MEASUREMENT OF PERIPHERAL PLASMA CORTICOSTEROID CONCENTRATIONS BY MEANS OF GAS-LIQUID CHROMATOGRAPHY

A Thesis presented in part fulfillment of the requirements for admittance to the degree of Doctor of Philosophy of the University of Glasgow.

Peter A. Mason, B.Sc., 1976

ACKNOWLEDGEMENTS

I would like to thank the staff of the M.R.C. Blood Pressure Unit, Western Infirmary, Glasgow, for their help and encouragement during the work of this thesis. In particular, I wish to thank Dr. A.F. Lever (Director) and Dr. R. Fraser for valuable advice and guidance. I wish also to thank Professor R.M.S. Smellie under whose auspices this work was conducted.

I would like to thank Mrs. Evelyn Cruikshank for her help in the typing of this thesis.

Finally, I would like to thank my wife, Pamela, for her patience and encouragement during the period of this work.

SUMMARY

1. A method has been devised and evaluated for measuring the plasma concentrations of six major corticosteroids - aldosterone, 18-hydroxy-11-deoxycorticosterone (18 OH DOC), corticosterone, 11-deoxycorticosterone (DOC), cortisol and 11-deoxycortisol - using gas liquid chromatography (GLC) with electron capture detection. After extraction from plasma and both chromatographic and chemical purification, thermostable derivatives of the compounds were formed; γ-lactones of the 18-oxygenated steroids, aldosterone and 18 OH DOC; androstenes of the 17α-hydroxylated steroids, cortisol and 11-deoxycortisol. Esterification of these derivatives with heptafluorobutyric anhydride (HFBA) formed enyl-heptafluorobutyrates which could be detected in quantities ranging from 0.3 pg for androstenetrione HFB to 2.3 pg for corticosterone 3, 21 bis HFB.

2. Accuracy, specificity, precision and convenience have also been assessed for each compound and discussed in relation to similar aspects of the performance of methods based on other techniques such as radioimmunoassay. Recovery of added steroid appeared to be quantitative in all cases and replicate variations of steroid values from a plasma pool were low in relation to the ranges of concentration experienced. Use of several GLC columns with differing separative/

separative properties indicated that discrete compounds were producing the chromatographic responses. The method has slightly lower capacity than the simplest steroid radioimmunoassay and a high degree of operator skill is needed to maintain efficient performance of the GLC. However, a physico-chemical method, such as GLC, for estimation of steroid levels has several distinct advantages over those based on saturation analysis. The principal one of these is the ease of setting up a method for a steroid which may be of only transient interest. In this case it would be a fairly simple matter to "add in" other steroids, such as 18-hydroxy-corticosterone or cortisone, whereas with radioimmunoassay, considerable effort and expense would be required to produce the necessary antigens and antibodies.

3. Investigation of the effects of some factors which influence adrenocortical secretion was undertaken. This allowed further evaluation of the method and also yielded new data. Levels of all steroids measured except aldosterone followed the diurnal rhythm of ACTH, although an effect on aldosterone may have been masked by changes in posture. Similarly, stimulation of endogenous ACTH by hypoglycaemia, induced by insulin administration (insulin stress test), caused a rise in all steroid plasma levels. Infusion of exogenous ACTH uncovered a possible role of the hormone in electrolyte metabolism./

metabolism. Infusion rates designed to produce levels in the high physiological range caused very marked stimulation of DOC and corticosterone secretion - steroids with known mineralocorticoid properties. Angiotensin II, however, whether administered exogenously or raised by physiological manipulation caused stimulation of secretion of aldosterone only.

4. Analyses were made in several pathological states; in 7 analyses of bilateral adrenal venous and also concurrent peripheral plasma samples of patients suffering from primary hyperaldosteronism; in two analyses of peripheral plasma from patients suffering from Cushing's syndrome due to an ectopic ACTH-producing tumour. It was possible, using the method, to predict from the adrenal venous results the site of the lesion in the 7 cases analysed. It is thought that the method may be of diagnostic value, also, in helping to distinguish primary hyperaldosteronism due to an adenoma from that due to bilateral hyperplasia. Results from the two patients with Cushing's syndrome indicated yet again that high levels of ACTH cause gross elevation of DOC and corticosterone secretion and considerable disruption of electrolyte metabolism.

5. The "multisteroid" approach has advantages over individual steroid estimation methods in that it is possible to monitor the secretory pattern of adrenocorticoids in investigation of both normal adrenal physiology and abnormal disease processes.

It/

It is thought that this type of measurement may be of particular value in research into causes of hypertnesion, where many factors are interrelated.

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1 INTRODUCTION

INTRODUCTION

Assessment of the role played by the adrenal cortex in electrolyte homeostasis and control of intermediary metabolism is a complex problem because, unlike many other endocrine glands such as the Islets of Langerhans or the thyroid, the adrenal synthesises and secretes a wide variety of biologically active compounds (Figure 1), varying not only qualitatively, but also quantitatively. For example, cortisol, a glucocorticoid, affects the metabolism of protein and carbohydrate in the liver, but has little direct effect on renal sodium conservation, whereas aldosterone appears to act primarily on electrolyte metabolism (i.e. a mineralocorticoid) and although relatively potent in terms of activity per unit mass, by virtue of its low secretion rate, may play little significant part in intermediary metabolism. Corticosterone has both mineralocorticoid and glucocorticoid properties but the former is less potent than aldosterone and the latter less potent than cortisol. In addition to the corticosteroids, the adrenal cortex also secretes small quantities of oestrogens, large quantities of androgens such as dehydroepiandrosterone (DHA) and its sulphate, and a certain amount of progesterone. No studies have been carried out on these latter compounds in this thesis.

Evaluation/

Evaluation of adrenocortical activity is further complicated by the susceptibility of its secretion to changes provoked by direct sampling which in any case is rarely possible in patients and never possible in normal human subjects. Stresses of many kinds affect secretion of the various corticosteroids to a different extent leading to alterations not only in the overall rate of secretion but possibly also in the nature of its total effect.

The hormones of the adrenal cortex are chemically similar and relatively dilute in plasma which may be the most appropriate medium to study. Thus, methods of measuring concentrations of individual corticosteroids must be at once specific and sensitive. There follows an account of attempts to establish and validate methods for estimating the plasma concentrations of the major corticosteroids in man based on the technique of gas liquid chromatography with electron capture detection. These methods have then been used to study some of the changes which occur in normal subjects.

1.1 Nomenclature.

The following trivial names have been used.

Table 1 (a)

Trivial Name	Systematic Names
Aldosterone	11β, 21-dihydroxy-4-pregnene-3, 20-dione-18, al.
Corticosterone	11β, 21-dihydroxy-4-pregnene-3, 20-dione.
Deoxycorticosterone (DOC)	21-hydroxy-4-pregnene-3, 20-dione.
18-hydroxy-corticosterone	11β, 18, 21-trihydroxy-4-pregnene-3, 20-dione.
18-hydroxy DOC (18OHDOC)	18, 21-dihydroxy-4-pregnene-3, 20-dione.
Cortisol	11β, 17α, 21-trihydroxy-4-pregnene-3, 20-dione.
11-deoxycortisol	17α, 21-dihydroxy-4-pregnene-3, 20-dione.
Spironolactone	β-(7α-acetylthio-17α-hydroxy-3-oxandrost-4-en-17-yl) propionic acid lactone.
Dexamethazone	9α-fluoro-16α-methyl-11β, 17α, 21 trihydroxy-1, 4-pregnadiene-3, 20-dione.
Aldosterone-γ-lactone	11β, 18$\in_2$-epoxy-17β-carboxyandrost-4-en-3-one, 17^1, 18-lactone.
Androstenedione	4-androstene-3, 17-dione
18 OH DOC-γ-lactone	17β-carboxyandrost-4-en-3-one, 17^1, 18-lactone.

Trivial Name	Systematic Names
Androstenetrione	4-androstene-3, 11, 17-trione.
11-ketoprogesterone	4-pregnene-3, 11, 20-trione.
17α -hydroxyprogesterone	17α -hydroxy-4-pregnene-3, 20-dione.
Deoxycorticosterone bis-heptafluorobutyrate (DOCHFB)	3, 21-bis (heptafluorobutoxy)-3, 5-pregnadiene-20-one.
Corticosterone bisheptafluorobutyrate	11β -hydroxy-3, 21-bis (heptafluorobutoxy)-3, 5-pregnadiene-20-one.
Aldosterone-γ-lactone heptafluorobutyrate.	3-(heptafluorobutoxy)11β, 18 ϵ epoxy-17β-carboxyandrost-3, 5-diene, 17^1, 18-lactone.
18OHDOC-γ-lactone heptafluorobutyrate	3-(heptafluorobutoxy)-17β-carboxyandrost-3, 5-diene, 17^1, 18-lactone.
Androstenedione heptafluorobutyrate	3-(heptafluorobutoxy)-androst-3, 5-diene-17-one.
Androstenetrione-heptafluorobutyrate.	3-(heptafluorobutoxy)-androst-3, 5-diene-11, 17-dione.
11-ketoprogesterone-heptafluorobutyrate.	3-(heptafluorobutoxy)-pregna-3, 5-diene-11, 20-dione.

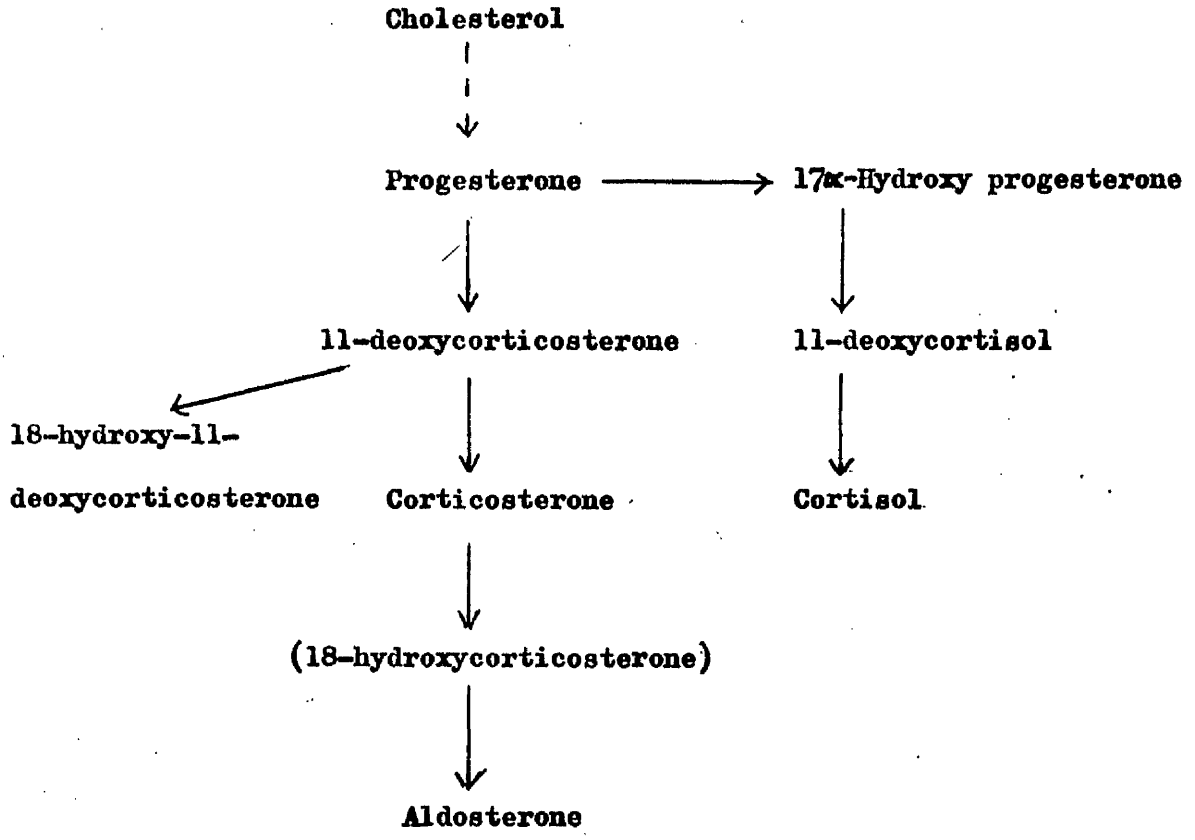


Figure 1 (a)

Biosynthesis of mineralocorticoids and glucocorticoids

2. APPLICATIONS OF GAS-LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION TO PLASMA CORTICOSTEROID ANALYSIS.

2.1 LITERATURE SURVEY

2.1.1 Introduction

Chromatography employs small differences in the physical properties of compounds, particularly solubility, to effect their separation. The effect is of a continuous partition between two phases, one stationary, the other mobile. The stationary phase may be solid or liquid while the mobile phase must be gaseous or liquid. There are therefore four possible combinations of phases which can be used and these have been summarised in Table 1. Where the stationary phase is solid, a mixture of compounds dissolved in the mobile phase will separate as they pass over the solid surface according to their different tendencies to adsorb on it. This type of chromatography is called "adsorption chromatography" and is subject to the laws of adsorption as laid down by scientists such as Freundlich and Langmuir. If, however, the stationary phase is liquid, then the laws of solubility and partition between solvents, such as those of Henry or Nernst, apply and the technique is called "partition chromatography". The technique can be subdivided into liquid chromatography and gas liquid chromatography (GLC) depending on the physical nature of the mobile phase. It is with GLC that this thesis is concerned. GLC is conducted in separatory or fractionating columns/

columns which are packed with a solid supporting material such as kieselguhr, impregnated with a liquid (e.g. a silicone oil). The compounds to be separated are mixed in gaseous or vapour form with the mobile phase, an inert carrier gas such as nitrogen, which travels through the bed of inert support coated with stationary phase. Depending on their affinity for and their solubility in the stationary phase, the compounds are retarded to a variable degree as they travel through the column as separate fractions.

Table 1(b) Chromatographic Techniques.

Mobile Phase	Stationary Phase	
GAS	Solid (adsorption)	Liquid (partition)
	Gas-solid chromatography	Gas-liquid chromatography
LIQUID	Liquid-solid chromatography (adsorption chromatography)	Liquid-liquid chromatography (paper chromatography)

2.1.2 Historical development

The study of natural products began to occupy the attention of chemists and biologists at the beginning of the 20th century. Tswett (1906) first applied the term chromatography (chroma, colour; graphein, to write) to the separation of plant pigments on columns of alumina. However, 25 years elapsed before liquid-solid adsorption chromatography received much further attention, when Kuhn, Winterstein and Lederer (1931) rediscovered it and applied it to the separation of carotenes.

Liquid-liquid chromatography was first described by Martin and Synge (1941) and later developed into paper chromatography. As early as 1941, Martin and Synge showed the feasibility of using partition chromatography with gases and laid down some guiding principles for the development of GLC. Although many studies of the related technique, gas-solid chromatography, were known at this time, it was not until 1952 that James and Martin put these principles into practice. Since then, gas-liquid chromatography has developed rapidly and has completely overshadowed gas-solid chromatography in its scope and flexibility.

The first practical separation of steroids by GLC was achieved by VandenHeuvel and Horning (1960). However, to apply GLC to steroids the compounds must first be converted to a gaseous form. Steroids have relatively high boiling points/

points and many of them are unstable at high temperatures. For example, cleavage of the side chain of corticosteroids may occur at high temperatures, giving rise to the corresponding 17 ketosteroids (Luetscher and Gould, 1964). The different ways in which this problem of instability has been solved will be dealt with in detail later. General methods by which most groups of steroids can be separated by GLC have been described and reviewed by many groups of investigators, including Wotiz and Clark (1966), Eik-Nes and Horning (1968) and Wotiz and Chattoraj (1973).

2.1.3 Theoretical considerations

As compounds emerge from the column, their presence and mass is assessed by a detector (see below) and converted by a potentiometric recorder to a peak approximating to an isosceles triangle. The efficiency of separation of compounds by GLC will obviously depend on the distance between the apices of these peaks (i.e. peak centres), but where the peak centres are close, only peaks of narrow base will be separated. Thus in order to achieve maximum separation it is necessary to understand the factors which influence the shape of the GLC peak, in addition to the column properties which determine the efficiency of partition (i.e. separation of peak centres).

