by angiotensin infusion, although in one subject high initial

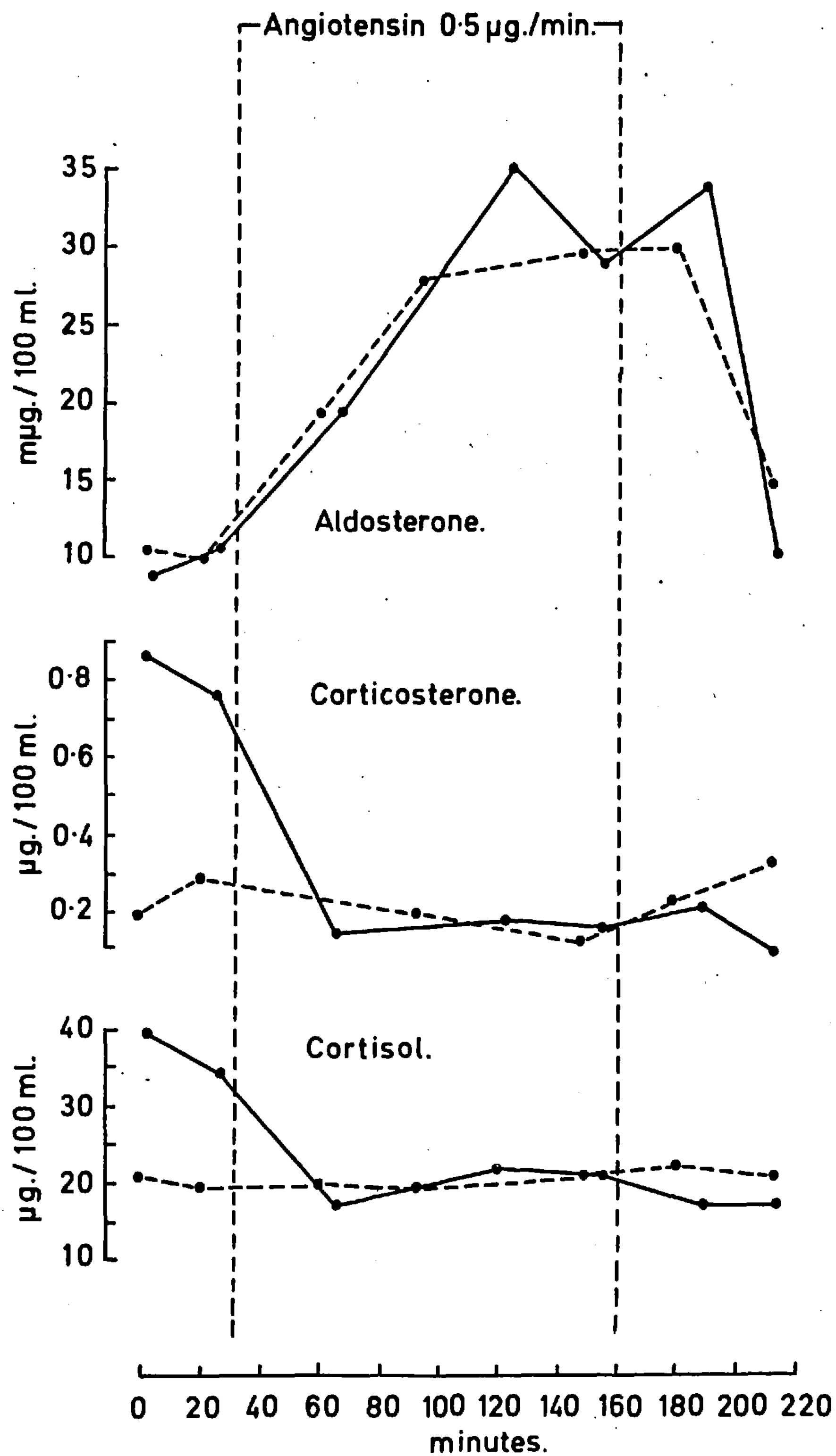


Fig. 8: The effect of angiotensin on the peripheral plasma concentration of corticosteroids.

concentrations of each substance were found which, however, had returned to normal before infusion of the pressor peptide. In contrast, in both subjects plasma aldosterone concentration had approximately doubled within 30 mins. of angiotensin infusion, attaining a maximum level of between 27 and 34 mμg. per 100 ml., which was maintained until about 30 mins. after the infusion was terminated. The concentration of aldosterone then fell rapidly to resting levels.

(b) The effect of angiotensin in normal man with suppressed pituitary function.

The experiment described in section 3.3.1 was repeated in two normal male volunteers who had received 1 mg. of dexamethasone at midnight and again at 8.0a.m., before commencing a control infusion of dexamethasone which then continued throughout the experiment. Angiotensin (0.5μg. per hr.) was infused for about 1½ hrs. Plasma steroid concentrations were measured by double isotope derivative assay and blood pressure readings were taken at frequent intervals.

The results are shown in Fig. 9 (a) and (b). Administration of dexamethasone had no effect either on the control concentration of aldosterone or on its response to

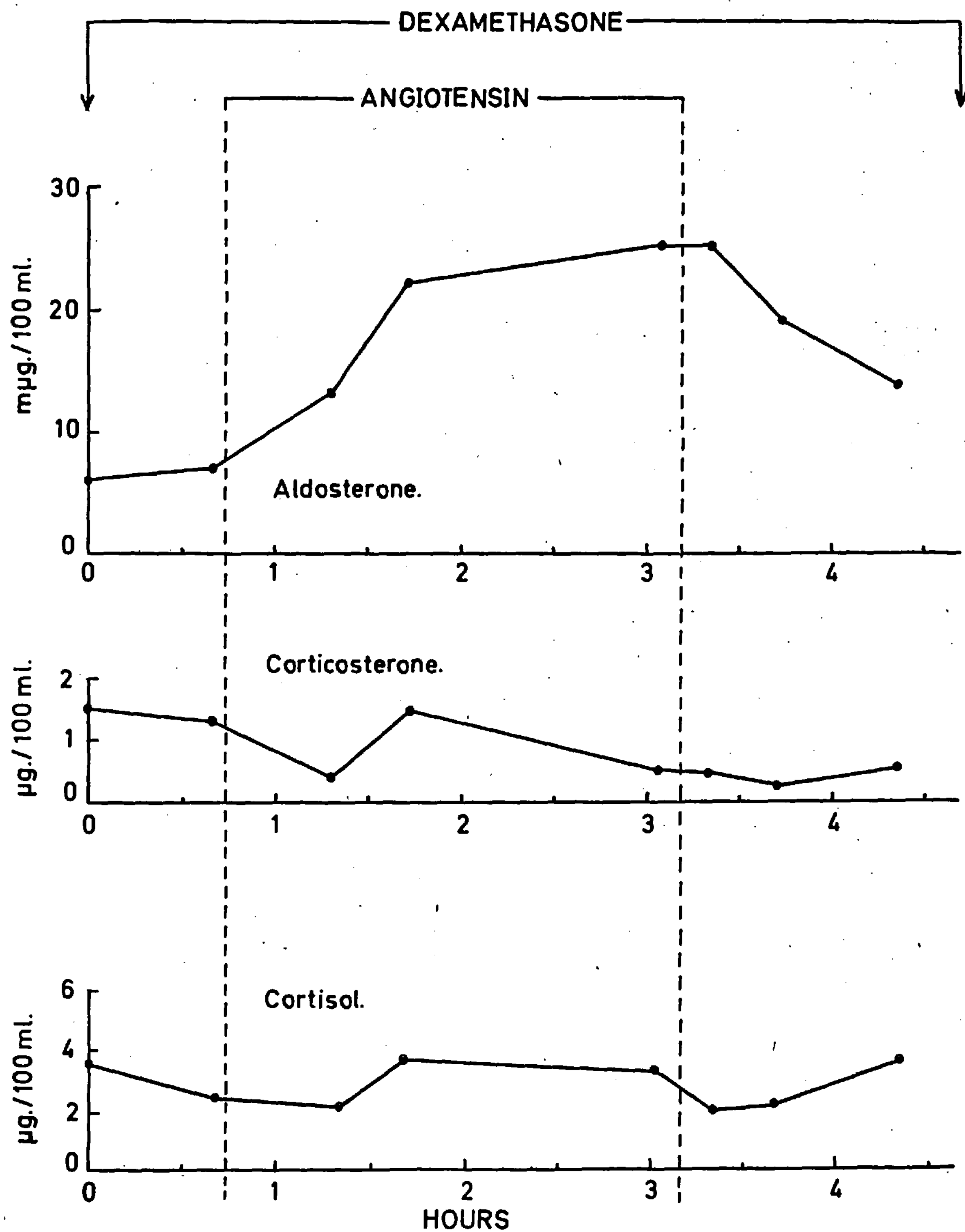


Fig. 9(a): The effect of angiotensin on peripheral plasma corticosteroid concentrations in the absence of pituitary function.