Peak/

Peak shape

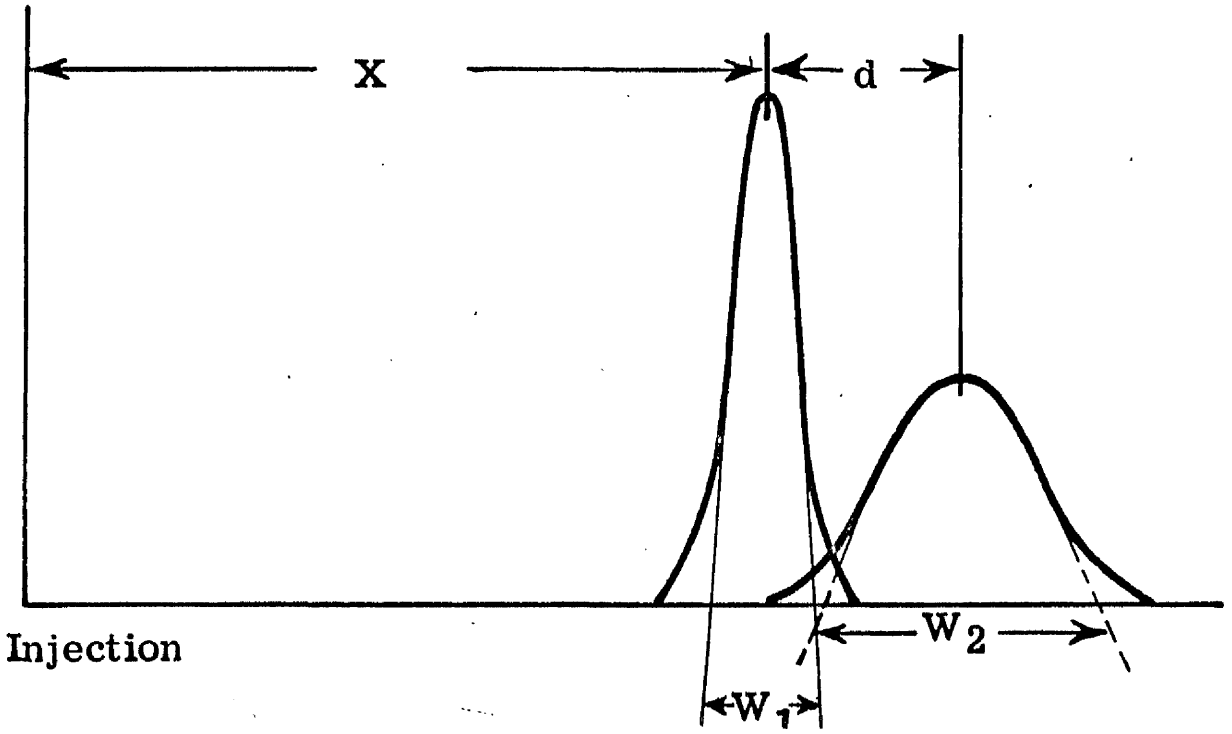
Peak width or spread is determined by mass transport and kinetic phenomena which are related to the efficiency of the GLC column. This is expressed in terms of theoretical plates in the column. In a separation process, such as countercurrent extraction, which can be carried out in discrete steps, perfect equilibration of the solute between two phases is established at each step, when phases can be separated. Each of these steps is termed a theoretical plate. However, in a chromatography system such as GLC, the solid is in constant motion down the column as an infinite number of partitions occur and perfect equilibrium is not established at any one point. Consequently the best that can be done is to calculate the height of the column that will give a separation equivalent to one theoretical plate, that is, the height equivalent to a theoretical plate (HETP). The HETP of a given GLC column can be calculated from information obtained from a potentiometric recording of separations by such a column (Figure 1b). Tangents are drawn to the peak at the points of inflection and 'N' for the first peak is given by the equation:-

$$N = 16\left(\frac{x}{w}\right)^2$$

where 'x' is the distance from the point of injection to the peak/

Fig. 1(b)

CALCULATION OF N AND R FROM A CHROMATOGRAM



peak maximum and 'w' is the length of the base line enclosed between the two tangents. Both the numerator and denominator must be in the same units, usually centimetres. HETP is then the effective column length, measured in the same units as in the equation and divided by 'N'. A good column has a large number of theoretical plates and a small HETP (less than 1 mm). The HETP may be different for each solute on a given column. HETP is related to a number of physical factors governing the characteristics of gas flow and diffusion which are in turn largely governed by the rate of the flow of gas through the column (average linear gas velocity, μ). As the volatilized sample passes through the column, the sample peak will tend to spread as the molecules take different paths through the packed column. This is described as the multiple path or eddy diffusion effect (A). To optimise column efficiency and HETP, the size of the particles (dp) of the column packing should be as small as possible consistent with an adequate gas flow and a low pressure drop across the column. Uniformity of size (λ) is also essential.

Diffusion will also affect the spread of the sample peak. The effect of normal longitudinal molecular diffusion of the solute in the carrier gas due to the concentration gradient across the sample zone (B) will decrease with increasing rate of gas flow and will also fall as the molecular/

molecular weight of the carrier gas falls, i.e. the coefficient of diffusion of the solute in the gas phase (Dg) will be low. Finally, there is a resistance to mass transfer of the solute (C), again proportional to the flow rate of the carrier gas. The effects of this can be minimised by choosing a liquid stationary phase which has a low viscosity and coefficient of diffusion (di). The depth of the film of stationary phase (df) should be as thin and uniform as possible. However, the need to maintain a reasonable ratio between the capacities of the stationary and mobile phases (K) may dictate that some compromise be made between df and K to obtain optimum column behaviour. In practice, the optimum carrier gas flow rate is determined by plotting HETP against μ and choosing a value at or slightly above the minimum in the curve. Since the outlet pressure is usually one atmosphere, the flow rate chosen will dictate the inlet pressure to be used. The whole of the complex relationship of factors affecting gas flow has been synthesised into an equation by van Deemter, Zuiderweg and Klinkenberg (1956).

$$HETP = 2\ dp + \frac{2\ Dg}{\mu} + \frac{8K\ df}{\pi^2(1+K^1)^2 D_1}\mu$$

Fortunately this equation has been simplified into the following form -

$$HETP = A + \frac{B}{\mu} + C\mu$$

The/

The efficiency of separation of adjacent peaks (Figure 1b) is measured by the resolution (R).

$$R = \frac{2d}{W_1+W_2}$$

When comparisons of the efficiency of resolution of different columns are being made, it is important that the same standard compounds are used.

Peak centres

The location of the peak centre for the given compound, usually referred to in terms of retention time, is determined by the solutes distribution (partition) coefficient and the temperature. The interactions between the solute and the solvent which lead to dissimilar partition coefficients are hydrogen bonds, Debye forces, van der Wals forces and specific chemical interactions. Careful choice of phases and temperature maximise the differences in the behaviour of solutes on the column and thus increase resolution. Some examples of this approach are shown in section 2.2.3.

2.1.4 The gas liquid chromatograph.

The GLC consists of four basic components -

the column

the detector

the amplification system for the detector signal

the recorder system

There/

There are many variations of each of these from which to select and there follows a discussion of the first two components - the column and the detector. The technology of the amplifier and recorder systems is beyond the scope of this thesis.

(a) The column

The column is a tube of uniform diameter packed with an inert, particulate support which is coated with a non-volatile liquid stationary phase. The length is varied according to the resolution required (see section 2.1.3). The complete system is housed in an oven at constant temperature. The sample to be analysed, which may be a gas, liquid or solid, is introduced into the beginning of the column and is swept through it by an inert carrier gas. For this to be possible, the column must operate at a temperature at which the sample is immediately volatilized on entering the column. With compounds of high molecular weight, a supplementary heater at the point of sample injection may be required. On leaving the end of the column, the sample may pass through a detector (analytical GLC) and thence to the atmosphere or alternatively it may be trapped and collected for further analysis (preparative GLC, e.g. Weinstein, Lai and Xenakis, 1971).

(i) Composition

GLC columns have been constructed of many different materials,/

materials, the main requirements being malleability and resistance to heat. Columns may be of metals such as stainless steel, copper and aluminium. However, the catalytic effects of hot metal surfaces on steroids restrict the choice for steroid analysis to glass.

(ii) Geometry

Both the diameter and the shape of the column can be varied depending on the chromatographic requirements. For example, much interest has been shown in the use of capillary columns. The lengths of these vary from 50 to 250 feet, increasing length tending to increase resolution. Instead of coating an inert packing material, the inner walls of the capillary are coated with stationary phase. Unfortunately, application of samples to capillary columns is fraught with difficulties and the choice of detectors is severely restricted. As explained below (b) the dead space in the detector must be minimised and this is difficult when the diameter of the column is small compared with the detector internal volume. Since high detector sensitivity is essential for the type of work described in the experimental section, the use of capillary columns was not considered. Indeed, packed columns have so far been much more widely used in steroid work.

The shape of the column is dictated by both practical

and/

and economic factors. Coiled columns have the advantage of compactness so that long columns can be accommodated in relatively small ovens. This retains the resolution advantages of length while keeping the cost of the oven low. Coiled columns are, however, more difficult to pack effectively and there is a tendency, as the column is used at its working temperature, for the stationary phase to settle to the bases of the turns of the coil giving an apparent increase in dead space between the stationary phase and the wall of the column, preventing the establishment of an equilibrium in the partition of sample between mobile and stationary phases and thus reducing the number of theoretical plates. Similar changes occur in straight horizontal columns, but settling in uncoiled, vertically positioned columns will obviously not affect partition in this way, although the effective length of the column will be slightly reduced.

The resolving power of the vertical straight column is superior to that of a coiled or spiral column of equivalent length and this discrepancy is exaggerated where the coils are small and the column tubing of wide diameter. In practice, however, the cost of ovens to house long straight columns and the practical problems of ensuring the absence of temperature gradients in large ovens, enforces a compromise and, in the experiments described in this thesis, a Pye 104 gas/

gas chromatograph employing a 1.5 m column shaped into coils of relatively wide diameter (17 cms) was used. A fan mechanism within the oven eliminated temperature gradients and constant vigilance was maintained to ensure that column resolution did not deteriorate as a result of the factors discussed above.

(iii) The support material

The supporting material must meet the following requirements:-

It should be porous so as to present the greatest possible surface per unit volume.

It should not adsorb the materials to be separated through the liquid film.

The two supports most commonly used, Kieselguhr, a diatomaceous earth, and Ground Fire Brick (e.g. C22 and the Chromosorbs), possess these qualities to a large degree. Both of these silicate supports are able to absorb large quantities of solvent and still remain free flowing, thus facilitating uniform packing of columns. The importance of using finely divided, uniform support materials has been discussed in connection with the Van Deemter equation (page 7).

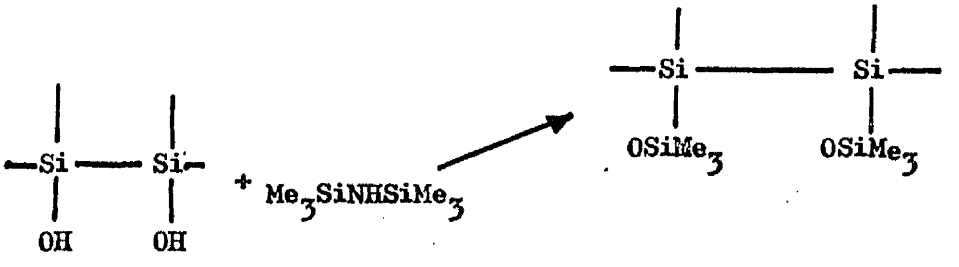
Finely divided packing yields columns with the highest number of theoretical plates, but again in practice a compromise/

compromise must be reached between this factor and the tendency of such supports to impede gas flow through the column. In order to maintain the same flow obtainable with coarser packings, it is necessary to increase the inlet pressure. This leads to a higher ratio of inlet to outlet pressure and consequently to flow rates which are different in different parts of the column. If the ratio of inlet to outlet pressure exceeds 1.5 - 2.0, sections of the column will not be used efficiently since the rate of flow along its length will not be uniform owing to the compressibility of the carrier gas. In practice, a compromise is again easily arrived at, for impedance to flow continues to increase with decreasing particle size, whereas column efficiency tends to level off. Packing materials with a narrow range of particle size will give better results than those with a wide range of particle size even although the average size is the same, because small particles will fill the voids between large ones and thus increase impedance to gas flow.

(iv) Support materials for steroid analysis

For steroid work, where thin film column packings are used, particular attention must be paid to the choice of support material. A very important consideration is its purity and inertness, particularly in regard to the solute molecules to be analysed. Diatomaceous earth supports of mesh/

mesh size 80-100 or 100-120 which have been washed with both acid and alkali are most commonly used. The supports, even when purified by this severe treatment, are silicates which contain active sites on their surfaces, which must be blocked in order to minimise adsorption of the solute molecule. Deactivation of such sites is usually accomplished by siliconising the support with hexamethyldisilazane or dichlorodimethylsilane which eliminates the major part of the chemical reactivity, which is particularly essential when dealing with hydroxy and ketonic compounds such as corticosteroids. When glass columns are used, it is necessary to subject them to the same pre-treatment before packing (Hanaineh 1964).



(v) The stationary phase

The selection of a stationary liquid is the most important choice to be made in developing a gas chromatography method, for it determines whether any given pair of solutes can be separated; that is, it determines the location of the peak centres of solutes and also, to some extent, their spread.

The/

The liquid selected as the stationary phase must be substantially non-volatile and thermostable at the operating temperature of the GLC. Generally, its boiling point should be 250°-300° above the operating temperature of the GLC so that evaporation is negligible and does not affect the sensitivity of the device used for detection.

In the case of extremely sensitive detectors, such as electron capture detectors (see below), even a small tendency for the liquid phase to evaporate effectively precludes its use. As the operating temperature of a column is increased, the choice of stationary phase becomes more and more restricted and eventually may become a limiting factor. Until the very recent advent of silicoborane polymers, only irradiated asphaltenes, polyphenyl tars and eutectic mixtures of inorganic salts could be used at temperatures above 350° for any length of time.

The melting point and viscosity of the stationary phase are also important. The phase should obviously be liquid at the column temperature. If it is highly viscous at operating temperatures, the time required for equilibration of the solute between the mobile and stationary phases will be increased and column efficiency will be impaired. Liquid phases used in steroid analysis fall roughly into two categories:-

Non-selective/

Non-selective phases which separate compounds primarily according to the molecular weight and volatility of the molecule. These are relatively non-polar liquids, the most common of which are the siloxane polymers with methyl or phenyl substituents (e.g. SE30, OVI).

Selective liquid phases which show characteristic behaviour towards specific functional groups. Polymers of the polyester type and methylsiloxane polymers with polar groups (e.g. fluorine or cyanoethyl) fall into this category. SP2250, for example, is one of the most polar high temperature phases. Retention times of polyfunctional steroids with these polar phases are very long indeed, but the phases are useful for the analysis of less polar steroids such as androstanes or those with polar groups which have been substituted with aliphatic radicals.

The column is the heart of the GLC. Separation of similar solutes is only made possible by careful choice and preparation and this in turn is only possible if the importance of the effects of all the foregoing variables is recognised.

(b) The detector

As stated earlier, the separated compounds leave the column in a gaseous mixture and pass through a detector. This device should be as reliable and as sensitive as possible. Several different principles have been employed in constructing GLC detectors, some of which are discussed below.

(i) Thermal conductivity

The thermal conductivity of a solute and the carrier gas differ. The thermal conductivity detector uses this difference to measure the quantity of solutes emerging from the column. It is capable of measuring a very wide range of compounds which it does not destroy in the process, but is relatively insensitive ($2\text{-}5 \times 10^{-6}$ g) and its reliability is affected by small changes in column temperature and gas flow rate. Although it has achieved the most widespread general use, it is of little value for corticosteroid analysis.

(ii) Flame ionisation

The flame ionisation detector depends on changes in the electrical conductivity as the sample traverses the detector. Such changes are maximised by burning the compound in a hydrogen flame as it enters the detector. The process is therefore destructive. Flame ionisation is more sensitive (2×10^{-11} g) than thermal conductivity as a means of monitoring column effluent and the range of linear response is wide.

Moreover,/

Moreover, because the internal volume of the detector is small, it is ideal for use with capillary columns (see section a (ii) above) and can, with rare exceptions such as carbon disulphide and carbon sulphoxide (Sherma 1972) be used to detect most compounds. Although the flame ionisation detector has been used to study steroids in many biological fluids, its sensitivity is inadequate for analysis of human plasma corticosteroids unless very large samples are taken. For physiological reasons (see section 3.3, 3.4) this is not possible.

(iii) Electron capture

The electron capture detector employs a low energy electron source and a collector electrode. The carrier gas, usually nitrogen, passes between but has little affinity for electrons and an undisturbed potential is set up across the detector cell (equation 1). When solutes pass through the cell, those with a higher affinity than nitrogen for electrons will alter this potential difference (equation 2) and the change can be registered on a potentiometric recorder.

1. $N_2 + \beta \longrightarrow e^-$ (potential set up)

2. e^- + sample molecule $\longrightarrow$ loss of signal.

Obviously, only those solutes which accept and stabilise electrons can be detected by this device and the greater the avidity of the compound for electrons, the greater the sensitivity/

sensitivity of detection. Sensitivity can reach levels of 5×10^{-13} g for some compounds. Thus it is a non-destructive and selectively sensitive detector.

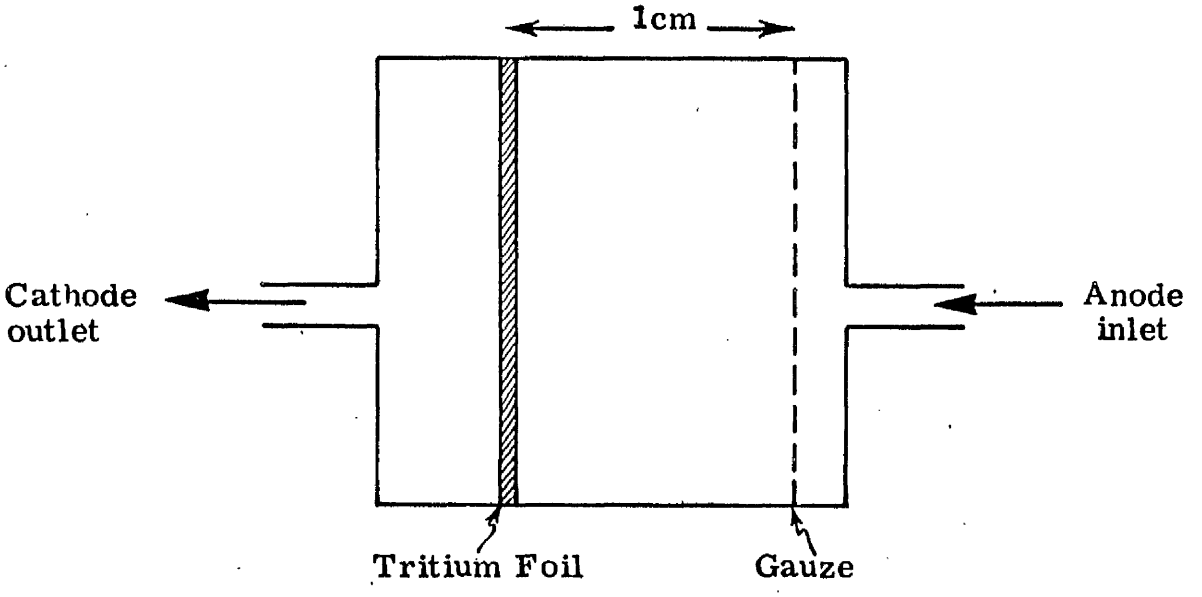
Electron sources

The early electron capture detectors used tritium foil as a source of electrons (Lovelock and Lipsky, 1960). Although this formed the basis of a sensitive detector, tritium was slowly lost from the foil, even at relatively low temperatures, and the device could not be used at all above 220°C. ^{63}Ni detectors are slightly less sensitive but, since they can be used up to 350°C, they have a wider scope. In any case, the detector temperature must be kept well above (c 50°) that of the column to prevent condensation within the detector cell and consequent impairment of sensitivity.