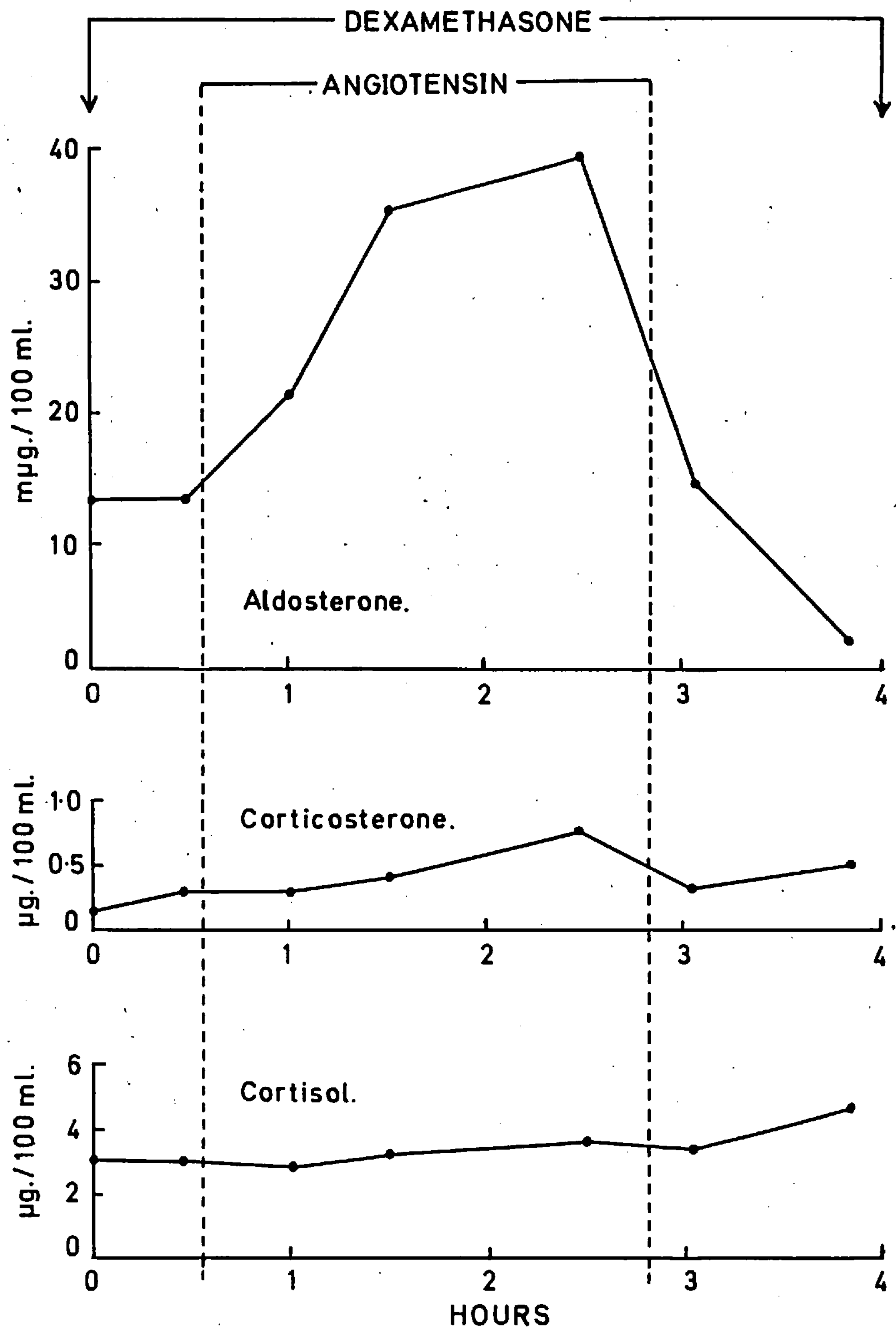


Fig. 9(b): The effect of angiotensin on peripheral plasma corticosteroid concentrations in the absence of pituitary function.

angiotensin, neither did the concentrations of cortisol or corticosterone rise in response to the pressor hormone. It should be noted that, while the control level of cortisol was obviously suppressed below normal by dexamethasone, corticosterone concentration was not noticeably reduced (cf. section 3.2.1. (b)). As in the previous experiment, blood pressure was not markedly elevated during angiotensin infusion.

(c) Angiotensin infusion into patients with Addison's disease.

Two patients with adrenal insufficiency were subjected to infusions of angiotensin at a rate of 0.058ug. per Kg. per min, in the first case (A), and 0.0078ug. per Kg. per min., in the second (B). Control blood samples were compared with those taken at the termination of infusion. Plasma 'cortisol' concentrations were estimated fluorimetrically (Mattingly, 1962).

In case B, a small, submaximal rise in aldosterone concentration (cf. 3.3.2 (a)) occurred, but in case A no response was obtained, both resting and post-infusion concentrations being insignificantly different from zero. No changes in 'cortisol' concentrations were noted and changes in plasma corticosterone levels were inconsistent. The results are shown in Table 21.

TABLE 21. THE EFFECT OF ANGIOTENSIN IN PATIENTS WITH ADDISON'S DISEASE

	Patient.	Aldosterone. (mμg./100ml)	Corticosterone. (mμg./100ml)	'Cortisol' (μg./100 ml)
Control	A	* 4.2	0.4	5.9
	B	* 2.1	* 0.1	5.9
After Infusion	A	* 0.0	* 0.1	6.3
	B	10.1	0.4	5.3

(* Values not significantly different from zero.)

(d) The effect of angiotensin in a patient with primary aldosteronism

The treatment described in section 3.6.2(a) was repeated in a patient with diagnosed primary aldosteronism, and previously shown to have an elevated plasma concentration of aldosterone (see section 2.3.6(e)). Again, plasma 'cortisol' concentration was estimated fluorimetrically.

Control concentrations of aldosterone measured at an interval of 10 mins., were within the normal range, but infusion of angiotensin caused a rapid rise which was reversed after termination of the infusion. Neither corticosterone nor 'cortisol' concentrations, which were both low throughout, responded significantly to the infusion.

The results are shown in Fig. 10.