The mechanism of electron capture detection, as introduced by Lovelock and Lipsky (1961), is illustrated in Fig. 1(c). As the inert carrier gas passes through the detector an average current of between 10^{-9} and 10^{-8} amps, is produced between the tritium foil (cathode) and the gauze (anode). This current is due to secondary electrons formed by a collision between primary electrons or beta particles and molecules of carrier gas (see above). The current may be increased by applying a higher potential until a current plateau/

Fig. 1(c)

DIAGRAM OF AN EARLY ELECTRON CAPTURE DETECTOR



plateau is reached, when all the electrons in the cell are collected. This resting condition is disturbed when an electron capturing compound enters the cell. Moving into the area between the electrodes, where there is an abundance of low energy free electrons, its molecules react with electrons as explained below:-

$$e^- + AB \longrightarrow AB^- + \text{energy} \qquad (1)$$

$$e^- + AB \longrightarrow A + B^- - \text{energy} \qquad (2)$$

In equation 1, the energy of electron capture is either liberated as radiation or shared with other molecules on collision. The final result is the removal of an electron from the system and its substitution by a negative ion of far larger mass. These may then combine with positive ions in the plasma and a rapid decrease in current then occurs. This ionic recombination will occur about 1 million times faster than the recombination of electrons and positive ions. A compound capturing free electrons is therefore readily observed by this increased recombination. As can be seen from figure 1(c), the carrier gas flow is in the opposite direction to the flow of negative charge. That part of the negative charge due to free electrons will be unimpeded by the gas flow because the electron drift velocity is so much greater than the linear gas velocity. However, the/

the negative ions which make up the remainder of the negative charge, move more slowly towards the anode, are slowed down by the gas flow and thus their chances of encountering a positive ion are increased.

Application of current to the detector

Direct current can be applied to the detector continuously (direct current sampling) or in pulses of duration (width) and frequency (pulse mode) which can be varied. Direct current sampling produces electrons in the detector of higher kinetic energy, proportional to the strength of the electric field, than the carrier gas molecules. Therefore the electrons are not in thermal equilibrium with the carrier gas and are thus less available for capture. Moreover, Lovelock (1963) has shown that the direct current-operated cell is capable of generating erroneous and even totally false responses. This is due to the fact that a cloud of positive ions near the cathode sets up a potential in opposition to that applied to the chamber, hindering the collection of free electrons.

Under pulse conditions, providing that the pulse period is large compared with its width, the electrons are virtually in thermal equilibrium with the carrier gas molecules. A pulse with a voltage between 40 and 80 volts for about 0.5% of the time (1 microsecond per 200 microseconds) is sufficient to collect all the electrons to the anode (Exley, 1967). Thus, the electron concentration varies in a saw-tooth manner with the applied potential, the concentration building up to a maximum just before the pulse is applied. Current pulsing is therefore/

therefore the technique of choice.

(c) Summary

The electron capture detector is not without its disadvantages. It has a relatively large internal volume and is therefore unsuitable for use with capillary columns. Even under pulsed conditions, the range of linear response is narrow, although this range is perfectly adequate for the measurement of very small masses of steroids (0 to 200 pg for halogenated steroids). Unlike the flame ionisation detector which is self-cleaning, the electron capture detector is extremely susceptible to contamination. Thus, slow evaporation of stationary phase (column bleed) severely affects detector performance and impurities in the phase or the support will be similarly detrimental. The detector therefore tends to dictate the type of phase and support which can be used. Obviously, only the most highly purified supports are inert enough not to decompose, releasing pg quantities of contaminating material. This problem is amplified in steroid analysis where high column temperatures must be used in the separation (200 to 250^{o}). Similarly, only the highly non-volatile liquid phases can be used, the OV and SP silicones and carboranes (Dexsil) can be employed.

However, when used in pulsed mode with compatible liquid phases and ultra pure supports, the electron capture detector is more sensitive than other devices when suitable steroid derivatives are used. Also the detector is relatively insensitive to hydrocarbons used as solvents in steroid analysis, adding to the sensitivity of the system by reducing background noise. The following section discusses the type of compounds and derivatives for which this technique of detection can be used to most advantage.

2.1.5 APPLICATIONS OF GAS LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

As previously stated, only a restricted range of compounds possess the ability to attract electrons and, not surprisingly, many of the early applications of electron capture detection were concerned with studies of this group of substances. Careful examination of their properties, however, gives an insight into the chemical characteristics required for electron avidity and this information can then be used to modify compounds lacking the requisite properties, thus extending the usefulness of the detector.

(a) Compounds with high electron affinity

(i) Halogenated compounds

Compounds containing halogen atoms, as might be expected, have a high affinity for electrons, although there is often no clear relationship between the number of halogen atoms and the sensitivity of electron-capture (EC) detection. The earliest applications of this form of detector were in the field/

field of halogenated insecticides and related toxic compounds, a use which still continues to be important (Goodwin, Goulden, Richardson and Reynolds, 1960; Goodwin, Goulden and Reynolds, 1961). Residues of insecticides in crops (Burke, 1965; Giuffrida, Ives and Bostwick, 1966) and metabolism of herbicides (Getzendaner, 1969; Benvenue and Ogata, 1970; Hall, Giam and Merkle, 1970) and fungicides (White, Kilgare and Mallet, 1969; Day, Decker, Koons and Holzer, 1970) have all been studied by this method. Of important sociological implication are the studies of contamination of human tissues with toxic substances such as D.D.T. (Bonderman, Choi, Hetzler and Long, 1969; Dale, Miles and Gaines, 1970; Henderson, Deboer and Stahr, 1971) and the presence of these substances in foods of animal source such as milk (Lawrence and Burke, 1969; Carr, 1970), meat (Ramodski and Rey, 1970) and fish (Armour and Burke, 1970). Environmental chemical pollution studies have also been facilitated by this method (Devine and Zweig, 1969). The quantities of halogenated metabolic residues in tissues are extremely small and such studies would not have been possible without/

without the extreme sensitivity of the electron capture detector to this type of compound.

Many important drugs also contain halogens. Examples of applications of GLC with electron capture detection to drug metabolism, again previously limited by lack of sensitivity to existing techniques, include tranquillizers, anaesthetics and sedatives, such as chlorpromazine (Curry, 1968), some benzodiazepine compounds (Desilva and Puglisi, 1970), halothane (Brachet-Lierman, Ferrus and Caroff, 1971) and ethchlorvynol, a non-barbiturate sedative (Cummins, Martin and Scherfling, 1971). The metabolism of the halogenated antibiotics by analysis of blood can also be studied in this way (e.g. chloramphenicol: Resnick et al, 1966; thiamphenicol: Aoyama and Iguchi, 1969). Antihistamine drugs have also been monitored in blood and urine (Bruce, Pitts and Pinchbeck, 1968). Finally, few natural compounds are halogenated but important exceptions are the thyroid hormones. Studies of these substances have been made possible by the use of electron capture detection technology. It is therefore probably true to say that halogenated compounds present the most promising approach to obtaining sensitivity of detection. There are, however, a number of other possible molecular characteristics which might be employed instead of, or in addition to, halogenation.

(ii)/

(ii) Sulphur-containing and Nitrogen-containing compounds

A number of compounds of agricultural importance contain electronegative sulphur and/or a nitrofunction, both of which are detected by electron capture. For example, the electron affinities of a group of nitrobenzene derivatives investigated by Hoffsommer (1970), have allowed the estimation of small quantities of the irritant alkyl nitrobenzenes in tobacco smoke (Hoffman and Rathkamp, 1970). Studies of the 2-nitropropanol content of cotton seed (Devine, Fletcher and Zweig, 1969), ipronidazole in animal feed stuffs (Dervitter and Osadca, 1971) and insecticides such as accothion and its analogues in crops and milk (Bowman and Berozoa, 1969) have all been studies by gas-liquid chromatography with electron capture detection.

(iii) Conjugated carbonyl groups

Of particular relevance to studies of corticosteroids are the electron capturing properties of conjugated carbonyl groups. Some use has already been made of this phenomenon in the analysis of plant tissue for such compounds as caffeic acid (Mitruka and Alexander, 1969), flavone compounds (Anderson and Vaughn , 1970) and abscisic acid (Seeley and Powell, 1970).

(b) Compounds with low electron affinity.

(i) Non-steroidal compounds

There/

There has been considerable interest in GLC/EC by investigators in biochemical research. Because of its potential sensitivity, GLC/EC can be useful in measuring biologically important compounds which are often present at very low concentrations. Unfortunately, relatively few possess structures which show high electron affinities. Thus, techniques have been extensively employed which involve preparation of suitable derivatives.

Carcinogenic nitrosamines have been determined following oxidisation to their corresponding nitramines (Althorpe, Goddard, Sissons and Telling, 1970). Analysis of picogram quantities of dimethylnitrosamine in fish has been reported (Sen, 1970). Pentafluoropropionamide derivatives were utilised in the assay of 1- and 2-naphthylamines in cigarette smoke (Masuda and Hoffmann, 1969).

GLC/EC has been applied to studies dealing with catecholamines and their metabolism. Clarke, Wilk, Gitlow and Franklin (1967) developed a method for dopamine in urine following conversion of its trifluoracetate. Trimethyl silylether-hepta-fluorobutyrate derivatives have been suggested for catecholamine analysis (Horning, Moss, Boucher and Horning, 1968). GLC/EC of dopamine and noradrenaline as their trimethylsilylether-pentafluoro-benzaldehyde Schiff's bases has also been effected (Moffat and Horning, 1970). A systematic study of the trifluoroacetate, pentafluoropropionate/

pentafluoropropionate and heptafluorobutyrate derivatives of several noradrenaline metabolites has been described (Anggard and Sedvall, 1969). The higher molecular weight derivatives were more thermally stable and gave greater EC responses. GLC/EC has been utilised in the determination of 3-methoxy-4-hydroxy-phenylethylene glycol, the principal metabolite of noradrenaline in certain brain tissues. (Dekirmenjian and Maas, 1970).

Methods relying on the synthesis of derivatives have been developed for other centrally active amines. Moffat and Horning (1970) evaluated thirteen derivatives of β-phenylethylamine. Indole amines and their metabolites (Vessman et al, 1969), amphetamines and related compounds (Bruce and Maynard, 1969), and pseudoephedrine (Cummins and Fourier, 1969) have been analysed as their heptafluorobutyrate derivatives; the latter two groups report analysis of some of these from blood.

(ii) Steroids

Many hormonal steroids, particularly the corticosteroids, are unstable at temperatures required for evaporation in a GLC column. Moreover, concentrations of these compounds in biological materials are very low. The main purposes of derivative synthesis are therefore to induce molecular stability at high temperatures and to maximise the response induced in the electron capture detector by the compound.

These/

These factors are not necessarily separate and a number of electron capturing substituents tend also to stabilise the molecule. A number of practical factors must also be borne in mind. For example, poor yields of a derivative will obviously detract from sensitivity of estimation in biological extracts, and thus only reactions which give approximately quantitative yields need be considered. Retention time (see above) of the derivative is also important. Very short retention times are undesirable because the derivative will be eluted from the column with complex mixtures of highly volatile contaminants of biological extracts. However, long retention times are inconvenient for economic use of machine time and in any case may result in peaks of poor shape and difficult to quantitate.

Ketone and hydroxyl groups afford the main possibilities of substitution in the steroid molecule. In corticosteroids, those positions which readily yield to attack are the $\Delta 4$, 3-ketone in ring A and the primary alcohol group at position 21. Androgens such as testosterone and androstenedione share with corticosteroids the structure of ring A but, lacking a side chain, present an alcohol or ketone group respectively at position 17 for substitution. Progesterone is only readily substituted in ring A, the 20-ketone being sterically hindered.

Halogenated derivatives

Formation of haloacetate esters of cholesterol was studied/

studied by Landowne and Lipsky (1963) who compared the relative sensitivity of detection of the three types of haloacetate. Paradoxically, the monochloracetate was detected with greater sensitivity than the trichloracetate. They suggest that this may be due to an increase in polarity of the double bond resulting from the greater interspacial electron attraction of the halogen atom in the monochloracetate and causing attachment of the electrons to the carbonyl carbon atom. Methods for the estimation of concentration of testosterone (Brownie, van der Molen, Nishizawa and Eik-Nes, 1964), progesterone (van der Molen and Groen, 1965) and oestradiol (Aakvaag, Hagen and Eik-Nes, 1964) in human plasma have been based on this derivative.

Some increase in sensitivity of detection is obtained by the use of various halogenated silyl ethers. For example, chloromethyl dimethyl silyl ethers of steroids have formed the basis of steroid methods (Thomas, Eaborn and Walton, 1966; VandenHeuvel, 1967) and the iodoanalogue of testosterone is slightly more electron capturing. These ethers are, however, less convenient than the haloacetates because they are readily hydrolysed in the presence of traces of moisture (Wotiz and Clark, 1966). Another ketonic reagent, pentafluorophenylhydrazine, has been used to estimate oestrone concentrations by GLC with electron capture detection (Attal, Hendeles/

Hendeles and Eik-Nes, 1967), and oestradiol, first oxidised to oestrone, can be analysed in the same way (Mead, Haltmeyer and Eik-Nes, 1969).

Heptafluorobutyrates

Given the correct conditions, heptafluorobutyric anhydride reacts with both alcohol groups and ketone groups, producing derivatives with shorter retention times than monochloracetates and capable of detection at much lower concentrations by the technique of electron capture detection (Clark and Wotiz, 1963; Exley and Chamberlain, 1967). It is the enyl esterification of the $\Delta 4$, 3-ketone in ring A, applicable to all hormonal steroids except the oestrogens, which is most effective at increasing electron avidity. Unfortunately, these derivatives are unstable on thin layer chromatography and many may also break down during paper chromatography (Exley and Chamberlain, 1967) making purification difficult. Fortunately, corticosteroid enyl heptafluorobutyrates seem unaffected by paper chromatography (Wilson and Fraser, 1971, see also experimental section) and most other such compounds can be purified by gel filtration (Challis and Heap, 1969, 1970). While the esters formed by reaction with groups such as the 17-α-hydroxyl of C 19 steroids or the 21-hydroxyl group of corticosteroids do not result in such a marked increase in electron capturing ability, they are superior in this respect to/

to haloacetates and also increase the thermostability of some steroids. Methods have been developed using these derivatives for the estimation of plasma testosterone (Exley, 1967, 1968; Vermeulen, 1968; van der Molen and Groen, 1965), oestrogens (Wotiz, Charransol and Smith, 1967; Charransol and Wotiz, 1966) and androstenedione (Kirschner and Coffmann, 1968). Oestriol has been determined in urine by forming the heptafluorobutyrate (Honda, Ostergard and Kushinsky, 1969).

Other derivatives

Longer chain, polyhalogenated substituents have been used by Kirschner and Taylor (1969). These reagents, pentadecafluoro-octanoic acid (PFO), hexadecafluorononanoyl chloride (HFN) and eicosafluoroundecanoyl chloride (EFU), readily esterify hydroxysteroids giving steroid derivatives, such as those of testosterone and androstenedione which can be detected at levels as low as 100 pg. Unfortunately, good yields of the enyl ester are not easy to achieve. Oestrogen 17-pentafluorobenzoyl derivatives have been used in analysis of biological material (Zmigrod, Ladany and Lindner, 1970).

Addition of more than two polyhalogenated derivatives to a steroid molecule does not appear to enhance the response to electron capture detection (Lovelock, 1963). This was also a main conclusion of the work of Exley and Dutton (1969) who/

who compared the behaviour of 27 oestrogen derivatives both on thin layer chromatography and gas liquid chromatography with electron capture detection. The derivative which had the most desirable properties, stability on thin layer chromatography and eliciting an excellent response from the electron capture detector, was the rather complex oestradiol-3-(2-iodomethyl-dimethylsiloxy) propyl ether-17β-iodomethyl silyl ether which the authors employ as the basis of a plasma oestradiol method. The necessary reagent is not generally available and is likely to be expensive.

(c) Gas liquid chromatography of corticosteroids

Thus, in spite of the increases in electron affinity of some of the newer derivatives, the availability of heptafluorobutyric anhydride and high yields of its derivatives, together with the sensitivity with which they can be detected by electron capture detection, makes the steroid heptafluorobutyrate the derivative of choice. Many corticosteroid heptafluorobutyrates have been prepared and characterised by van der Molen and Groen (1965) and Dehennin and Scholler (1969). The instability of the 3-enyl substituent may not be an insuperable problem.

The remaining problem is to produce a derivative of the corticosteroid which is not destroyed at temperatures in the region of 200°C at which the derivatives are vapourised.

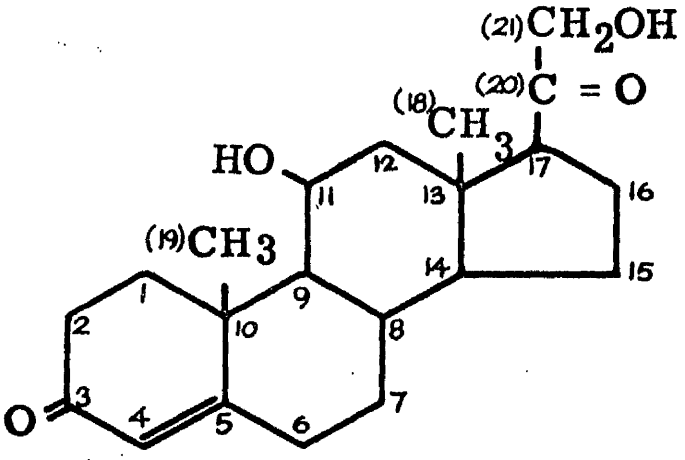
Simple/

Simple esterification at positions 3 and 21 (see Fig. 2) is sufficient to produce a thermo-stable derivative of deoxycorticosterone (Exley and Chamberlain, 1967; Dehennin and Scholler, 1969; Wilson and Fraser, 1971). It is possible that this may also be true for corticosterone (see Experimental section). The remaining corticosteroids fall into two groups, those with an oxygen function at carbon 18 and those possessing a 17α- hydroxyl group.

Derivatives formed by direct esterification of aldosterone and 18 hydroxy-deoxycorticosterone, both with oxygen functions at carbon 18, are unstable during gas liquid chromatography. However, periodate oxidation of both steroids forms internal lactones which are extremely stable (Merits, 1962) in high yields. These γ-lactones are electron capturing in their own right and have been used in assays of tissue (Kittinger, 1964, 1968), urine (Aakvaag, 1971; Rapp and Eik-Nes, 1966; Bravo and Travis, 1967) and even blood from the rat (Rapp, 1970). Sensitivity of detection is not, however, adequate for detection in human peripheral plasma (Brodie, Shimizu, Tait and Tait, 1967), where concentrations are lower, unless a polyhalogenated substituent is attached at position 3 (Nicolis, Wotiz and Gabrilove, 1969; Fabre, Fenimore, Farmer, Davis and Farrell, 1969). Other derivatives, including/

Fig. 2

CORTICOSTERONE SHOWING NUMBERING SYSTEM FOR THE CARBON ATOMS



including methoximes and silyl ethers of aldosterone, have been compared with the heptafluorobutyrate, but in terms of performance or convenience they are probably less satisfactory (Horning and Maume, 1969).

The 17α- hydroxylated corticosteroids, cortisol and 11-deoxycortisol, are similarly unstable (Luetscher and Gould, 1964) and must be modified before esterification. Little information is available about derivatives of these compounds.

This discussion is perhaps best summarised by the conclusions of Kittinger (1968) and Norymberski (1971) that gas liquid chromatography of corticosteroids depends for its success on the initial preparation of derivatives that are more volatile and more stable by protecting vulnerable oxygen functions or by selective, oxidative removal of labile parts of the molecule such as the side chain. In spite of the wealth of information concerning steroid derivatives and their behaviour, few attempts have been made to apply this knowledge to analysis of human peripheral plasma for corticosteroids. The following experimental section is an account of attempts to evaluate the usefulness of some corticosteroid derivatives in this respect and to use them in methods of estimating plasma concentrations of 6 major corticosteroids.

2.1.6 EXTRACTION AND PURIFICATION OF CORTICOSTEROIDS

(a) The Sample

The concentration of corticosteroids in the peripheral circulation reflects more accurately than secretion or excretion rate studies the effective concentration of the hormones, i.e. the concentrations at tissue level. It is a measure of the dynamic equilibrium between secretion and metabolism and is therefore affected not only by kidney function but also by a function of the liver (Brown, Englert, Wallach and Simons, 1957; Colvin and Bondy, 1958; Peterson, 1959) and thyroid (Eik-Nes and Brizzee, 1956; Hellman, Bradlow, Zumoff and Gallacher, 1961). Accordingly, for the purpose of this study, plasma was chosen.

Conditions for taking plasma samples from individuals were standardised for reasons which will be discussed in section 3.3 and 3.4. Plasma is particularly suitable for analysis as it may be stored conveniently in deep freeze for several months without alteration of levels of corticosteroids (Fraser, 1967). Péron (1962) recommends that plasma should be separated by refrigerated centrifugation as soon as possible after withdrawal of the blood and this has been carried out throughout the study.

(b) Extraction of corticosteroids

Corticosteroids are lipophilic compounds dissolved in plasma/

plasma or bound to plasma proteins. Consequently they may be easily extracted using organic solvents. The properties of the solvents commonly used for extraction from urine and plasma have been reviewed by McLaughlin, Kamicki and Gray (1958), Johansson (1969) and Braunsberg and James (1961). Most workers prefer methylene chloride as this is relatively stable after purification and has a low boiling point which facilitates evaporation from plasma extracts at temperatures low enough to avoid thermal degradation of the corticosteroids. Recoveries of cortisol and corticosterone from plasma by a single extraction with methylene chloride (ratio plasma:solvent, 1:5 v/v) are in excess of 90% (Braunsberg and James, 1961). This procedure also rapidly denatures plasma proteins, breaking steroid-protein bonds, and good recoveries of all the biologically active corticosteroids are obtained (Bush, 1957).

Partial purification of the extract may be achieved by solvent partition which removes less polar substances such as cholesterol or by alkali washes which remove phenolic compounds (Venning, 1954). However, since high concentrations of alkali will cause structural alteration of the corticosteroids (Mason, 1938; Wendler and Graber, 1956), the excess alkali must be removed by means of a dilute acid wash.

(c)/

(c) Chromatographic purification

Having extracted the corticosteroids from the mixture of solutes in plasma, further purification techniques must be employed in order that the steroids may be separated, not only from other lipophilic substances, but also from each other.

Techniques used include thin-layer chromatography (TLC) (Adamec, Matis and Golvoneck, 1962; Ertel and Ungar, 1964), paper chromatography (Neher, 1958; Bush, 1961; Zaffaroni, 1953; Eberlein and Bongiovanni, 1955), gas-liquid chromatography with fraction collectors (Weinstein, Lai and Xenakis, 1971), Sephadex gel filtration (Murphy and Pattee, 1964) and more recently high pressure liquid-liquid chromatography (Thomas, 1972).

Although it is capable of separating the majority of corticosteroids (Vinson and Whitehouse, 1969) there are several disadvantages of chromatography on thin layers of silica gel. The principal one of these is that the Δ 4, 3-ketone group of corticosteroids, particularly aldosterone, is said to be quite labile in silica gel. Another drawback is that TLC materials are difficult to clean thoroughly.

Gas-liquid chromatography is not suitable for purification of plasma corticosteroid extracts for reasons previously discussed. High pressure liquid-liquid chromatography would/

would seem from initial experiments to be perhaps the method of choice for purification of corticosteroids but as yet the equipment is very expensive and the technique has not been fully proven for use in this field. Paper chromatography, however, is an extremely well documented technique with regard to corticosteroids. Paper can be washed very easily and while recovery of material from the chromatograph is not so easily achieved as with GLC or liquid-liquid chromatography, it can be made almost quantitative with less difficulty than TLC. Also, paper chromatograms may be conveniently run overnight.

(d) Location and elution of compounds on paper chromatographs.

Some of the corticosteroids are present in human peripheral plasma in such low concentrations that vast amounts of plasma (about 100 litres for aldosterone) would need to be extracted before the endogenous steroids could be detected on paper (or thin layer) chromatographs. Almost 10 µg of steroid is needed before it can be detected by U.V. absorption or colour reactions (e.g. antimony trichloride). In order to solve this problem, the use of radioactive tracers has become widespread. The type of label normally used is a tritium (^{3}H) isotope which is usually inserted into the steroid molecule in place of hydrogen at positions 1, 2 and less commonly 6 and 7 (see Fig. 2).

Substitution/

Substitution with (^{3}H) at these positions does not markedly affect the physico-chemical properties of the molecule. The label to be used must have a very high specific activity (i.e. high disintegration:mass ratio) so that the amount of labelled steroid added to the sample does not substantially increase the amount of steroid present in the sample, but still possesses enough radioactivity to be detected on a paper chromatograph. The labelled steroid added to the plasma sample can also be used to assess the recovery of steroid through a purification procedure by estimation of percentage loss of the label. Losses can then be corrected for in estimations of steroid concentration. This is particularly important when labile steroids such as aldosterone and 18-hydroxy-11-deoxycorticosterone (18OHDOC) are to be estimated by use of methods employing multiple chromatographic and chemical purification steps.

Removal of the purified steroids from a paper chromatograph is normally achieved by washing the respective strip of paper with a solvent of high polarity such as methanol. Descending chromatographic elution with methanol is found to be quantitative and convenient to perform on a large number of samples.

These observations influenced the development of a method for the simultaneous estimation of the concentration of corticosteroids/

corticosteroids in human peripheral plasma by GLC with electron capture detection. The method is described in the following section.

2.2 EXPERIMENTAL

2.2.1 Materials

Solvents

Dichloromethane, ethanol and petroleum ether (80–100°C BP range) (B.D.H. Ltd) were redistilled immediately prior to use. Pyridine (Silylation grade) was obtained from Pierce Chemical Co. in sealed ampoules and used without further purification. Glacial acetic acid A.R. grade (B.D.H.) was refluxed over chromium trioxide for 3 hours before being redistilled. All other solvents (acetone, methanol, benzene, hexane and toluene) were of Nanograde quality (Mallinkrodt Chemical Works, St. Louis). Glass-distilled water was used throughout.

Steroids

Aldosterone (Ciba Ltd.), 18-hydroxy-11-deoxy-corticosterone (Searle, Mexico Ltd.), 18-hydroxycorticosterone, aldosterone -γ- lactone (Ikapharm, Israel), cortisol, 11-deoxycorticosterone, 11-deoxycortisol, corticosterone, androstenedione and androstonetrione (Sigma Chemical Co.) were examined for purity on paper chromatography on systems B_1 and B_3 of Bush (1961).

(^3H) aldosterone, corticosterone, cortisol, DOC, 11-deoxycortisol (25–35 Ci/mM New England Nuclear Corp) were/

were diluted in benzene: methanol 9:1 and stored at 4°C. The (^{3}H) steroids were purified in the appropriate paper chromatography systems immediately prior to use. (^{3}H) 18-hydroxy-11-deoxycorticosterone was prepared, purified and specific activity calculated as described on page 75 Latterly however, it has become available at a higher specific activity from the Radiochemical Centre, Amersham, and this preparation is now preferred.

Reagents

Methoxylamine hydrochloride (Applied Science Labs) Heptafluorobutyric anhydride, perfluoropropionic anhydride, pentadecafluoro-octanoyl chloride, hexadecafluorononanoyl chloride, eicosafluoroundecanoyl chloride (Pierce Chemical Co.) and monochloroacetic anhydride (Kochlight Labs) were used without further purification. Acetic anhydride (B.D.H.) was refluxed over calcium carbide and then redistilled before storage in glass ampoules at 4°C. Chromium trioxide (B.D.H., A.R. Grade) was dissolved in glacial acetic acid (2 mg/ml) immediately before use. Periodic acid (BDH, GPR) was stored in a dessicator at 4° and a solution made up in water: pyridine (100:1) (22.7 mg/ml) when needed.

Incubation medium

Adrenal glands were incubated (see below) in "Minimum/

"Minimum Essential Medium" (Biocult Laboratories Ltd) lacking L-glutamine but containing Hank's balanced salt solution. Immediately before use, NADPH + H^+ (Sigma Ltd ; 100 µg/ml final concentration) and gamma-irradiated, membrane-filtered calf serum (Biocult Laboratories Ltd; final conc. 10%) were added.

General methods

(a) Scintillation counting

A Nuclear Chicago Mark 1 Liquid Scintillation System was used for all samples. The scintillation mixture (diphenyloxazole (PPO) 10 g and methanol (40 ml) in toluene (2 l) was stored in the dark at room temperature.

(b) Paper chromatography

Chromatography paper (No. 2 Whatman Ltd) was washed for 24 hours with methanol in a Soxhlet reflux condenser before use. Steroids were chromatographed in Bush systems A, B_1, B_3 and B_5 (Bush 1961).

A : Petroleum ether (80-100°) :methanol:water (100:80:20)

B_1 : Petroleum ether (80:100°) :toluene:methanol:water (25:25:35:15)

B_3 : Petroleum ether (80-100°) :benzene:methanol:water (33:17:40:10)

B_5 : Benzene : methanol : water (100:50:50)

Steroids/

Steroids labelled with tritium (^{3}H) were located on paper chromatograms by use of a Panax chromatogram strip scanner. Once located, these were eluted chromatographically with methanol.

(c) Glassware

All glassware was submerged in a detergent solution (Decon 75, Pharmaceutical Developments Ltd) for several hours and then rinsed with water followed by methanol. Test tubes were subjected to cleaning in the detergent solution for at least fifteen minutes in a Mettler Corporation Ultrasonic Cleaner. They were then rinsed, dried and silanised (Repelcote, Hopkins & Williams Ltd) and rinsed with methanol prior to use.

(d) Gas-liquid chromatography

Pye 104 model 84 gas-liquid chromatographs (Pye Unicam Ltd.) were used equipped with (^{63}Ni) electron capture detectors and suitable potentiometric recorders (Leeds & Northrup Speedomax XL683 and Honeywell Electronik 194). Pyrex glass columns (1.5 m x 4 mm) were siliconised and packed with the support on which was coated the required phase. The column packings were obtained with the phase already coated on the support (Supelco Inc.). Unless otherwise stated, conditions were as follows:-

Temperatures/

Temperatures

Flash heater : 270°C

Detector : 350°C

Column : 210 - 250°C

Nitrogen flow

Carrier gas : 75 - 100 ml/minute

Quench gas : 20 ml/minute

Electron capture supply

Pulse space : 150 μsecs

Pulse width : 0.75 ± 0.25 μsec

Pulse amplitude : 47 - 60 v positive

High purity nitrogen (< 7 p.p.m. oxygen Air Products Ltd.) was passed through molecular sieves which were reactivated when necessary. This state was made obvious by the inclusion in the nitrogen supply line of a molecular sieve containing an indicator compound (Hydro Purge, Coast Engineering Lab. California).

Detector by-pass valve

Contamination of the detector by components of high volatility from plasma samples was reduced by use of a detector by-pass valve as described by Wilson & Fraser (1971). While being of immense value in preventing contamination of the detector, the valve prevents use of the column above 250°C. However, the latest model rotary vales (Carle Inc.) have operated very efficiently at 250°C for at least 18 months.

2.2.3 EVALUATION OF CORTICOSTEROID DERIVATIVES FOR GAS LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

(a) Introduction

There are two primary objects in forming corticosteroid derivatives for gas liquid chromatography: to induce, if necessary, a sufficient level of thermo-stability for GLC conditions and to introduce affinity for electrons which will allow detection at very low concentrations. In the following section, the 6 compounds under investigation have been divided into groups mainly on the basis of the steps needed to induce thermostability and a comparison has been made of the effect of a number of polyhalogenated substituents on the electron capturing properties of one of the compounds, deoxycorticosterone. Formation of stable, polyfluorinated derivatives of the remaining corticosteroids is then examined. The groups of compounds are as follows:

(i) Deoxycorticosterone and corticosterone which require little stabilisation apart from masking the 3-ketone and 21-hydroxyl groups.

(ii) The 18-oxygenated compounds, 18-hydroxy deoxycorticosterone and aldosterone, in which some modification is necessary.

(iii) The 17α-hydroxylated compounds which are relatively unstable/

unstable and must be radically altered.

(b) Method

The 18-oxygenated compounds were converted to γ-lactones by periodic acid oxidation and the 17 α - hydroxylated compounds to their respective androstenes by chromium trioxide oxidation. (^{3}H) standard steroids were incorporated in all reactions so that yields of derivative could be assessed. Following paper chromatography on Bush systems A and B_3 respectively, the products were esterified with the appropriate reagents as described in section 2.2.4 (b). Deoxycorticosterone and corticosterone were esterified directly. Aliquots of the esterified compounds were injected into the gas liquid chromatograph.

(c) Results

Table 2 Properties of polyhalogenated derivatives of DOC

Substitution		Yield %	Relative* Response	Relative ≠ Retention Time
C 3	C 2 1			
HFB	HFB	95	1.0	1.0
HFB	PFO	90	1.0	1.4
HFB	HFN	86	0.9	1.6
HFB	HFU	74	0.8	2.1

* Relative Response: peak height compared to that produced by equal amounts of DOC bis HFB under identical conditions.

≠ Relative Retention Time: time taken for elution of derivative from column compared to that of DOC bis HFB

Table 2 lists the four derivatives of deoxycorticosterone formed and compares the responses of the electron capture detector to these compounds. As can be seen, no advantage in terms of sensitivity of detection results from the use of the higher molecular weight ester substances at carbon 21 (see Fig. 2) as compared with the 3, 21 bis-heptafluorobutyrate. It will also be noted that their synthesis is more complicated, requiring an extra esterification stage and possibly an extra paper chromatographic stage. In addition, as would be expected, retention times become increasingly extended and consequently less convenient. It is probable that corticosterone derivatives would behave in this way also.

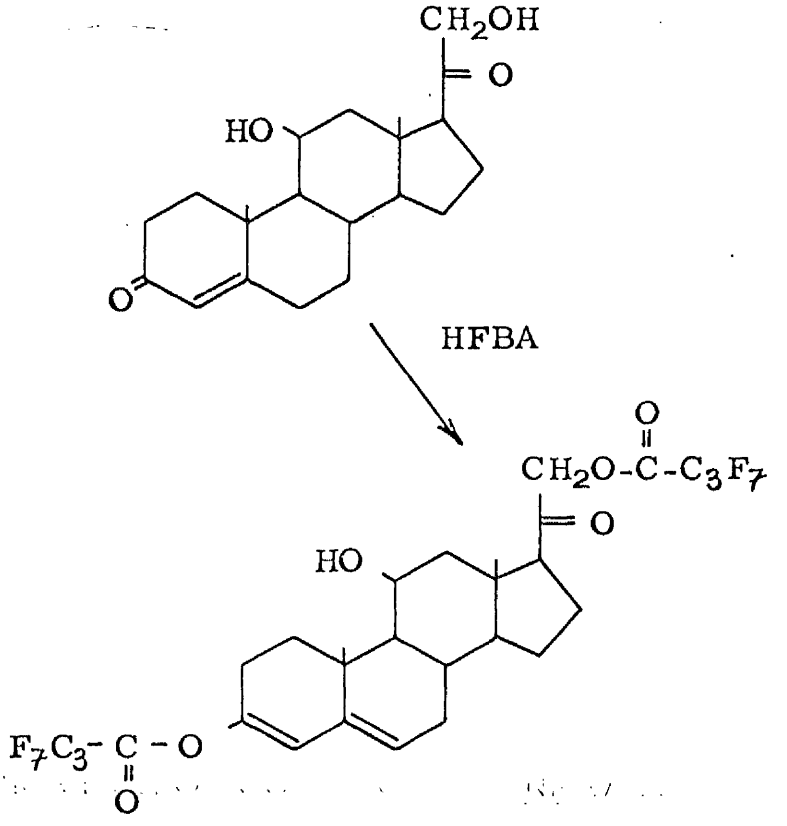
Table 3 (see page 79) lists the properties and yields of the corticosteroid heptafluorobutyrate derivatives. Figures 3 - 7 illustrate the structure of the compounds and their derivatives. Figure 8 shows the linearity and sensitivity of response for each derivative. All the derivatives were formed in almost 100% yield, and, with the possible exception of the corticosterone derivative, were detectable by the electron capture detector at picogram levels or less. Moreover, all had convenient retention times (see Table 3).