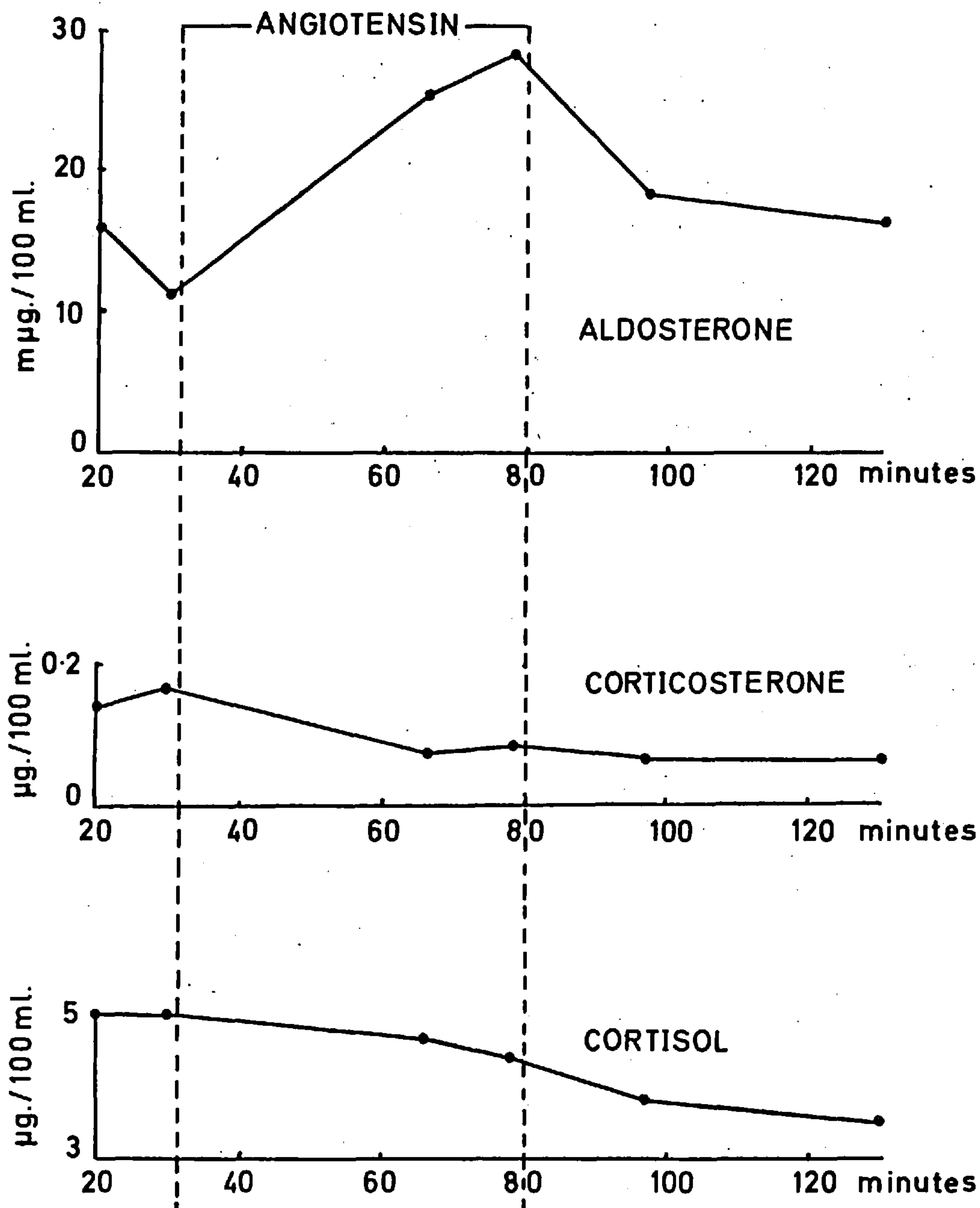


Fig.10: The effect of angiotensin in a patient with primary aldosteronism.

3.6.3. The effect of haemorrhage

(a) The effect of serial blood sampling

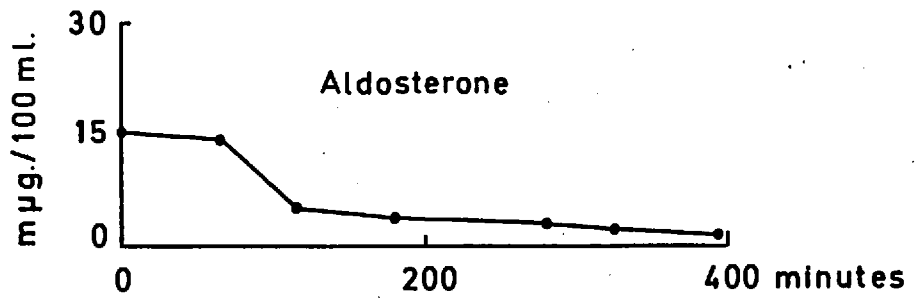
Blood samples were withdrawn from a single male volunteer via an indwelling needle at intervals and volumes approximating those used in other experiments in which serial estimations were made. The plasma corticosteroid concentrations measured are shown in Fig. 11.

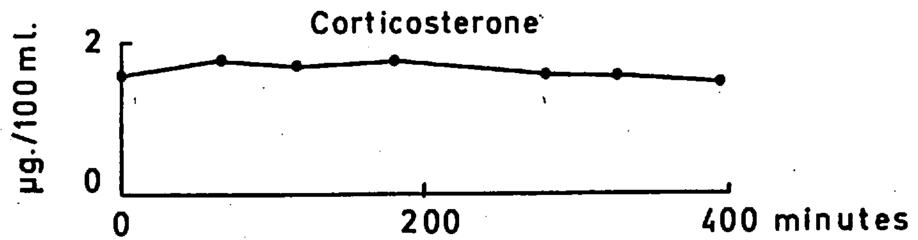
This type of haemorrhage had little effect on the concentrations of cortisol and corticosterone and, although aldosterone concentration appeared to fall during the experiment, the last four values were not significantly different from zero.

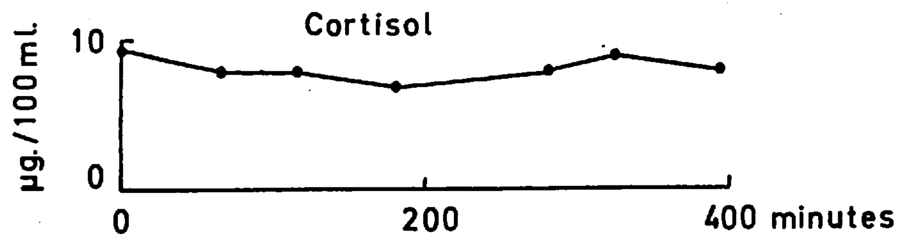
(b) The effect of acute haemorrhage

Two normal male volunteers were subjected to a 500ml. haemorrhage, the first and last 50ml. of which were used for corticosteroid determinations. Two further samples (100ml.) were taken from each subject at 35 min. and 60 min. The results are shown in Fig. 12.

No consistent alteration in the plasma concentration of any of those corticosteroids determined had occurred at 35 mins., and in one subject, this was also true at 60 mins. Small increases were seen in the second subject at 60 mins., in aldosterone, corticosterone and cortisol.