(d)/

Fig. 3

ESTERIFICATION OF CORTICOSTERONE WITH HFBA.

CORTICOSTERONE

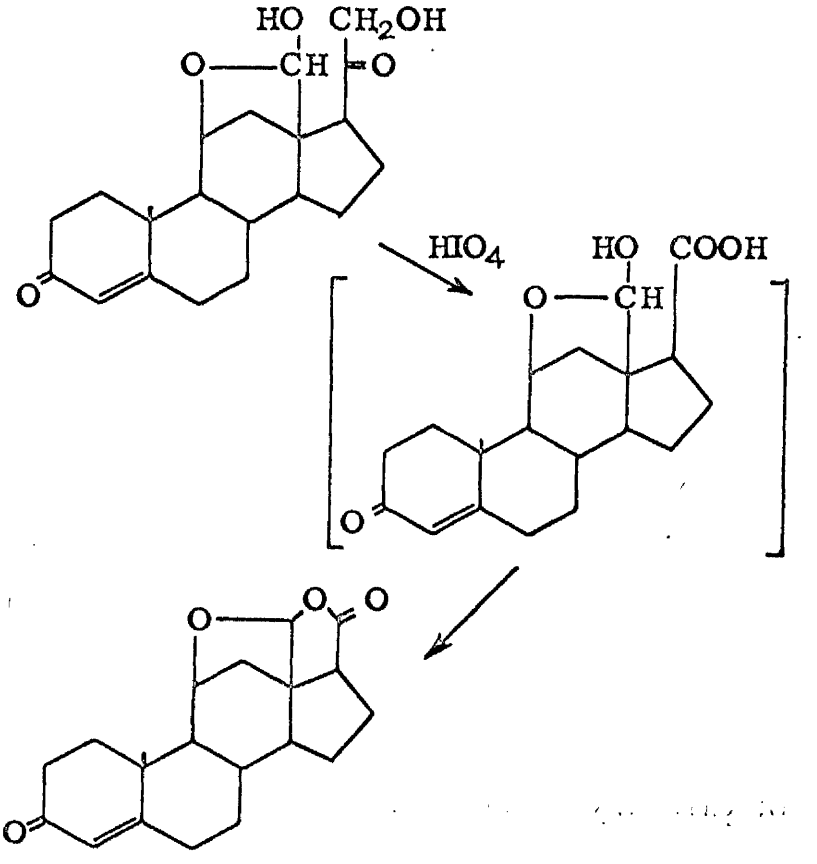


CORTICOSTERONE - 3ENYL, 21 - BIS. HFB

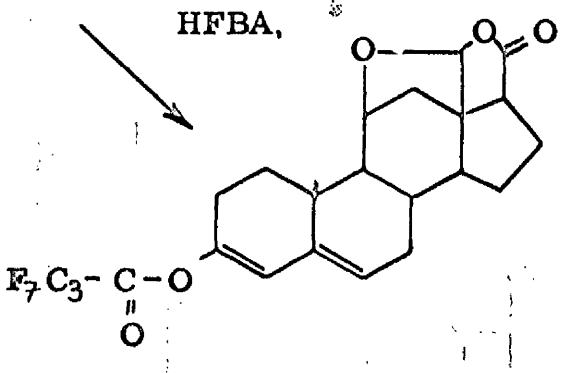
Fig. 4

FORMATION OF ALDOSTERONE-γ-LACTONE AND SUBSEQUENT ESTERIFICATION WITH HFBA.

ALDOSTERONE



'ALDOSTERONE γ LACTONE'

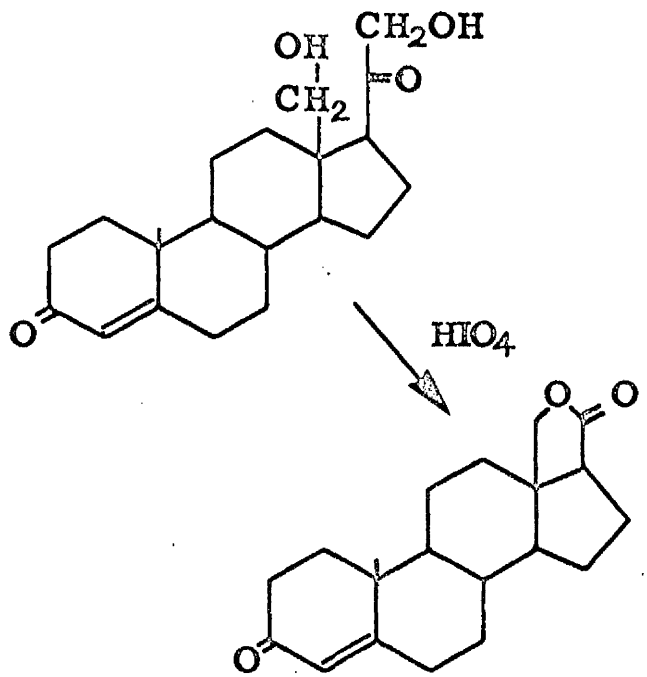


'ALDOSTERONE-γ-LACTONE' 3ENYL-HFB

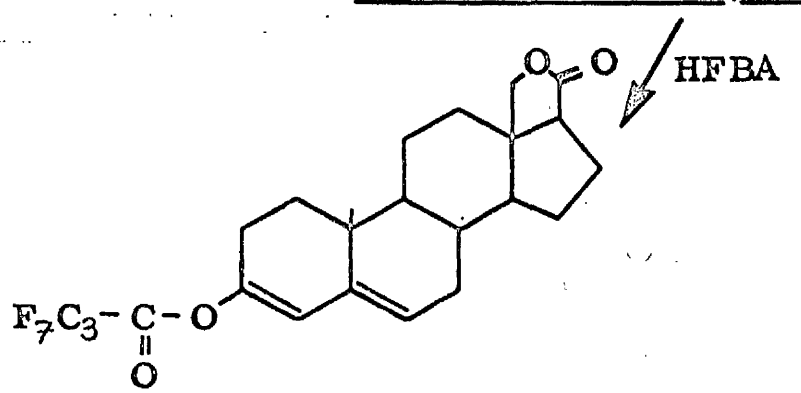
Fig. 5

FORMATION OF 18 OH DOC-γ-LACTONE AND SUBSEQUENT ESTERIFICATION WITH HFBA.

18. HYDROXYDEOXYCORTICOSTERONE



'18. HYDROXY DOC-γ-LACTONE'

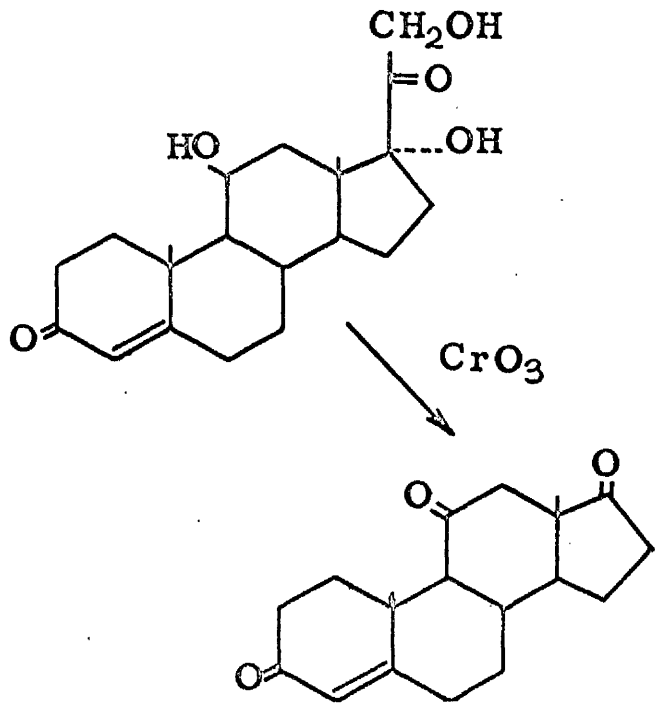


18 HYDROXY DOC-γ-LACTONE- 3ENYL HFB

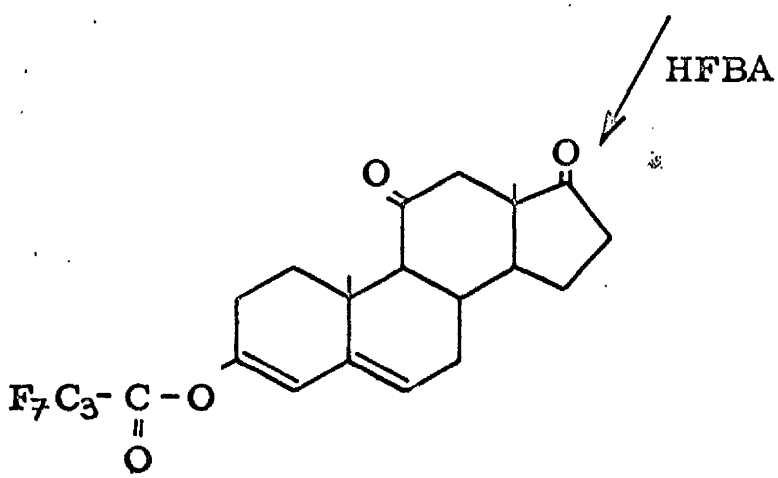
Fig. 6

OXIDATION OF CORTISOL TO ANDROSTENETRIONE AND ESTERIFICATION WITH HFBA.

CORTISOL



ANDROST-4-ENE-3, 11, 17. TRIONE

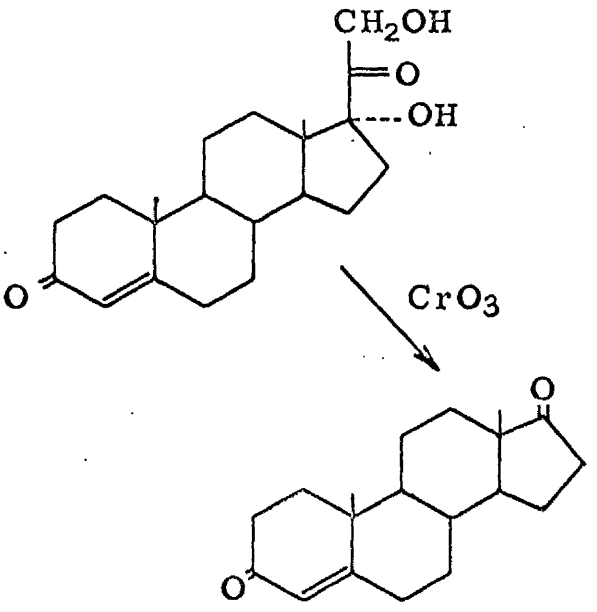


ANDROST-5(6)-ENE-11, 17-DIONE-3ENYL HFB

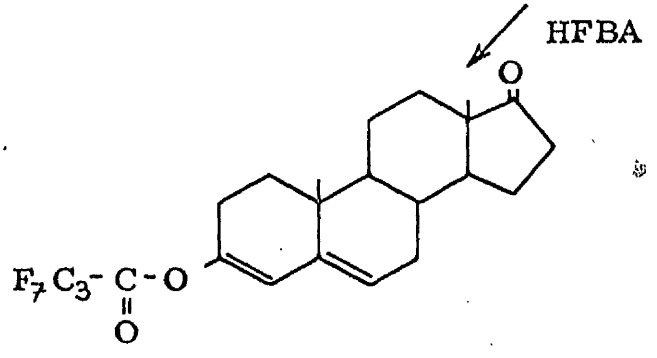
Fig. 7

OXIDATION OF 11-DEOXYCORTISOL TO ANDROSTENEDIONE AND SUBSEQUENT ESTERIFICATION WITH HFBA.

11 - DEOXYCORTISOL



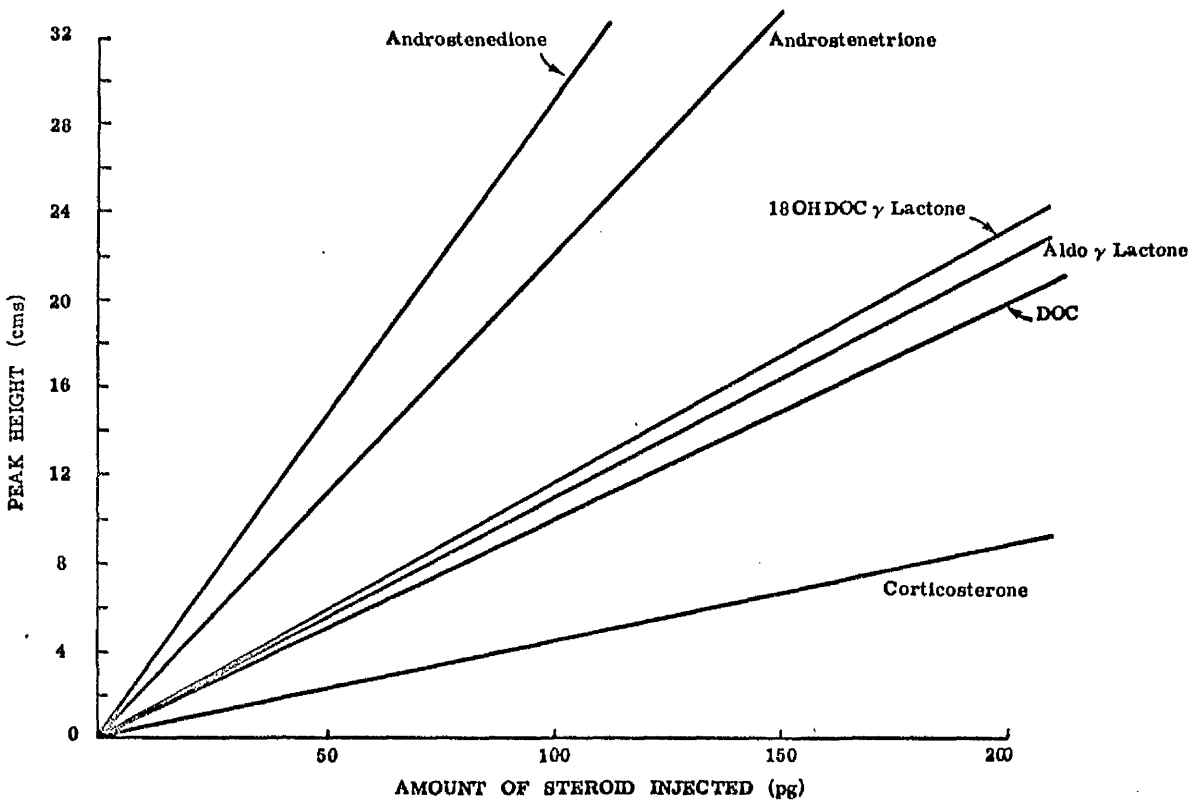
ANDROST - 4 - ENE - 3,17 - DIONE



ANDROST-5(6)-ENE-17-ONE-3ENYL HFB

Fig. 8

COMPARISON OF LINEARITY AND SENSITIVITY OF RESPONSE TO CORTICOSTEROID HFB DERIVATIVES



(d) Discussion

The 3, 21 bis-heptafluorobutyrate derivative of deoxycorticosterone has previously been used as the basis of a method for estimating plasma deoxycorticosterone concentrations in man (Wilson and Fraser, 1971). Although the higher molecular weight substituents, EFU, HFN and PFO, are said to have greater affinity for electrons than heptafluorobutyrate (Kirschner and Taylor, 1969), this is not borne out, at least when a primary alcohol group is esterified, by the results in Table 2. However, it is clear that for corticosteroids at least, esterification of the C21 primary alcohol group, while conferring considerable thermostability on deoxycorticosterone and corticosterone, is relatively ineffective in augmenting electron capturing ability and that formation of the enyl ester at carbon 3, forming the conjugated double bond system illustrated in Figures 3 - 7, is far more important in this respect.

Unfortunately, no comparison was made of the various substituents in position 3 because the reagents were available as acids or acid chlorides as opposed to anhydrides and could not therefore be used to attack the Δ 4, 3 ketone. It is at least possible that the relative potency of the substituents in position 3 may be the same as in position 21 and that there would/

would have been no advantage over heptafluorobutyrate to compensate for the additional inconvenience and expense.

Comparing the mixed substitution with the bis-heptafluorobutyrate, no evidence of increased sensitivity was obtained. Retention times of the higher molecular weight derivatives were frequently inconveniently long. It was therefore decided to retain the simpler, unified approach of using heptafluorobutyrates as the gas-liquid chromatography derivatives for all corticosteroids.

2.2.4 MEASUREMENT OF ALDOSTERONE, 11-DEOXYCORTICOSTERONE, 18-HYDROXY-11-DEOXYCORTICOSTERONE, CORTICOSTERONE, CORTISOL AND 11-DEOXYCORTISOL IN HUMAN PLASMA BY GAS-LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION.

This section describes the development and assessment of a method for measurement of peripheral plasma steroid concentrations using gas-liquid chromatography with electron capture detection of thermostable fluorinated derivatives of the respective steroids.

(a) Preparation of (^{3}H) 18-hydroxy-11 deoxycorticosterone

Radioactive 18 OH DOC was prepared from (^{3}H) DOC by a modification of the method of de Nicola and Birmingham (1968). Individual decapsulated rat adrenal glands were quartered and kept in incubation medium (2 ml) for 30 minutes at 37°. The substrate depleted glands were transferred to fresh aliquots of medium containing 5 µCi of (^{3}H) DOC and incubated for a further 2 hours. The tissue was then discarded and the medium extracted with dichloromethane (10 volumes), the extract evaporated to dryness and the residue chromatographed consecutively on paper systems B_1, B_3 and B_5 (Bush 1961). As in all further cases of paper chromatography, the steroid region was located by radioscanning equipment (Panax Ltd.) and eluted chromatographically with methanol (5 ml). Twenty batches/

batches of (^{3}H) 18.OH.DOC have been prepared in this way and the mean yield was 19%. Specific activities varied from 15 to 20C/mM.

(b) Preparation of samples for gas-liquid chromatography.

Plasma samples were divided into two aliquots of 5 ml.

Sample 1: Cortisol, corticosterone, 18.OH.DOC, aldosterone.

The sample, to which the appropriate (^{3}H) steroids had been added, was extracted with dichloromethane (1 x 8 volumes) and the extract washed consecutively with one volume each of sodium hydroxide (0.1 M), acetic acid (0.1 M)and water before being evaporated to dryness at 35° under a stream of nitrogen. All further evaporations were conducted in this way. The residue was chromatographed on system B_1.

Sample 2: DOC and 11-deoxycortisol.

The sample was processed as described above but the resulting residue was chromatographed in system B_3.

Of the six compounds under investigation DOC and corticosterone required no further purification prior to gas-liquid chromatography and aliquots (10% by volume) were taken at this stage for measurement of (^{3}H) yield. The remaining four compounds were oxidised as described below.

Aldosterone, 18.OH.DOC

These eluates were combined and evaporated to dryness.

The/

The residue was redissolved in methanol (0.1 ml) and periodic acid solution (0.5 ml of 0.1M containing 1% pyridine) added. The mixture was left in the dark at room temperature for 1.5 hours, diluted with water (1 ml) and the steroid products extracted with dichloromethane. The extract was washed with water (1 x 1 ml). After evaporating the solvent, the residue was chromatographed on system B_3. The oxidation products of aldosterone and 18.OH.DOC (γ-lactones) were located and eluted separately. The eluates were evaporated to dryness. Aliquots for assessment of (^{3}H) yield were taken at this stage.

Cortisol, 11-deoxycortisol

These eluates were also combined and evaporated to dryness. The residue was dissolved in chromium trioxide, acetic acid solution (0.25 ml) and left at room temperature for 30 minutes. Water (1 ml) was then added and the mixture extracted with dichloromethane (1 x 10 ml). The extract was washed (1 x 1 vol. of water) and evaporated to dryness, the residue then being chromatographed on system B_3 for 4 hours. The products of cortisol and 11-deoxycortisol were located, eluted separately and the eluates evaporated to dryness. Aliquots for assessment of (^{3}H) yield were taken at this stage.

Formation/

Formation of heptafluorobutyrate derivatives

Each residue was dissolved in benzene (30 µl) and heptafluorobutyric anhydride (30 µl) and heated at 60° for 30 minutes. The reagents were then evaporated and a known quantity of the appropriate internal standard added (see Table 3) prior to injection of aliquots into the gas-liquid chromatograph.

(c) Results

(i) Paper chromatography

The properties of the six corticosteroids and, where relevant, their oxidation products, are shown in Table 4. From these data, the difficulty of separating corticosterone and 11-deoxycortisol reliably without derivative formation is apparent. Similarly, the markedly faster migration of DOC compared to the other compounds made complete separation of all the compounds on a single chromatogram impossible. For this reason the compounds were arranged in two separate groups for analysis.

(ii) Yield of steroid from the purification procedure

The mean recovery of (^{3}H) DOC and (^{3}H) corticosterone from the purification stages was in excess of 60% (see Table 3) but where an oxidation stage was necessary the recovery fell to/

Table 3 Derivatives for gas-liquid chromatography of corticosteroids

Compound	Aldosterone	18-OH-DOC[x]	Cortico-sterone	DOC	Cortisol	Deoxy-cortisol	Oxopro-gesterone
Derivative	(1) γ-lactone HFB[x]	(2) γ-lactone HFB	(3) HFB	(4) HFB	(5) Androstene-trione HFB	(6) Androstene-dione HFB	(7) HFB
Yield from esterification %	93.7	94.1	94.7	93.6	95.1	94.2	93.1
S.D.	4.7	5.3	3.9	3.8	3.5	4.8	5.2
n	8	8	8	8	8	8	8
Relative * retention time	3.76	2.75	1.03	1.00	1.06	0.55	1.62
Column temperature (°C)	245	245	225	225	225	218	225
Gas flow (ml/min)	100	100	80	80	80	80	80
'Sensitivity' + (pg)	1.44	1.68	2.50	1.56	0.45	0.33	0.56
Internal standard	18-OH-DOC HFB	aldo-γ-lactone HFB	oxoproges-terone HFB	oxoproges-terone HFB	oxoproges-terone HFB	androstene-dione HFB	-

* Retention time relative to DOC HFB. (Column: 3% OV17, 230°C)

\+ Quantity required to give a 1 cm peak at full detector sensitivity.

x HFB, heptafluorobutyrate; 18-OH-DOC, 18-hydroxy-11-deoxycorticosterone; DOC, 11-deoxycorticosterone.

Table 4 Paper chromatography of corticosteroids and their derivatives

Compound	System B_1 (Bush 1961) $R_{cortisol}$	System B_3 (Bush 1961) R_{DOC}
Aldosterone	1.46	0.08
Aldosterone-γ-lactone	11.50	0.62
18-Hydroxydeoxy-corticosterone (18-OH-DOC)	3.56	0.19
18-OH-DOC-γ-lactone	21.10	1.14
Corticosterone	4.81	0.26
Deoxycorticosterone (DOC)	18.50	1.00
Cortisol	1.00	0.05
Androstenetrione	16.10	0.87
11-Deoxycortisol	4.80	0.26
Androstenedione	29.20	1.50

to between 30 and 40%, partly due to losses during chemical manipulation and also to the additional chromatography stage required after modification. However, these recoveries were more than adequate for gas-liquid chromatography.

(iii) Yield of steroid from esterification with heptafluorobutyric anhydride

A relatively large quantity (50 µg) of each steroid or derivative, containing 250,000 dpm of (^{3}H) compound was esterified with heptafluorobutyric anhydride as described above. The products were chromatographed in paper chromatography systems together with parallel, unesterified standards. Areas corresponding to steroid and ester were located and eluted. No radioactivity was recovered from the region corresponding to the unesterified compound and recovery from the ester region was almost quantitative (see Table 3). Representative compounds, aldosterone-γ-lactone HFB, DOC bis HFB and androstenetrione-HFB, were subjected to a second chromatography and the yield of (^{3}H) in the region of the derivative reassessed. Results indicated that losses were largely due to chromatography and not to incomplete esterification.

(iv) Gas chromatography

The conditions used for GLC are shown in Table 3. Column temperatures and rates of column gas flow were chosen/

chosen in order to give conveniently short retention times while avoiding contamination of the peaks both from volatile contaminants associated with the solvent front, which could then be voided by means of the by-pass valve, and also from other halogenated compounds. Sensitivity of detection under these conditions is also shown in this table and has been expressed as that quantity of steroid required to produce a peak 1 cm high. It can be seen that the technique possesses a high sensitivity potential, varying from 0.3 to 2.5 pg.

(v) Reliability

Specificity: Blank assays, using water instead of plasma, were included in all batches of analysis and always gave undetectable steroid levels (Table 5). In duplicate samples of plasma taken from an adrenalectomised subject maintained on 9α -fluorocortisol, levels were again undetectable with the exception of 18 OH DOC of which a small but reproducible quantity was found (Table 5). It was, however, negligible when compared to the normal range. Specificity was further evaluated, as shown in Table 6, by comparing the retention times of the steroid HFB derivatives on a number of columns, coated with stationary phases covering a wide range of polarity. The concentration of stationary phase was also varied. The retention times for plasma/

Compound	Aldosterone	18-OH-DOC	Corticosterone	DOC	Cortisol	Deoxycortisol
^{3}H Yield %	37	34	62	68	34	32
S.D.	10.2	10.3	9.9	10.0	8.6	8.9
n	179	184	183	189	185	189
Blank: water ng/100ml	undetectable	undetectable	undetectable	undetectable	undetectable	undetectable
plasma	"	1.2±0.3 S.D.	"	"	"	"
Replicate variation						
mean *	7.0 ng	54.6 ng	0.25 ug	10.0 ng	5.6 ug	99.3 ng
S.D	1.24	7.71	0.02	0.21	0.69	11.60
n	20	18	19	21	20	19
Accuracy$^{\mp}$ (%) 1	106	94	96	93	104	98
2	101	101	106	100	110	106
3	103	97	103	94	110	99
Normal range /100 ml	4-18 ng	20-160 ng	0.08-0.8 ug	2.8-16.0 ng	2.5-10.0 ug	40-400 ng
Concentration/100 ml after dexamethasone (n = 7)	4.1±1.48 ng	37.2±17.85 ng	0.11±0.04 ug	1.88±0.82 ng	0.71 ±0.48 ug	23.0±14.40 ng

Plasma from an adrenalectomised subject was used in the blank.

*Plasma pool obtained from normal subject, concentrations expressed per 100 ml of plasma

Accuracy for aldosterone and DOC: 1 = 5 ng/100 ml, 2 = 10 ng/100 ml, 3 = 20 ng/100 ml

Accuracy for corticosterone, 18-OH-DOC and 11-deoxycortisol: 1 = 50 ng/100 ml, 2 = 100 ng/ml, 3 = 200 ng/100 ml

Accuracy for cortisol: 1 = 0.5 ug/100 ml, 2 = 1.0 ug/100 ml, 3= 2 ug/100 ml

(18-OH-DOC, 18-hydroxy-11-deoxycorticosterone; DOC, 11-deoxycorticosterone).

Table 6 Retention times of steroid derivatives and plasma steroids on columns with a variety of properties

Support	% Phase	Gas flow ml/min.	Temp. °C	Relative retention time (a)						
				1	2	3	4	5	6	7
Chromosorb WHP 80-100 mesh	1% OV 22	90	225	4.2	3.2	0.53	0.56	0.65	0.44	1.0
"	3% OV 225	85	245	4.2	2.4	0.52	0.70	0.65	0.33	1.0
Supelcoport 100-120 mesh	1% Dexsil 300 GC	100	245	3.0	2.5	0.78	0.82	0.68	0.36	1.0
"	3% "	100	250	2.5	1.9	0.82	0.92	0.55	0.42	1.0
Supelcoport 80-100 mesh	3% OV 17	90	245	4.6	2.2	0.76	0.81	0.64	0.44	1.0

(a): Retention times relative to 11-oxoprogesterone-HFB

1 - 7: Steroid derivatives, see Table 3. Retention times for pure and plasma-derived steroids were identical.

plasma product and standard compound were identical in all cases and injection of mixtures of plasma products and standard compound into the column invariably produced a single peak.

Precision: Samples of a normal plasma pool were included in each batch of assays (Table 5). Coefficients of variation ranged from 7% for corticosterone to 17% for aldosterone. The replicate variation for this study was for aldosterone: 6.1 ± 0.6 ng/100 ml; corticosterone: 0.25 ± 0.03 ug/100 ml; and DOC: 11.8 ± 0.7 ng/100 ml.

Accuracy: Recovery of added steroid from plasma was tested in duplicate at three levels within the range expected in clinical investigation (Table 5). Means of the duplicate estimations indicate a high degree of accuracy. Linearity of response for three compounds was examined by analysing in quadruplicate a range of plasma volumes (2, 5, 7.5 and 10 ml). For those compounds tested no volume effect was apparent.

Calculations from a chromatogram: Plasma steroid concentrations were calculated by substitution into the equation:

$$\frac{\text{Sample peak height (mm)}}{\text{Std. peak height (mm)}} \times A \times B \times \frac{10}{9}^{*} \times \frac{100}{C} \times \frac{100}{D} = \text{pg/100 ml plasma.}$$

A = mass of standard injected (pg) B = fraction of sample injected

C = % recovery of $[^{3}H]$ label D = volume of plasma sample (mls)

* Factor to correct for removal of $\frac{1}{10}$ sample for liquid scintillation spectrometry.

Normal ranges: The normal ranges shown in Table 5 are based on analysis of plasma taken randomly through the working day from normal healthy males and females aged from 20 to 40 years. None of the subjects was pregnant or was receiving medication of any kind.

(d) Discussion

While the literature abounds with descriptions of methods of estimating the concentrations of individual corticosteroids in human peripheral plasma, and many report simultaneous assays of small groups of compounds, particularly cortisol and corticosterone (Ely, Hughes and Kelley, 1958; Braunsberg and James, 1961; Cameron and Kilborn, 1964; Eechaute, 1966; Martin and Martin, 1968; Butte and Noble, 1969) or aldosterone, corticosterone and cortisol (Stachenko, Laplante and Giroud, 1964; Coghlan and Scoggins, 1967; Fraser and James, 1968; Underwood and Williams, 1972) few methods have attempted to cover all the major compounds secreted by the adrenal cortex and none, so far as we are aware, have included the estimation of 18-OH-DOC concentration. Fraser (1967) and Oddie, Scoggins and Coghlan (1971) used the double isotope derivative technique for plasma aldosterone, corticosterone, DOC, cortisol and 11-deoxycortisol and Newsome, Clements and Borum (1972) and Clements and Newsome (1973), using a competitive protein binding method, added cortisone to this list but omitted aldosterone. The value of the "multisteroid" approach is widely recognised and the problems of establishing such a procedure fall mainly into three/

three categories, effective separation of steroid compounds, adequate purification and sufficiently sensitive quantitation.

(i) Separation: Although it is capable of separating the majority of corticosteroids (Vinson and Whitehouse, 1969) the disadvantages of chromatography on thin layers of silica gel as a prelude to gas liquid chromatography have been discussed previously (Wilson and Fraser, 1971). Gel filtration, particularly using Sephadex LH20, has been recommended as a means of separation (Carr, Mikhail and Flickinger, 1971; Newsome et al, 1972) and is reported to give an adequate separation of aldosterone, corticosterone, DOC and 18-OH-DOC (Shapiro and Péron, 1972) although cortisol and 11-deoxycortisol were not tested. While gel filtration is undoubtedly a clean and rapid method of isolating steroid fractions, rates of elution may vary from column to column and constant monitoring of individual eluates is not easy.

Paper chromatography has received much wider application. Although development times are considerably longer than for gel filtration, the development is usually an overnight procedure and thus does not consume working time. When radioactive standards are used, as described here, positive location of individual sample fractions by radioisotope scanning equipment is simple and its routine use ensures maximum yield of compound while avoiding contamination by other/

other adjacent substances. Moreover, the ease with which paper can be cleaned by solvent extraction makes it ideal as a preparation for gas liquid chromatography. Unfortunately, as can be seen from Table 1, corticosterone and 11-deoxycortisol do not separate with the paper chromatography systems used, unless a derivative such as an acetate (Fraser, 1967; Oddie et al, 1971) is first formed. The newer techniques of high pressure liquid-liquid chromatography (Landgraf and Jennings, 1973) affinity chromatography (Cuatrecasas and Anfinsen, 1971) or immunologic purification (Gomez-Sanchez , Kem and Kaplan, 1973) may eventually prove to be more convenient than those at present available.

(ii) Purification: The extent to which a steroid-containing fraction of a biological extract requires to be purified is closely related to the specificity of the technique eventually used to assay the compound. The double isotope derivative technique is relatively non-specific and multiple chromatography, usually coupled with structural modification of the compound to alter its chromatographic properties, is always necessary, with the consequence that methods become extremely laborious and lengthy. Conversely, preparation for radioimmunoassay, which may be highly specific (see below) involves minimal purification (Mayes, Furuyama, Kem and Nugent, 1970; Ito, Haning and Horton, 1972; Gross, Ruder,/

Rader, Brown and Lipsett, 1972; James, Arnold, Rippon and Marie, 1972; Jowett, Slater, Piyasena and Ekins, 1973) or none at all (Vetter, Vetter and Siegenthaler, 1973 a, b; Martin and Nugent, 1973). Gas-liquid chromatography is itself a powerful means of separating the individual components of a mixture of steroids and is therefore an effective method of purification in its own right. Indeed, Weinstein, Lai and Xenakis (1971) have used this facility to prepare samples for the double isotope derivative assay of DOC.

Providing that the problem of non-specific detector contamination of the electron capture detector can be prevented, for example by the use of a bypass valve as described here and by Wilson and Fraser (1971), only a single chromatography may be required before the gas-liquid chromatographic assay of those corticosteroids, DOC and corticosterone, for which a suitable derivative is available. The remaining corticosteroids are not stable at high temperatures. The formation of suitable derivatives of these is of necessity followed by a second purification stage.

(iii) Quantitation: Few techniques are sufficiently sensitive to detect the quantities of the more dilute corticosteroids, aldosterone and DOC, present in normal human peripheral plasma. Of these, the double isotope derivative assay has the disadvantages that it requires the use/

use of massive quantities of radioactive isotopes with the attendant risks of cross-contamination of samples, that it most frequently is forced to employ relatively low specific activity (^{14}C) steroids to assess yield and that it may require relatively large plasma samples. However, most double isotope derivative methods are chemically simple, are readily adaptable to multisteroid analysis and, while the number of samples handled at any one time may be small (i.e. low capacity), the range of concentration detected is unlimited. Specificity can be checked by physico-chemical methods.