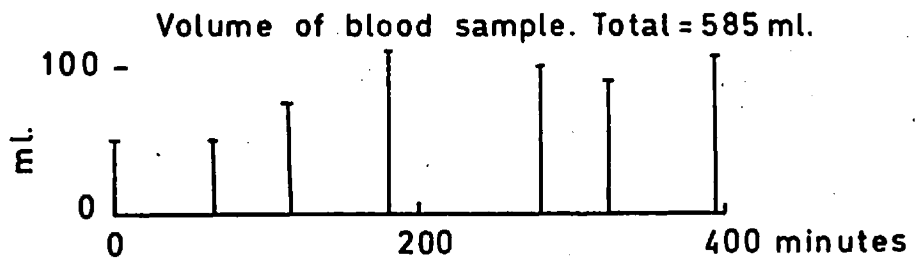
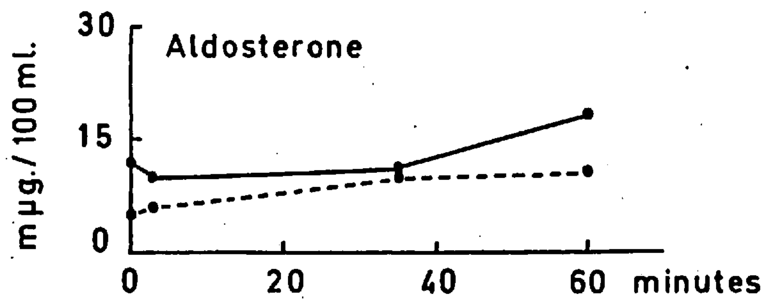
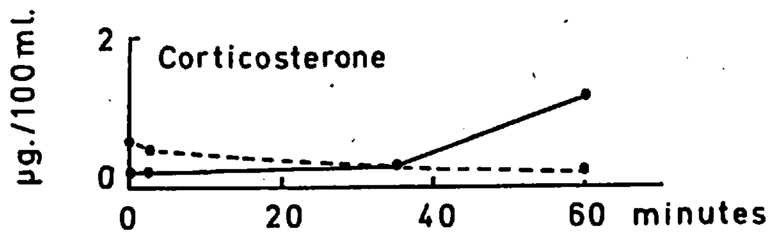
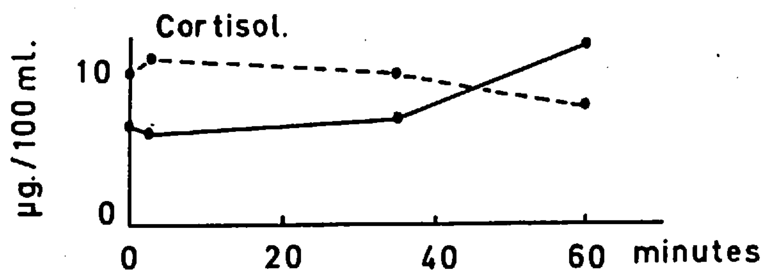


Fig. 11: The effect of serial blood sampling on plasma corticosteroids.







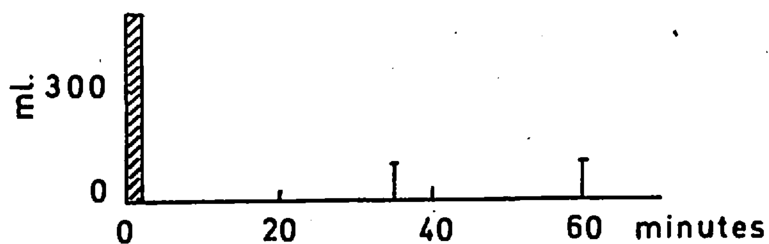


Fig.12: The effect of haemorrhage on plasma corticosteroids.

3.6.4. The effect of 'exercise' in patients with hypotension

Fig. 13 shows the results of an experiment in which four patients with orthostatic hypotension carried out 'exercise' which was graded to the severity of their condition, and which varied from sitting up in bed to going for a walk. Blood samples were taken before and after this exercise.

Plasma aldosterone concentrations in the recumbent patients were not significantly different from zero, and were increased in each patient by 'exercise', but in no case did the concentration exceed significantly the upper limit of normal. Little change occurred in the concentrations of cortisol or corticosterone after 'exercise', with the exception of one patient in whom the levels of both steroids rose slightly.

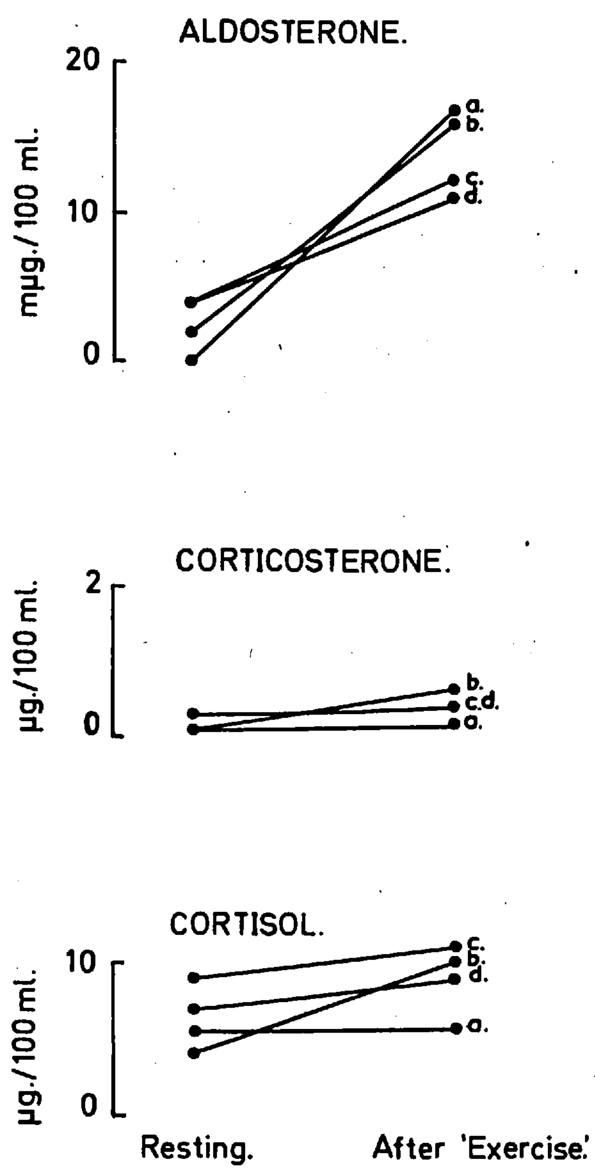


Fig.13: Plasma corticosteroids in patients with orthostatic hypotension.

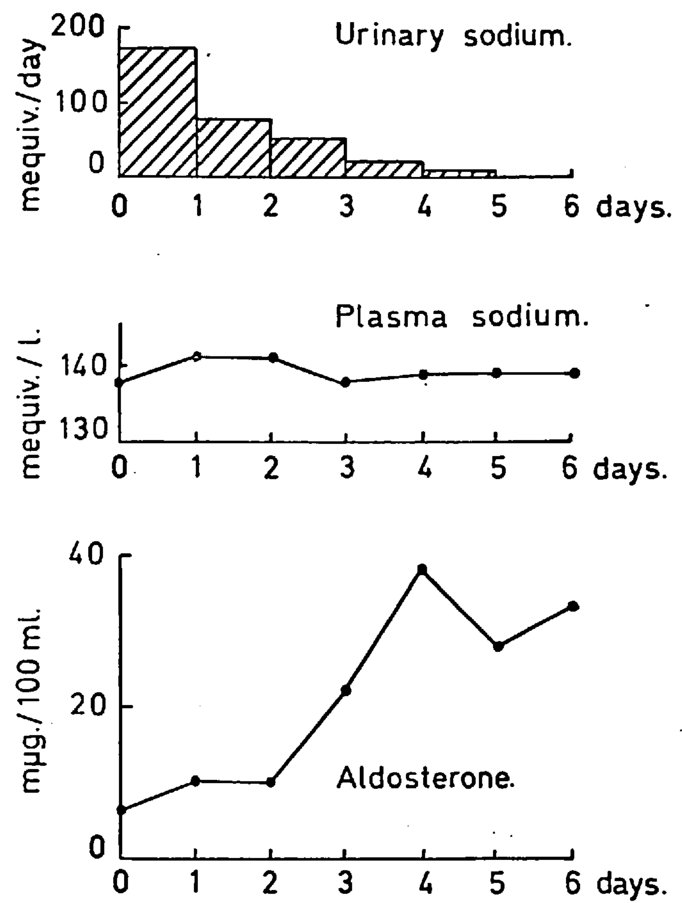
3.6.5. The effect of sodium depletion

(a) The effect of dietary sodium restriction in a normal man

A normal male subject was placed on a diet containing 10 mequiv. of sodium per day for a period of 6 days. Blood samples (100 ml.) were taken daily at approximately 3.0p.m., immediately before and during sodium deprivation, and 24 hr. urine collections were also made. Urinary electrolyte excretion and plasma electrolyte concentrations were measured by staff of the Medical Unit, St. Mary's Hospital. These results, together with the plasma corticosteroid concentrations, measured by double isotope derivative assay, are illustrated in Fig. 14.