The use of antibodies to steroid haptens for saturation analysis, a technique which has received widespread acceptance in a remarkably short time, has a high degree of implied specificity and the majority of steroid methods based on this technique have sensitivities in the range capable of measuring plasma aldosterone and DOC concentrations with ease in small samples. The possibility that complete specificity may be achieved, by raising antibodies to unique parts of the steroid molecule, is real (Weinstein, Linder, Friedlander and Bauminger, 1972) and thus high capacity by means of automation could be achieved. Unfortunately, demonstration of absolute or even high specificity for radioimmunoassay is not easy. The majority of published methods rely entirely on the cross-reaction of relatively few closely related compounds, while

others/

others have attempted to calibrate their methods against methods based on other principles (Ito et al, 1972; Fraser, Guest and Young, 1973; Fraser, Wilson and Holmes, 1973). Since the assay is carried out on a crude or minimally purified biological extract, assessment of specificity by cross-reaction ignores possible interference, specific and non-specific, of unknown steroid compounds, steroid drugs and their metabolites such as aldadiene (Vecsei, P, and Joumaah, C. personal communication) and possibly of non-steroidal compounds also.

Considerable improvement can result from the assay of a unique steroid derivative. For example, Varsano-Aharon and Ulick (1973) describe a method for the radio-immunoassay of aldosterone in which aldosterone is first oxidised to the γ-lactone, thereby destroying many of the steroid competitors present in biological extracts, and is then assayed using an antibody to this derivative. Farmer, Roup, Pellizzari and Fabre (1972), use a similar approach. While many data have been accumulated on plasma corticosteroids in man by the use of other techniques with which to assess radioimmunoassay methods, where, as in the case of 18 OH DOC, this is not the case, evaluation of specificity is of considerable importance. The finite range of detection of radioimmunoassay methods may be a marginal inconvenience.

Gas-liquid/

Gas-liquid chromatography with electron capture detection falls between these extremes. As shown in Table 2, subpicogram levels of some corticosteroids can be detected and, at worst, sensitivity of detection is equivalent to that of radioimmunoassay. Like the double isotope technique, there is no upper limit of detection and specificity can be assessed in a positive way rather than by a process of elimination as in radioimmunoassay. Also, being chemically simple, this type of method possesses a high degree of flexibility. Gas-liquid chromatography is more sensitive and requires less preliminary preparation than the double isotope methods.

(iv) Corticosteroid derivatives for gas-liquid chromatography.

The choice of derivative is determined initially by the need to prevent destruction at the high temperature required for gas chromatography. The reasons for the choice of the 3,21-bis HFB derivative of DOC have already been discussed (Wilson and Fraser, 1971) and this reasoning has also been extended to corticosterone. The sensitivity of the electron capture detector to this derivative is rather less than to DOC.HFB but the plasma concentration of corticosterone may be as much as a hundred times greater than that of DOC. Simple esterification of aldosterone, 18-hydroxy DOC and the 17α-hydroxy corticosteroids does not prevent their thermal decomposition. The γ-lactone derivatives of

aldosterone/

aldosterone and 18-OH-DOC are stable at temperatures of 200° (Merits, 1962) and attract electrons with sufficient avidity to form the basis of methods for measuring the aldosterone content of tissue (Kittinger, 1964, 1968) and urine (Bravo and Travis, 1967; Palem, Lapiere, Coninx and Margoulies, 1970). Although the derivative has been used in the analysis of rat plasma (Rapp, 1970), sensitivity of detection is not high enough for use in man unless electron-capturing ability is augmented by esterification with heptafluorobutyric anhydride, directly as described here or following reduction to the tetrahydro-aldosterone derivative (Nicolis and Gabrilove, 1969), or with other polyhalogenated substituents (Nicolis, Wotiz and Gabrilove, 1969; Fabre, Fenimore, Farmer, Davis and Farrell, 1969). The relative merits of a number of derivatives of aldosterone for gas-liquid chromatography have been compared by Horning and Maume (1969).

Thermostability of the 17α- hydroxylated compounds, cortisol and 11-deoxycortisol, is most simply achieved by oxidation which removes the side chain (Holzbauer and Newport, 1968). The resulting androstenes can again be rendered highly avid of electrons by formation of the 3-enyl heptafluorobutyrate. It is this derivative which confers sufficient sensitivity on all the corticosteroids, or the products/

products of their oxidation, to allow a unified and yet flexible approach to multisteroid analysis.

(v) Performance: Using the method, one person can produce sixty steroid results (6 different steroids on 10 plasma samples) in four days. This represents a slightly lower capacity than the simplest radioimmunoassay methods but is a considerable improvement on the majority of methods based on physicochemical techniques. The main disadvantage of gas-liquid chromatography is the skill required to maintain high standard performance of the chromatograph, although this is simplified by use of the bypass valve.

Assessment of (^{3}H) yield to correct the mass measurement for recovery from plasma was carried out prior to esterification because of the reported instability of steroid 3-enyl heptafluofobutyrates during thin layer chromatography (Exley and Chamberlain, 1967). Paper chromatography does not appear to be so destructive (Wilson and Fraser, 1971). From Table 2 it can be seen that the esterification procedure is virtually quantitative and that the small losses encountered in the experiment were probably due, not to the esterification but to subsequent paper chromatography.

The precision of estimation of plasma concentrations of/

of aldosterone, DOC, corticosterone, 11-deoxycortisol and cortisol is similar to those based on other techniques and the variability is small in relation to the ranges of concentration experienced. No previous data on 18.OH.DOC in plasma are available but precision is of the same order as for the other compounds.

The specificity of estimation is illustrated by the gas chromatographic data in Tables 5 and 6. It is extremely unlikely that two or more compounds could behave with such similarity on such a variety of columns. The evidence of the behaviour of mixed injections is also significant. Similarly, the absence of detectable steroid derivatives in plasma and water blanks may be evidence of lack of non-specific interference. No explanation could be found for the small but definite and reproducible quantity of 18.OH.DOC in plasma from an adrenalectomised subject but this interference was considered to be negligible.

Accuracy was checked at several points in the ranges of concentration expected to be encountered in human peripheral plasma and recovery of corticosteroids added to plasma was quantitative in all cases.

Normal ranges for the concentrations of cortisol, corticosterone, 11-deoxycortisol, DOC and aldosterone are available/

available from many sources (e.g. Braunsberg and James, 1961; Coghlan and Scoggins 1967; Brodie, Shimizu, Tait and Tait, 1967; Fraser and James, 1968; Arnold and James, 1971; Wilson and Fraser, 1971; Oddie et al, 1972), and those listed in Table 4 are similar. Although the values of plasma 18.OH.DOC concentration predicted by Melby, Dale, Grekin, Gaunt and Wilson (1972) on the basis of secretion and metabolic clearance rate estimations are lower than those shown in Table 5, this discrepancy may be explained by the bound state of a proportion of the hormone in plasma.

In addition to its reliability, the extreme flexibility of the method makes it possible to add to the list of compounds other compounds of interest such as cortisone with minimal effort, time and expense. It should prove valuable in studies of the changing pattern of adrenocortical secretion under conditions of stress and electrolyte imbalance in normal people and in pathological states involving abnormalities of adrenocortical secretion.

3. FACTORS AFFECTING THE SECRETION OF ADRENOCORTICAL HORMONES.

3.1 Introduction

Adrenocortical secretion is directly or indirectly influenced by several factors and brief reviews of these are presented in subsequent sections. It is evident that, although the secretion of particular corticosteroid hormones may be more responsive to one manoeuvre than another - cortisol secretion, for example, responds rapidly to ACTH stimulation but less markedly to angiotensin II - it is unusual, possibly because of the interrelated steroid biosynthetic pathways (see Fig. 1a) for trophic effects to be exclusive. Of more physiological significance, because the corticosteroids have mixed metabolic effects (see Section 1), each external influence on the adrenal cortex will alter to some extent the net mineralocorticoid and glucocorticoid role of the adrenocortical secretion. Thus, ACTH leads to a sharp increase in the secretion of the major glucocorticoid, cortisol, but the secretion rates of corticosterone and DOC, with marked mineralocorticoid properties, also rise and, in the long term, the situation is further complicated by secondary effects of this ACTH-induced sodium retention on the secretion of the major mineralocortical, aldosterone (see Section 3.2.2). It is this aspect of adrenocortical secretion which is the particular concern of the following sections of this thesis, illustrating the important additional information/

information which can be derived from concurrent estimations of as many as possible of the corticosteroid hormones in plasma - i.e. the multisteroid approach. More emphasis will be placed on relative changes in steroid secretion than on individual steroid responses. While the relative potencies of the effects of the hormones on intermediary and electrolyte metabolism have not yet been adequately quantitated, some attempt will be made to assess the changes in overall metabolic effect of adrenocortical secretion following the application of a number of standard stimuli.

The major factors controlling adrenocortical secretion are : ACTH, secreted by the anterior pituitary,

Angiotensin II, generated in plasma but directly related to renal events,

sodium balance,

potassium balance,

cardiovascular changes following changes in posture, blood volume, etc.,

These factors are, of course, interrelated and the list is not intended to be comprehensive as other factors may also be involved.

3.2 The effect of adrenocorticotrophic hormone (ACTH) on plasma corticosteroid concentrations.

3.2.1 Literature survey

The importance of the pituitary gland, particularly the anterior section, in the general control of the endocrine system, first suspected in the late nineteenth century, received considerable stimulus from the isolation of a number of active fractions from the gland in the second and third decades of the present century. Among these active principles was a fraction containing a hormone which increased the activity of the adrenal cortex and which was called adrenocorticotrophic hormone or ACTH (Smith, 1930). This discovery coincided roughly with a period of intense investigation of adrenocortical secretion and began a lengthy and, as yet incomplete, study of the control of ACTH secretion and its mechanism of action on the adrenal cortex.

(a) The nature of ACTH

The ACTH molecule is a single, unbranched polypeptide chain of 39 amino acids. While certain differences in the C-terminal sequence occur between species, the N terminal region of 24 amino acids is common to all species and contains all the biological activity, which resides in the 18 residues towards the N terminal end (Li and Oelofsen, 1967; Irvine et al, 1974). The α 1-24 ACTH molecule has been synthesised/

synthesised (Bell, 1954) and is commercially available under the name "Synacthen", providing a convenient and reproducible source for studies such as those described in section 3.2.2.

The antigenic effect of ACTH lies mainly in the C terminal end of the molecule. This may occasionally present problems when attempts are made to relate radioimmunological assay results with biological activity (Imura, Sparks, Grodsky and Forsham, 1965; Ratcliffe, Scott, Bennett, Lowry, McMartin, Strong and Walbaum, 1973). α 1-39 ACTH is secreted by the corticotrophs of the anterior pituitary (Dasgupta and Young, 1958). Of considerable clinical interest is the secretion of peptides or proteins with ACTH-like activity by neoplastic tissue, particularly bronchial carcinoma (Liddle et al, 1969; O'Neal, Kipnis, Luse, Lacy and Jarett, 1968; Rees and Ratcliffe, 1974). Sufferers from this condition exhibit supranormal adrenocortical activity and circulating levels of ACTH which are frequently very high indeed (Ratcliffe, Knight, Besser, Landon and Stansfeld, 1973). However, although blood ACTH activity is indisputably elevated, it now seems likely that much of the immunologically assayed ACTH is of the 'precursor' variety ('big ACTH') which is devoid of biological activity (Yalow, 1974). This may explain the lower potency of ACTH obtained from neoplastic tissue (Saffran, Matthews and Pearlmutter, 1971). The situation is further complicated by the secretion of immunologically/

immunologically active, biologically inactive fragments of ACTH (Ratcliffe et al, 1973; Rees et al, 1974; Orth, Nicholson, Mitchell, Island and Liddle, 1973).

(b) Control of ACTH secretion

ACTH is released from the anterior pituitary in response to a variety of stimuli generally classified as stresses, including pain, trauma, pyrexia, anxiety, anaesthesia and hypoglycaemia. These stresses have been subdivided into categories by Sayers (1950). In the normal human subject unaffected by stress, ACTH secretion is not constant but exhibits a well-defined periodicity-circadian or nycthemeral rhythm - which may be related to the sleep-wake pattern of the individual (Ceresa, Angeli, Bocuzzi, ~~Molinot~~ and Perotti, 1970; Orth, Island and Liddle, 1967; Perkoff, Eik-Nes, Nugent, Fred, Nimer, Rush, Samuels and Tyler, 1959). Secretion is at a minimum late at night and reaches a maximum in early morning in the normal man (Weitzman, Fukushima, Mogeire, Roffwarg, Gallagher and Hellman, 1971; Ney, Shimizu, Nicholson, Island and Liddle, 1963). Superimposed on this diurnal variation is an oscillation of much higher frequency, generally referred to as 'episodic secretion', which may indicate that ACTH is released from the hypophysis in a pulsatile manner rather than as a continuous secretion (Krieger, Allen, Rizzo and Krieger, 1971; Vagnucci, McDonald, Drash and Wong, 1974). Changes in plasma ACTH

ACTH concentration, which presumably relate most closely to adrenocortical effect, could be a function of either changes in frequency or amplitude or both.

Early theories of the control of ACTH secretion were derived from a consideration of the microanatomy of the hypophysis and hypothalamus (e.g. Harris, 1937). The pituitary stalk contains the vessels of a well defined portal system - the hypothalamo-pituitary portal system - arising from capillaries in the hypothalamus and leading to a capillary system in the anterior pituitary. Although at that time it was not easy to ascertain the direction of blood flow within this portal system, the hypothesis was formulated that communication between the central nervous system and the anterior pituitary was by means of humoral factors transported in the blood. This subject has recently been reviewed by Porter, Mical, Ben-Jonathan and Ondo (1973).

(c) Corticotrophin-releasing factor (CRF)

Extracts of hypothalamic tissue, when injected into animals such as the rat, cause synthesis and secretion of many of the anterior pituitary hormones. These hypophysiotrophic compounds have been called releasing factors or releasing hormones and one of them, CRF, is specific for ACTH release (Vernikos-Danellis, 1965). More recently, CRF activity/

activity has been demonstrated in blood removed from the hypothalamo-pituitary portal system of the rat and the dog (Porter et al, 1973).

The identity of CRF has not yet been established although it seems likely to be a small peptide. Lysine-vasopressin, a nonapeptide, was at one time suggested as a candidate for the role of CRF (Gwinup, 1965; Landon, James and Stoker, 1965). It is certainly capable of stimulating ACTH release (Landon et al, 1965; Saffran et al, 1971), but this effect is thought to be non-specific (Yates and Urquhart, 1962). Guillemin (1964) has listed a number of peptides which may be CRF.

(d) ACTH and adrenocortical secretion

Administration of ACTH *in vivo* or *in vitro* results in a rapid increase in steroid output, perceptible responses occurring within a few minutes. The effect is quantitatively different for the various corticosteroids (see below). The locus of action is probably early in the biosynthetic pathway before the formation of pregnenolone (Stone and Hechter, 1954; Karaboyas and Koritz, 1965). Graded doses of ACTH applied to *in vitro* incubations of rat adrenal tissue by the superfusion technique, reveal a sigmoid dose-response relationship where corticosterone release is taken as the response parameter and this effect can be reproduced if cyclic adenosine monophosphate (cAMP) is used in place of ACTH (Schulster, Tait, Tait and Mrotek, 1970; Richardson/

Richardson and Schulster, 1972). This confirms the earlier studies of Haynes and Berthet (1957), Haynes, Koritz and Péron, (1959), Birmingham, Kurlents, Muhlstock and Traikov (1960) and others suggesting that, in common with many other protein and polypeptide hormones (Sutherland and Robison, 1966; Robison, Butcher and Sutherland, 1971; Major and Kilpatrick, 1972; Garren et al, 1971), ACTH may depend for its activity on the 'second messenger' principle employing the adenyl cyclase enzyme system. In favour of this theory, it has been demonstrated that ACTH need not enter the cell in order to exert its effect on adrenal steroid metabolism. ACTH attached to polyacrylamide beads is as effective as the free hormone (Richardson et al, 1972). The fact that cAMP or its dibutyryl derivative is much less affective than ACTH (c25,000 times less, w/w) may be due to a relative inability to penetrate the cell membrane. In vivo, cAMP would, of course, be generated within the cell.

Current views on the mechanism of action of ACTH have been reviewed by Garren, Gill, Masui and Walton (1971). The major proportion of the adrenal cholesterol is stored in fat droplets in the form of the sulphate. To make this substrate available for corticosteroid synthesis, the cholesterol must be released from its conjugated state and transported into the mitochondria where conversion to pregnenolone takes place.

The/

The demonstration that ACTH causes a depletion of adrenocortical cholesterol sulphate, leaving mitochondrial free cholesterol unchanged or increased, (Davis and Garren, 1966) supports the argument that the hormone is responsible for altering hydrolysis and transport. There is much evidence that ACTH institutes changes in protein synthesis and, although long-term effects on nucleic acid metabolism have been reported (Grower and Bransome, 1970), the initial effects on synthesis are probably direct rather than through the mechanism of nuclear derepression, thought to be the mechanism of action of steroid hormones themselves (e.g. Edelman and Fimognari, 1968).

Cyclic AMP is bound to the microsomal and soluble fractions of adrenal cell preparations, a cAMP-dependent protein kinase has been demonstrated in the microsomal fraction and certain new proteins, reputedly synthesised in response to ACTH, have been isolated (Farese, 1967; Grower and Bransome, 1970). Garren et al (1971) summarise the evidence by means of the following hypothesis. ACTH binds to a specific cell membrane receptor and initiates cAMP synthesis from ATP by activating an adenyl cyclase enzyme system. This may regulate steroid biosynthesis by affecting the activity of a long-lived species of an RNA and may also bind to a receptor-protein kinase complex, releasing the kinase for the purpose of phosphorylating proteins of unknown function but probably enzymic in nature.

Protein/

Protein synthesis does not appear to be necessary for releasing cholesterol from its conjugated state in the fat droplets (Davis, 1969), but it may be involved in the procedure whereby cholesterol is transported across the cell and into the mitochondria. An alternative or possibly additional effect of ACTH may be to modify adrenocortical activity by altering adrenal blood flow (L'age, Gonzalez-Luque and Yates, 1970).

As stated above, the effect of ACTH on the various corticosteroid hormones is quantitatively different. For convenience, they will be divided into non-18-oxygenated (i.e. cortisol, corticosterone and their respective 11-deoxy compounds) and 18-oxygenated compounds. The latter group can be subdivided into 18-hydroxycorticosteroids and aldosterone.

(i) Cortisol, corticosterone, DOC and 11-deoxycortisol.