Dietary sodium restriction did not affect plasma sodium concentration, neither were the potassium concentration and urinary potassium excretion significantly altered. Urinary sodium excretion fell markedly, however, to reach very low levels on the last two days of the experiment. Aldosterone concentration began to rise after the third day of restriction, reaching a maximum after four days, after which it remained relatively constant.

The concentrations of both cortisol and corticosterone fell slightly during the early part of the experiment, rising



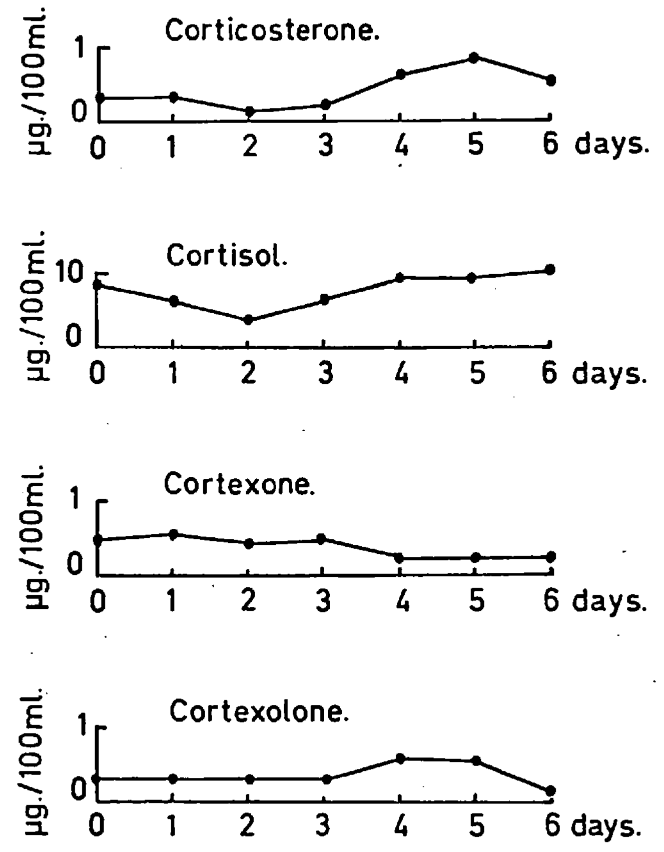


Fig.14: The effect of dietary sodium restriction on plasma corticosteroids in a normal man.

simultaneously on day 3. Whereas corticosterone concentration continued to increase to a final level significantly exceeding the initial control concentration, that of cortisol merely re-attained the concentration in the control sample. The concentrations of cortexone and cortexolone remained fairly constant throughout the experiment.

(b) The effect of dietary sodium restriction in a patient with sodium-losing renal disease

Sodium depletion was induced in a patient with recurrent urinary infection, bilateral renal calculi, and an inability to reduce sodium excretion, by placing her on a low sodium (10 m.equiv./day) diet. Blood samples were taken in the late morning with the exception of the second sample taken at 6.0p.m., and the patient continued her normal domestic duties throughout the period of the investigation. Electrolyte and blood pressure data were again obtained by the Medical Unit, St. Mary's Hospital, and are shown in Fig. 15, together with plasma aldosterone, and corticosterone concentrations measured by double isotope derivative assay and plasma 'cortisol' measured fluorimetrically (Mattingly, 1962).

During the period of sodium restriction, plasma sodium concentration fell markedly and this was associated with a very large increase in plasma aldosterone concentration, but

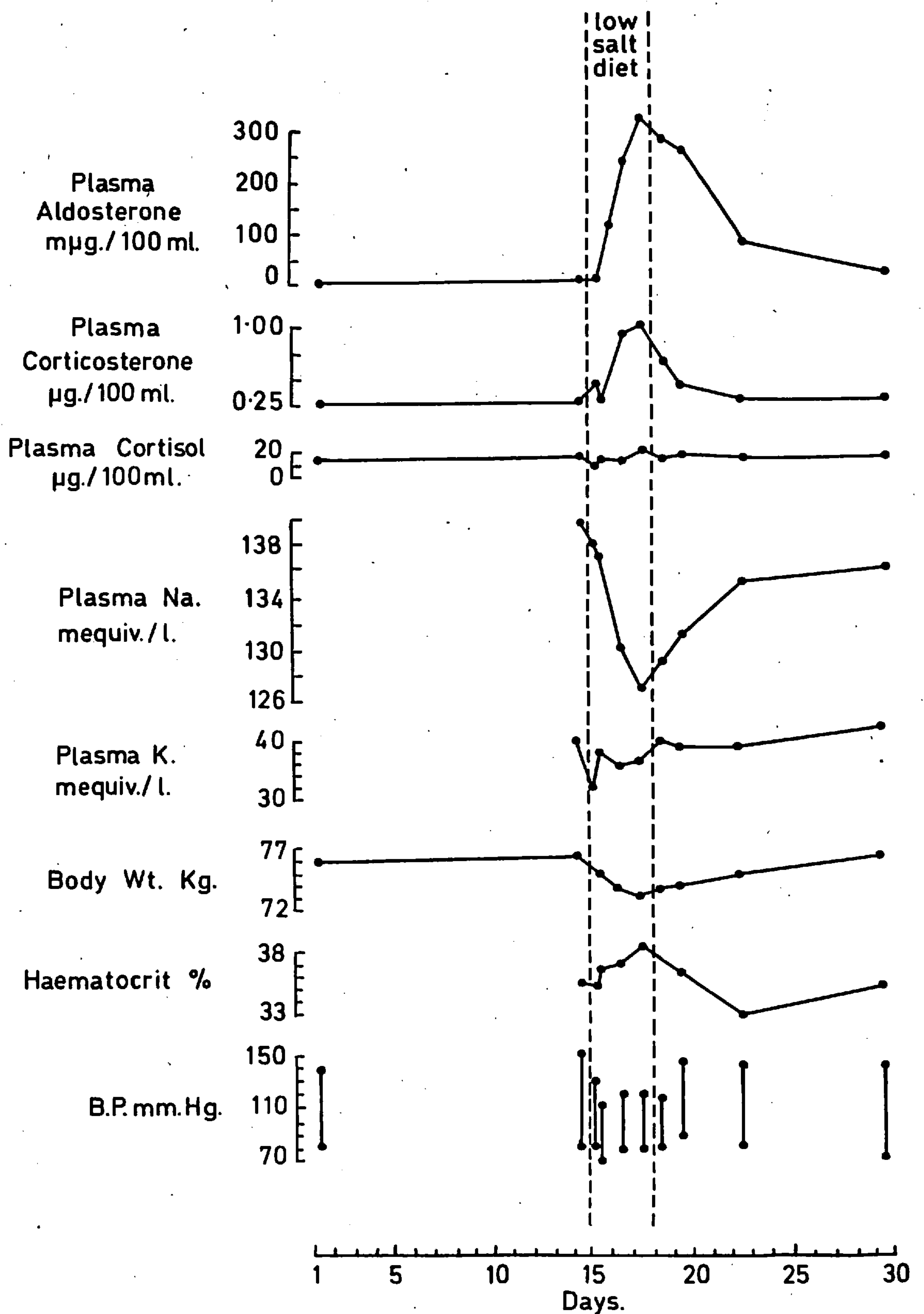


Fig.15: The effect of sodium depletion on plasma corticosteroids in a patient with sodium-losing renal disease.

these changes were readily reversed by replacing sodium in the diet of the patient. Plasma corticosterone concentration also rose significantly during the period of sodium depletion but plasma 'cortisol' levels were not affected, although these were consistently in the upper part of the normal range for this method (Wood, Frankland, James & Landon, 1965).

(c) <u>The effect of acute sodium depletion in normal man</u>

The effect of acute sodium depletion was studied in three normal male volunteers and, the effect of acute replacement of the sodium loss was also studied in one of these. The subjects emptied their bladders, and then drank 500 ml. of water. Two control blood samples (100 ml.) were collected and then 50mg. of Frusemide (4-chloro-N-(3-furfurmethyl)-5-sulphenoyl anthranilic acid) was administered intravenously. Blood samples (100 ml.) were taken at intervals from an indwelling needle. Urine was collected for electrolyte determination. In the third subject, urinary sodium loss, measured during the experiment, was then replaced by intravenous infusion of 0.9% sodium chloride solution.

All subjects showed a progressive increase in sodium loss during the experiment, although no change in plasma sodium or potassium levels was observed (Figs. 16 and 17).

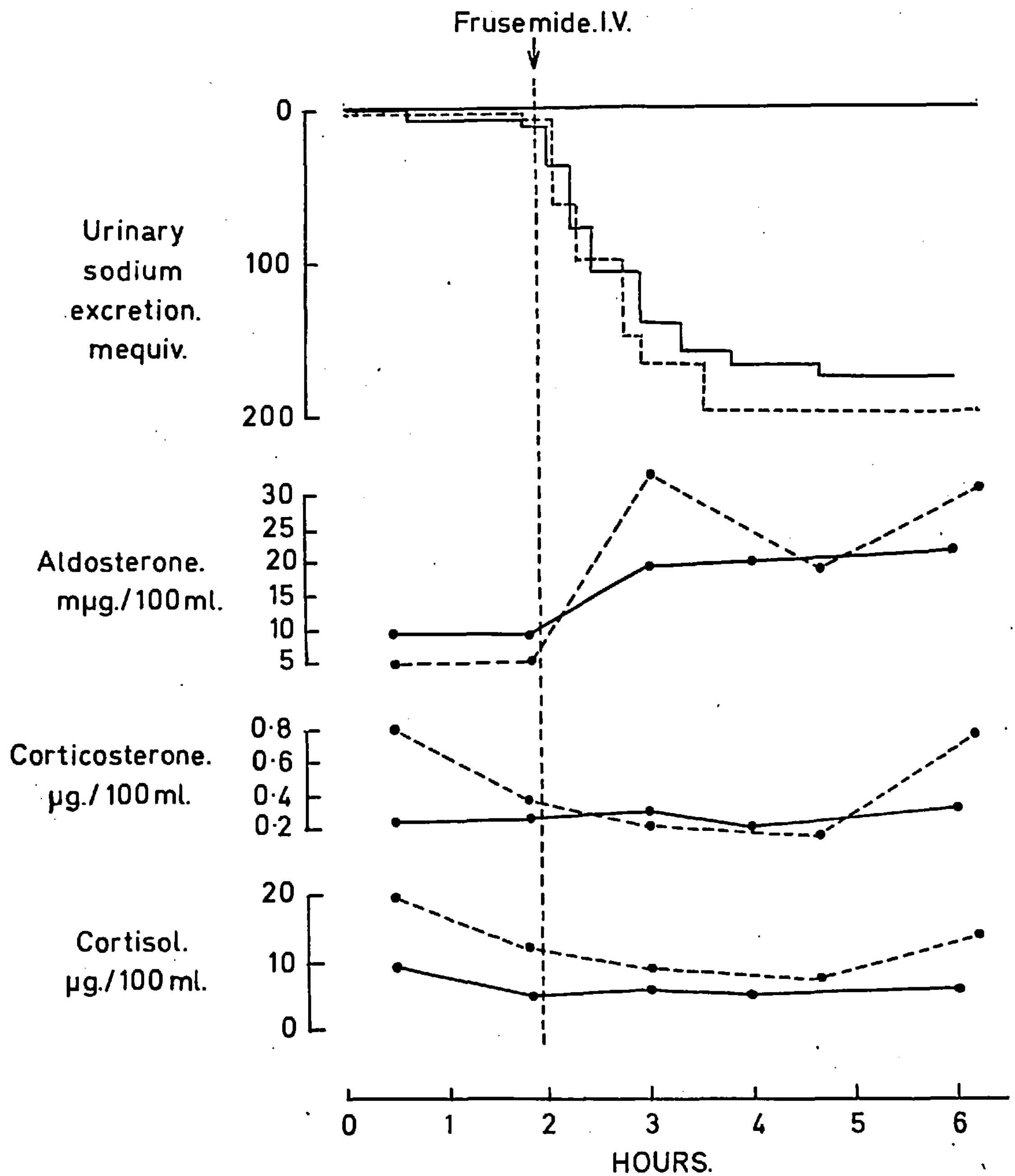


Fig.16: The effect of acute sodium depletion on plasma corticosteroids in normal men.

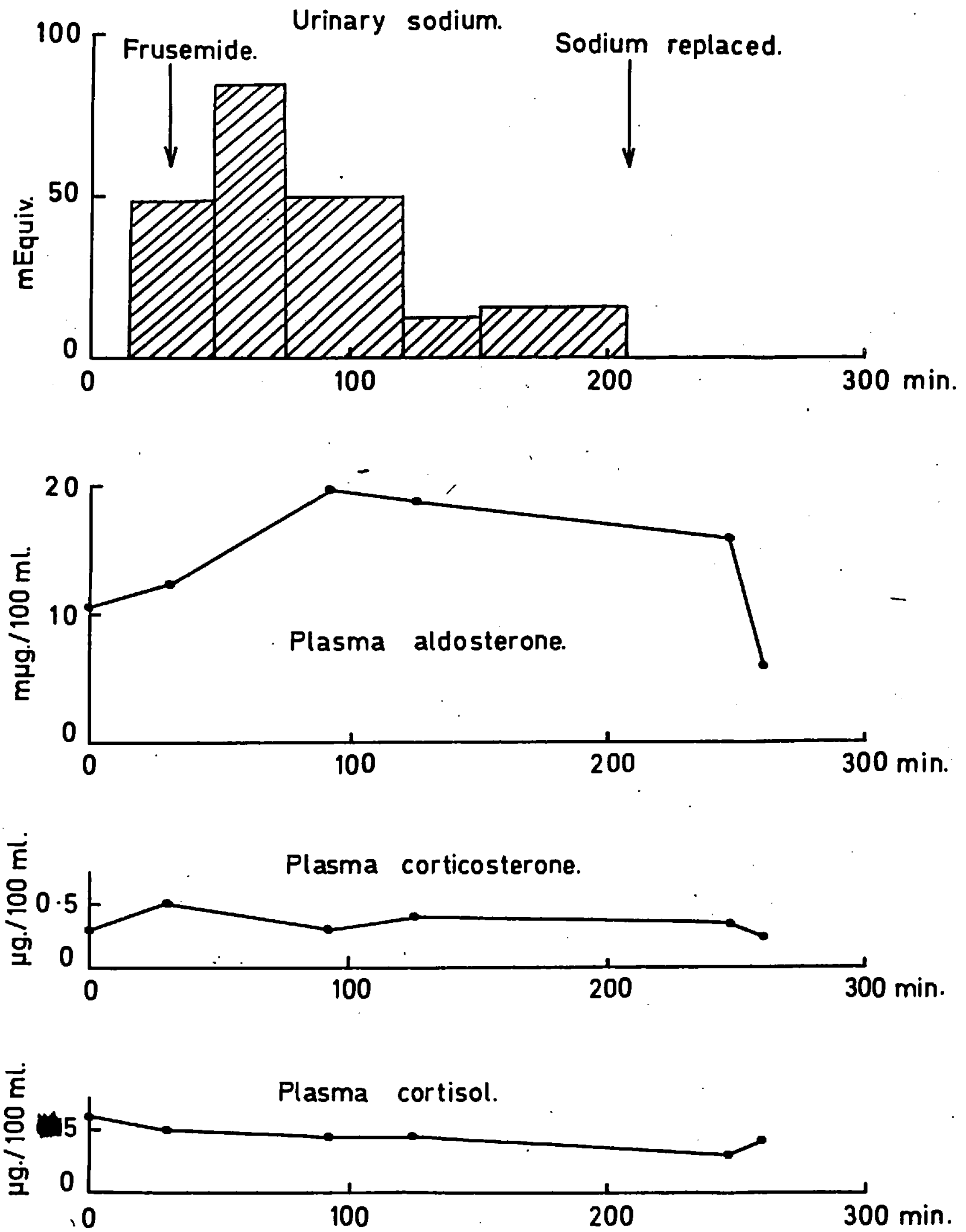


Fig.17: The effect of acute sodium depletion followed by replacement on plasma corticosteroids.

Neither cortisol nor corticosterone concentrations in plasma altered significantly during the experiments except in a single sample in one subject (Fig. 16) at 331 minutes in which both values increased. In none of the samples were the concentrations outside the normal range. The cortisol results in Fig. 16 were obtained fluorimetrically.

In the first two subjects (Fig. 16) no change in plasma aldosterone concentration occurred during the control period, but in both cases the sample obtained 60-70 minutes after the administration of Frusemide showed a significant increase to values above the upper normal limit. This coincided with a marked increase in sodium excretion, and for the remainder of the period of study, sodium excretion continued and plasma aldosterone concentrations remained elevated.

In the third subject (Fig. 17), a similar pattern of plasma aldosterone concentration was obtained, although the levels attained during sodium depletion were somewhat lower than those shown in Fig. 16. After re-infusion of sodium chloride, the plasma aldosterone concentration fell slowly during the following two hours but the decrease during the last 30 minutes of the experiment was more rapid.

3.7. Discussion

At the outset it must be emphasised that the preceding investigations were conducted on a small number of subjects and also that the experiments have not been repeated a sufficient number of times to allow more than tentative conclusions to be drawn. This is particularly true of the investigation of the role of ACTH in which all the experiments were carried out on the same volunteer. While this facilitates the comparison of experimental treatments, confirmation, using further normal subjects, preferably of different ages, sex, etc., is obviously desirable. The analysis of a single batch of plasma samples takes approximately five weeks, and the accumulation of data is consequently a slow process.

With the exception of the sodium restriction experiments, which lasted for a period of several days, only acute alterations in plasma steroid concentration were examined; the duration of the majority of experiments was between one and a half and six hours. It is possible that longer treatment may have resulted in patterns of corticosteroid secretion different from those described here.

The withdrawal of blood in volumes necessary for these studies had little effect on the plasma concentrations of the

three steroids examined and it seems unlikely, therefore, that this degree of haemorrhage could have contributed significantly to the alterations described in section 3.6. Even when larger blood volumes were removed from two normal subjects, no consistent changes in concentration were seen, although in one of these, small increases in the concentration of aldosterone, corticosterone and cortisol occurred one hour after haemorrhage. These results are in good agreement with the behaviour of renin in similar circumstances (Brown et al. 1965).

(a) ACTH.

The difference in the effect of ACTH at various rates of infusion is striking. Although all infusions produced a rise in plasma aldosterone concentration, this was only sustained at a high, pharmacological dose, the effect becoming increasingly transient at lower, more physiological doses - doses which were still adequate to cause maximum cortisol responses. At the lower ACTH dose levels it seems possible that the initial rise in plasma aldosterone concentration may have been due to an additive effect of endogenous and exogenous sources of ACTH, and that when the former source was inhibited by the negative feedback system, a decrease

in aldosterone concentration resulted.

Elimination of the endogenous secretion of ACTH, while it resulted in very low plasma cortisol and corticosterone concentrations, did not suppress the quantity of aldosterone in the plasma, indicating that the component of pituitary secretion suppressed by dexamethasone may not be necessary to maintain normal aldosterone production. Serial doses of β^{1-24} ACTH revealed that the dose of trophic hormone required to affect aldosterone is much higher than that which stimulates the secretion of cortisol or corticosterone in the human on an unrestricted diet.

It is possible that the effects of the tetracosapeptide on the individual adrenocortical steroid hormones may be different from those of the natural trophic hormone, making comparison of experiments 3.6.1(a) and (b) difficult. While no detailed comparison has yet been reported, neither Lamberg, Sandström & Pesonen (1966) nor Desaulles & Rittel (1967) could demonstrate any differences in the effects of the preparations on urinary steroids or total plasma corticosteroids, although the latter workers showed that substitution of D-serine for L-serine in the β^{1-24} ACTH molecule increased the potency and duration of action of the hormone.

The administration of small doses of peptide hormones is subject to error due to adsorption on to glass apparatus, etc. (Stouffer & Lipscomb, 1963), but this can be eliminated by acidifying the peptide solution to pH2 or below, injections of small volumes causing no discomfort to the experimental subject.

Judged by the submaximal effect of the doses of β^{1-24} ACTH on cortisol concentration, the preceding experiment may be assumed to imitate physiological conditions, but a more satisfactory procedure is to induce the release of endogenous ACTH by means of stress. Insulin-induced hypoglycaemia caused a rapid rise in both cortisol and corticosterone concentrations irrespective of the diet of the subject, which is indirect evidence for the release of ACTH, but the plasma aldosterone concentration was only affected during the experimental period when the subject was deprived of sodium. In this sodium-deprived subject the level of aldosterone concentration continued to rise during the experiment, whereas that of cortisol and corticosterone reached a maximum at one hour following insulin administration, after which cortisol concentration fell slightly. This increased sensitivity of aldosterone production confirms previous observations in

animals by Blair-West et al. (1962) and Ganong et al. (1966). It is therefore obvious that statements of threshold data such as those made above, must be accompanied by a description of the electrolyte status of the experimental animal, and also that animal experiments examining the control mechanism of aldosterone where surgery or adrenal vein cannulation is carried out will be difficult to interpret particularly when the animals are on low salt diets, unless the pituitary is extirpated or its secretion suppressed.

In man, therefore, it may tentatively be concluded that normal aldosterone secretion is relatively independent of ACTH control, but that in circumstances where aldosterone levels are already elevated, as in salt deprivation, the pituitary trophic hormone may play an important supportive role.

Administration of ACTH, both porcine and β^{1-24} preparations, and insulin-induced hypoglycaemia caused a predictable increase in plasma cortisol and corticosterone concentrations, and the threshold doses of β^{1-24} ACTH required to increase each of these steroids could not be distinguished. The response of cortisol concentration to stress appeared to be modified by diet, and it is tempting

to postulate that the reduced maximum level by the salt-deprived subject may be related to the alleged salt-excreting properties of this steroid hormone (Thorn, Engel & Lewis, 1941). More evidence is required before such a definite statement can be made.

The results of Bush (1953) and Cameron & Kilborn (1964) could not be confirmed here. With the exception of the administration of β^{1-24} ACTH, when the doses were at no time sufficient to raise the concentrations of either cortisol or corticosterone above the upper limit of normal, ACTH consistently produced a greater proportionate rise in corticosterone levels than in those of cortisol, consequently depressing the cortisol : corticosterone ratio. It is also relevant to point out that the ratios of the resting normal levels in this single subject varied between 22 and 59 (mean 36, N = 5), and that in a series of normal subjects the wider range of 6 - 59 (mean 21, N = 20) was obtained. It is clear, therefore, that in man, at least, there is no specific, genetically controlled ratio, neither is the ratio constant within a given individual. Since these steroid hormones have different physiological effects, it is also apparent that ACTH alters, not only the total output of the adrenal cortex, but

also the overall character of its secretion.

Stress, causing marked increases in plasma cortisol and corticosterone concentrations, resulted in only small rises in the cortexone and cortexolone content which were only significant at 90 mins. as opposed to 60 mins. for the other steroids examined. The doses of β^{1-24} ACTH used in the experiment had no significant effect on these 11-deoxy-steroids. It is possible that any increase in production within the adrenal cortex is effectively consumed by conversion to 11-oxy compounds such as corticosterone and cortisol, and that little escapes into the adrenal veins. It appears that only when the adrenocortical 11-hydroxylation system is inhibited, for example, with metapyrone, or when its capacity is exceeded, will the plasma concentration of 11-deoxysteroids rise in the normal human. The increase in response to stress, which occurred after the concentrations of cortisol and corticosterone had reached a maximum level, may provide evidence for this theory but a longer experiment or a higher level of stimulation is required for confirmation.

(b) <u>Angiotensin</u>

There is little doubt that angiotensin increases the concentration of aldosterone in the human peripheral circulation,

either in the presence or absence of ACTH, and that the effect is accomplished rapidly enough to accord with the postulate that angiotensin may be part of a natural control mechanism. Although the rapidity of response cannot be taken as positive evidence, a slow response would have eliminated the renin-angiotensin system as an important factor in the control of aldosterone secretion. From the preceding results it also appears that the effect of angiotensin is selective for aldosterone since little consistent elevation of corticosterone or cortisol concentrations occurred, even when ACTH secretion was suppressed by dexamethasone administration (cf. Slater et al., 1965; Ganong et al., 1966), although it must be admitted that the sensitivity of the methods of assay of these substances would have been inadequate to distinguish changes in concentration of the same order as those shown for aldosterone. Nevertheless, even if such changes occur, they would be of doubtful physiological significance.

Angiotensin reduces the splanchnic blood volume, and thus the rate of perfusion of the liver (de Bono, Lee, Mottram, Pickering, Brown, Keen, Peart & Sanderson, 1963). Some experimental situations, such as haemorrhage, in which

aldosterone secretion rate is increased may also be accompanied by a reduced splanchnic blood volume (Price et al., 1966), and the reduced metabolic clearance rate resulting from a fall in liver perfusion may be in part responsible for the increase in circulating aldosterone levels. However, it seems unlikely that this is wholly responsible, since angiotensin has been shown to have a direct effect on the adrenal gland (see section 3.3), and because proportionate increases in the concentrations of the other steroid hormones, for which metabolic clearance rates would also be reduced, are not found to occur.

Angiotensin failed to elicit a similar response in aldosterone concentration in either of the patients with Addison's disease, even though in one case a much higher rate of infusion was used. While the experiment was intended to test the specificity of the method of assaying aldosterone in plasma and as a control for the experiment in normal humans, it is possible that such a procedure could be used to measure adrenal reserve with respect to the mineralocorticoids.

The effect of angiotensin in the patient with primary aldosteronism is more difficult to interpret. It is possible, either that the adrenocortical adenoma subsequently revealed by surgery was not autonomous but able to respond to angiotensin,

or alternatively, that the tissue of the zona glomerulosa in the same gland or of the unaffected gland was responsible for the increase in secretion. Either is possible. The effect of angiotensin on normal zona glomerulosa tissue <u>in vivo</u> and <u>in vitro</u> has already been discussed. That adrenocortical adenomata may not necessarily be autonomous is demonstrated by the experiments of Davignon, Tremblay, Nowaczynski, Koiw & Genest (1961) who, by means of ACTH, induced an increase in the production of cortisol, corticosterone, cortexolone and aldosterone <u>in vitro</u> in a tumour consisting mainly of zona fasciculata tissue.

(c) <u>Hypotension</u>

The low resting aldosterone concentrations found in hypotensive patients confirms the results of Hall and Hökfelt (1966) and Gordon, Küchel, Liddle and Island (1967). Although the plasma concentration of this steroid increased when the patient assumed an upright posture, the rise was of the same order or less than those reported for normal humans (Bojesen, 1964), and the maximum concentration achieved remained within the normal range. These results contrast with those of Wolff et al. (1965) which showed marked elevation of plasma aldosterone concentration in a normal person subject to orthostatic collapse.

Conversely, Gordon et al. (1967), measuring urinary aldosterone excretion, were unable to find any increase in this parameter in a patient with postural hypotension resulting from an upright posture but comparisons of plasma concentrations and excretion rates are not easy to make.

(d) Sodium depletion

The consistent increase in plasma aldosterone concentration following dietary sodium restriction or sodium depletion in both normal and diseased subjects, provides good evidence of the specificity of the method of determining this steroid. The rapidity with which the human adrenal gland responds to acute sodium depletion could not easily have been demonstrated by any other technique.

Cortisol concentrations, on the other hand, do not seem to be affected significantly, either by dietary restriction or by acute depletion, although if Erlich's hypothesis (Erlich, 1966) that cortisol has salt-excreting properties is to be accepted, the concentration of the hormone would be expected to fall in these circumstances. In both experiments with dietary sodium restriction, in a normal male and in a patient with sodium-losing renal disease, a rise in the plasma concentration of corticosterone occurred during restriction

which, in the latter subject, returned to the control level when sodium was replaced. However, the highest concentrations did not exceed the upper limit of the normal range, and no such increase occurred during acute sodium depletion. It seems possible to conclude that a fairly severe stimulus of this type is required to affect corticosterone secretion but that in these circumstances, the steroid may contribute to the total retention of sodium.

Although cortexone has marked sodium-retaining properties, neither the concentration of this steroid nor that of cortexolone responded to dietary sodium restriction in a normal man. The role of cortexone in sodium homeostasis is therefore enigmatic, although it is possible that it provides a basic, constant level of sodium retention, while 'fine' control, in circumstances where body sodium is either increasing or decreasing, is accomplished by altering the rate of aldosterone secretion.

(e) General discussion

From these results, distinct changes in plasma corticosteroid concentrations in response to a number of stimuli can be discerned. Perhaps most striking are the effects of physiological doses of ACTH which both increased the

concentrations of cortisol and corticosterone and also markedly altered their ratio. Angiotensin, on the other hand, had no significant effect on these steroids but consistently and specifically increased the plasma concentration of aldosterone, thus contributing to the evidence that this pressor polypeptide may be of importance in the control of electrolyte metabolism in man. Whether quantities of angiotensin sufficient to cause increases in aldosterone concentration, such as are described in sections 3.6.2 and 3.6.5, are in fact produced endogenously is a question which must await the development of a sensitive and reliable method of determining the polypeptide.

The effect of sodium depletion and deprivation on aldosterone levels in plasma can only be considered as confirming previous secretion and excretion rate measurements. The effect on corticosterone concentration, however, is of new interest. The increases described in section 3.6.5 were small but significant and suggest that this steroid may, in some circumstances contribute to sodium retention.

The environment of the adrenal cortex is composed of several factors, a number of which may act simultaneously upon the gland, and the individual effects of these factors

may consequently be modified. This is strikingly illustrated by the contrasting response of aldosterone secretion in experimental subjects on diets containing different quantities of sodium. It seems probable that the effects of other stimuli may be modified in this way. For example, the response to angiotensin may also depend on the sodium status of the subject. There is much to be learned concerning the interaction of these environmental factors.

Much emphasis has been placed on the measurement of hormones in blood or plasma and comparatively few data are available from other body fluids. The corticosteroid pattern in the lymph draining from the adrenal cortex and the changes wrought in this pattern by ACTH, angiotensin and other factors are as yet unknown. Progress in this direction, and also in the study of factors inhibiting adrenocortical secretion, particularly aldosterone secretion, is limited by the lack of sensitivity of the methods of assay.

Adrenal vein blood may contain many steroid compounds other than those studied here, and their significance is little understood. Steroids, such as progesterone, and possibly also non-steroid substances, derived from other organs may play a modifying role in adrenocortical physiology. The double isotope

derivative assay of plasma steroids provides a powerful tool with which to study these situations. In addition, there are many problems in medicine, such as the etiology of hypertension, in which the application of this technique could well be fruitful.

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