ACTH increases the width of the zona fasciculata increasing RNA and protein synthesis and also the concentrations of several enzymes required for corticosteroid biosynthesis, but has little effect on the microanatomy of the zona glomerulosa (Symington, 1969). Conversely pituitary ablation or insufficiency leads mainly to zona fasciculata atrophy, at least in the short term (Farrell, 1959). It is the zona fasciculata which synthesises cortisol and 11-deoxycortisol and which probably supplies the major portion

of/

of the secreted DOC and corticosterone (see review by Beevers, Brown, Fraser, Kremer, Lever, Morton, Robertson, Schalekamp, Semple and Wilson, 1975). It might be inferred from this evidence that ACTH is a major factor in the control of the secretion of these hormones.

There is much direct evidence (see for example review by Braunsberg and James, 1961), that cortisol and corticosterone concentrations are extremely sensitive to ACTH. The pattern of response of these 11-hydroxysteroids is qualitatively similar in most situations (see however Fraser et al, 1966), but the magnitude of the response is greater for corticosterone (B) than for cortisol (F) so that the ratio F/B falls during ACTH administration (Krum and Glenn, 1965; Fraser, 1967), a fact which may have an important bearing on the physiological effects of ACTH (see below).

DOC concentrations also increase after ACTH administration or stress such as hypoglycaemia (Fraser, 1967; Biglieri, Schambelan and Slaton, 1969; Oddie, Coghlan and Scoggins, 1972; Wilson, 1973; Arnold and James, 1971; Irvine et al, 1974). High DOC levels in plasma or urine also occur in Cushing's syndrome when ACTH is increased (Biglieri, Slaton, Schambelan and Kronfield, 1968; Oddie et al, 1972). Hypopituitarism on the other hand, whether pathological or induced by dexamethasone suppression (see below) is followed by/

by a fall in DOC levels (Bledsloe, Island and Liddle, 1966; Schambelan and Biglieri, 1972; Wilson, 1973; Cope and Loizou, 1975). 11-Deoxycortisol concentrations also rise when exogenous (Oddie et al, 1972) or endogenous (Fraser, 1967) ACTH stimulation is applied.

Because they respond to ACTH, the plasma concentrations of the zona fasciculata-generated compounds follow the same nycthemeral pattern as ACTH with concentrations highest in the morning and lowest late at night (Hellman, Nakada, Curti, Weitzman, Kream, Roffwarg, Kilman, Fukushima and Gallagher, 1970). Moreover, several studies have also shown that plasma cortisol concentration also follows closely the shorter time scale, pulsatile pattern of ACTH secretion (Gallagher, Fukushima and Hellman, 1970; de Lacerda, Kowarski and Migeon, 1973) but no similar studies appear to have been carried out on other corticosteroids in this respect so far.

(ii) Negative feedback mechanism.

While ACTH controls the rate of secretion of cortisol, plasma cortisol concentration in turn acts as a modifier of ACTH release by means of a negative feedback mechanism. As plasma cortisol levels rise, they appear to act at the level of the hypothalamus which responds by secreting less CRF. ACTH secretion therefore falls (Yates, Leeman, Glenister and/

and Dallman, 1961). Adrenocortical sensitivity to ACTH is not impaired (James, Fraser and Landon, 1966). The feedback mechanism is not simple but can be subdivided into at least three components, a rapid feedback element responding to what might be termed the acceleration of plasma cortisol levels (Jones, Brush and Neame, 1972), a delayed feedback response appearing about one hour after rises in plasma cortisol, even although, at the time of the response, plasma cortisol levels have returned to normal (Hodges and Sadow, 1967) and finally a short feedback loop controlled by plasma ACTH levels themselves (Vernikos-Danellis, 1965; Motta, Mangili and Martini, 1965).

Only 11-hydroxylated corticosteroids, including some synthetic corticosteroids such as prednisolone and dexamethasone, are directly effective in suppressing ACTH secretion although 11-oxocompounds such as cortisone would be expected to have the same effect indirectly by virtue of reduction of the 11-oxogroup. In the liver, cortisone is converted to cortisol. This is amply demonstrated by the effects of 11-β-hydroxylase (see Fig. 1a.) deficiency, either pathological (Eberlein and Bongiovanni, 1956) or induced by the drug metapyrone (Cope, Dennis and Pearson, 1966). In this situation, plasma concentrations of cortisol and corticosterone are low and ACTH secretion, apparently/

apparently in an effort to restore normal concentrations, is raised (Crane and Harris, 1966; Beevers et al, 1975). Consequently, DOC and 11-deoxycortisol levels rise rapidly to concentrations far in excess of normal, but are ineffective in suppressing ACTH secretion. Metapyrone, for this reason, forms the basis of a valuable method of assessing anterior pituitary reserve (Kaplan, 1963; Buus , Binder and Petersen, 1962; James and Landon, 1968). In fact, there is some recent evidence that 11-deoxycorticosteroids such as DOC, 11-deoxycortisol and 18-hydroxy-DOC may antagonise the fast component of the feedback system (Jones, Tiptaft, Brush, Fergusson and Neame, 1974; Tiptaft and Jones, 1975), thus exagerating potential ACTH response to stress. However, the same authors also show that large quantities of DOC or 11-deoxycortisol but not 18-hydroxy-DOC, may operate the delayed negative feedback mechanism. These studies were carried out in the rat and their applicability to man is not yet established.

It is obvious that the level of plasma cortisol required to switch off ACTH secretion must vary with the physiological requirement for this steroid hormone, otherwise high plasma cortisol levels could neither be attained nor sustained, during stress.

The/

The fact that plasma cortisol varies diurnally must also be explained. High doses of glucocorticoids do not inhibit the rise of cortisol following surgical trauma and other stresses (Estep, Island, Ney and Liddle, 1963). It is suggested that the feedback mechanism is reset at a high cortisol concentration in these circumstances (James, Landon and Fraser, 1968), although this explanation has been questioned (Hodges, 1968).

(iii) 18-Hydroxy DOC.

No studies of the changes in peripheral plasma 18-hydroxy DOC concentration during the day or across physiological manipulation are available because reliable methods for its estimation have not been available until now (see below). Such information as is available for man is derived from analysis of adrenal vein blood or urine and has recently been reviewed by Melby et al (1972). Their conclusion that ACTH is a major factor in the control of 18-hydroxy DOC secretion was based on the observations that

dexamethasone reduced urinary excretion rates,

ACTH (i.v. or i.m.) increased adrenal venous and urinary levels.

It is pertinent to remember at this point that 18-hydroxy DOC is also produced mainly by the zona fasciculata/

fasciculata (Sheppard, Swenson and Mowles, 1963).

(iv) Aldosterone

Indirect evidence suggested, even before the isolation and characterisation of the major mineralocorticoid, aldosterone, that the anterior pituitary was not a major source of influence on adrenocortically-mediated electrolyte matabolism. For example, electrolyte metabolism was not severely impaired, in the short term at least, by hypophysectomy or hypopituitarism. Moreover, while deficiency of ACTH is followed by progressive atrophy of the zona fasciculata (see above), the zona glomerulosa, which synthesises aldosterone (Symington, 1962), is relatively unaffected. These early studies have been reviewed by Farrell (1959) and Ross et al. (1959). More recently, it has been reported that dexamethasone fails to depress the circulating levels of aldosterone (Fraser, 1967). Conversely, Cushing's Syndrome associated with high levels of ACTH secretion does not usually increase aldosterone production (Biglieri, Hane, Slaton and Forsham, 1963; Landon, James and Peart, 1967). Although these studies led inevitably to the conclusion that short-term control of aldosterone secretion relied on some extra-pituitary factor, some pituitary role could not be discounted. Liddle, Duncan and Bartter (1956), for example, discovered that long-term pituitary insufficiency in/

in human subjects resulted in a diminution of the normal response of aldosterone secretion to haemorrhage and sodium deprivation. Similar results have been obtained in animals (Binion, Davis, Braun and Olichney, 1965: Ganong, Biglieri and Mulrow, 1966; Lee, van der Waal and de Wied, 1968; Palmore and Mulrow, 1967). The anterior pituitary may therefore play a supportive or permissive role in controlling aldosterone secretion.

Direct study of the effects of ACTH, when preparations of porcine or synthetic ACTH became available, only added to the confusion, mainly because dose levels used by the various groups of research workers varied enormously but, in common, were all massively above the normal physiological range (see review by Fraser, Brown, Chinn, Lever and Robertson, 1969). Infusion of ACTH at physiological rates or induction of endogenous ACTH release by stress in normal, sodium replete subjects has only a transient effect on plasma aldosterone concentration (James, Fraser and Landon, 1966; James, Landon and Fraser, 1968) and the threshold dose required to elicit an aldosterone response is higher than that required to elicit a response of corticosterone or cortisol secretion (Fraser, 1967, see however Kem, Gomez-Sanchez, Kramer, Holland and Higgins, 1975). However, this state of affairs is completely altered by sodium depleting the subject. In these/

these circumstances, aldosterone plasma concentration is at least as sensitive as that of cortisol to ACTH release (James et al, 1968; Kem et al, 1975).

In recent years, with improved methods for assessing plasma aldosterone, interest in the relationship between ACTH and aldosterone secretion has been renewed although the question of the relative importance of ACTH in this respect has by no means been answered. These new methods allowing by their sensitivity more frequent plasma sampling, have revealed that aldosterone secretion, like that of cortisol (see above), may also be pulsatile or 'periodic'. There is some controversy as to whether the pulses are synchronous with those of cortisol. Vagnucci et al (1974), for example, found that the short term variability was superimposed on diurnal changes which were high soon after the onset of sleep whereas simultaneous assays of cortisol and corticosterone followed the pattern described in the previous sections. In contradiction, Vetter, Berger, Armbruster, Siegenthaler, Werning and Vetter (1974) and Kem et al, (1973), studying subjects with aldosterone-secreting adrenocortical tumours and Katz, Romfh and Smith (1975) using normal human subjects noted a pattern indistinguishable from cortisol, suggesting that, when the secretion of renin is suppressed (see below), the ACTH role is revealed. This situation is difficult to reconcile/

reconcile with the low values found by many authors when subjects have been recumbent overnight and it should be emphasised that the first of these studies employed an aldosterone assay which may have been affected by cortisol itself. Finally, Kem et al (1975) claim that the relative magnitude of the aldosterone response to ACTH is comparable with that of cortisol in dexamethasone-treated, sodium replete subjects. They also confirm the 'sensitisation' effect of sodium depletion.

Clearly there is still much to be learned in this field of research, particularly the importance of ACTH in comparison with other physiological stimuli such as renin (see below). If, as several recent publications have suggested, ACTH is indeed important, it is difficult to explain why aldosterone secretion cannot be sustained after nephrectomy (Blair-West, Coghlan, Denton, Scoggins, Wintour and Wright, 1967; Ganong et al, 1966), or in cases of isolated renin-deficiency (Brown, Chinn, Fraser, Lever, Morton, Robertson, Tree, Waite and Park, 1973). However, while evidence points to a minor role in the control of aldosterone secretion, nevertheless, ACTH could possibly exert a profound effect on electrolyte metabolism.

(e) <u>ACTH and modification of the adrenocortical role in electrolyte metabolism.</u>

There/

There is general agreement that naturally occurring rhythmical changes in ACTH secretion and more severe changes following stress or administration of the polypeptide are reflected in changes in plasma cortisol, 11-deoxycortisol, corticosterone, DOC and 18-hydroxy DOC concentrations. The effect on aldosterone may be relatively smaller unless the subject is first sodium-depleted. The role of cortisol in electrolyte metabolism is only vaguely understood (see Hierholze and Stolte, 1969) and 11-deoxycortisol probably has no effect (Travis and Sayers, 1965).

The major adrenocortical role in electrolyte metabolism is usually assigned to aldosterone, and the importance of these other corticosteroids discounted, except in rare cases of inborn errors of steroid metabolism (Biglieri, Stockigt and Schambelan, 1972; New and Seaman, 1970). However, now that endocrine treatment of inflammatory diseases such as rheumatoid arthritis has been changed from glucocorticoids such as prednisolone which caused many serious side effects, to ACTH which induces hypersecretion of anti-inflammatory steroids, the occurrence of hypertension and distortion of electrolyte metabolism is frequently reported, although plasma aldosterone concentration is not often raised. It seems not unreasonable to infer that such a response may be caused by increased secretion/

secretion of the ACTH-dependent, minor mineralocorticosteroids. Travis and Sayers (1965) give a comprehensive review of the relative pharmacological potencies of several corticosteroids and synthetic steroids with corticosteroid activity. The ratio of sodium-retaining activity, measured by the reduction in Na^+ excretion by the kidneys of an adrenalectomised animal, is as follows:-

aldosterone : DOC : corticosterone : cortisol =
3000 : 100 : 15 : 1. Birmingham et al (1968) report that the potency of 18.hydroxy DOC is roughly equivalent to that of DOC. Thus the individual quantities of zona fasciculata corticosteroids which would be equivalent to 1 ng/100 ml of aldosterone are therefore 30 ng of DOC, 30 ng of 18-hydroxy DOC, 200 ng of corticosterone and 3 ug of cortisol. Even at normal resting levels, there is therefore a considerable reservoir of non-aldosterone 'mineralocorticoid' and this will increase markedly when ACTH is secreted in quantities. Since it has been shown that the symptoms of primary hyperaldosteronism can appear if levels of 10-20 ng/100 ml of aldosterone are sustained for a sufficiently long period (Brown, Chinn, Davies, Düsterdieck, Fraser, Lever, Robertson, Tree and Wiseman, 1968) particularly in sodium replete subjects, the effects of ACTH are likely to be important. The relative potencies of the corticosteroids/

corticosteroids on carbohydrate metabolism show that although cortisol contributes most of this effect, the additional contribution from corticosterone and aldosterone is not negligible.

Thus, even if the direct effects of ACTH on aldosterone are discounted, ACTH may have a profound effect on both carbohydrate and electrolyte metabolism.

The following experiments were designed to test the 'multisteroid' GLC method in a physiological situation and to obtain some preliminary assessment of the dose-response relationship between ACTH and the corticosteroids.

3.2.2 EXPERIMENTAL

3.2.2.1 METHODS

(a) Effect of suppression of ACTH release by administration of dexamethasc

Seven normal subjects took 2 mg of dexamethasone orally at 23.00 h and a further 2 mg at 07.00 h. They remained fasting and were recumbent until blood samples were removed from an arm vein at 09.00 h. In all subsequent experiments where ACTH was infused, dexamethasone was administered in this way and the same conditions of fasting and posture were also observed unless otherwise stated. The results are shown in Fig. 9.

(b) Effect of exogenous ACTH

(i) Preliminary experiments

Control intravenous infusions of 5% dextrose solutions were given to two dexamethasone-treated subjects at rates of 2.5, 7.5 and 22.5 ml hr^{-1}, each rate being continued for one hour. Venous blood samples were continued from the contra-lateral arm at 0, 1, 2 and 3 hours to determine the effects of infused dextrose solution (Table 7).

(ii) To determine whether a one hour infusion period was sufficient to attain an equilibrium of adrenocortical secretion, experiment (i) was repeated, again with dexamethasone-treated, normal male subjects, infusing ACTH (Synacthen, CIBA) at a constant/

constant rate for 3 hours. One subject received 5 μg hr^{-1} and the other 10 μg. hr^{-1}. Samples were again taken hourly (Fig. 10).

(ii) Main experiments

Eight normal subjects, again treated with dexamethasone, were infused consecutively with graded doses of ACTH, each dose for one hour. Four subjects received 2.5, 5.0 and 10.0 μg. hr^{-1} and the remaining four received 0.25, 0.8 and 2.5 μg. hr^{-1}. Blood samples were again taken at the end of each infusion period for corticosteroid analyses (Figs. 11, 12 and 13).

(c) Effect of endogenous ACTH

(i) Effect of circadian variations in ACTH secretion

Venous blood samples were taken from a group of 12 normal men and women at 23.00, 07.00 and 12.00 h during the course of a normal working day. The 07.00 h sample was taken after overnight fasting and recumbency (Fig. 14). None of the female subjects was receiving contraceptive steroids.

(ii) Effect of insulin-induced hypoglycaemia

(see section 3.2.1). The insulin stress test as described by Landon, Wynn and James (1963) was performed on two normal male subjects. After taking a single basal blood sample, insulin (0.2 iu/kg) was given intravenously. Blood samples for/

for corticosteroid analysis were taken at 0, 30, 45 and 120 min (Fig. 15).

(iii) Effect of long-term stimulation by ACTH from a bronchial carcinoma

While in the process of studying the effects of ACTH on plasms steroid concentrations, the opportunity arose to obtain plasma from patients with carcinoma of the bronchus which were secreting ACTH. Both patients subsequently died and the diagnosis was confirmed at post-mortem (Table 8).

3.2.2.2 RESULTS

(a) Suppression of ACTH release

Fig. 9 shows the effect of overnight suppression of ACTH secretion. The rectangles represent the normal ranges for each compound and the ordinate is on a logarithmic scale. The mean concentrations of cortisol and 11-deoxycortisol fell to a level below the lower limit of normal while those of 18-hydroxy DOC, corticosterone and DOC were towards the lower end of the normal range. Plasma aldosterone concentration was also reduced to the lower limit of the normal range but this may have been due to the effects of recumbent posture rather than to lack of ACTH (see later).

(b) Exogenous ACTH

Table 7 illustrates that plasma concentrations of all the steroids studied remained constant during the period of the control dextrose infusion and no escape from dexamethasone suppression was apparent.

In the second preliminary experiment (see Fig. 10), maximum levels of all steroids except cortisol and aldosterone were attained by one hour. There was some evidence of a small further increase in cortisol and aldosterone concentrations during the second hour of infusion at 5 μg. hr^{-1} but this was considered to be negligible.

The/

Fig. 9

EFFECT OF DEXAMETHAZONE ON CORTICOSTEROID PLASMA CONCENTRATION

Rectangles indicate normal ranges; circles and bracketed lines indicate mean values ± SEM after dexamethazone treatment

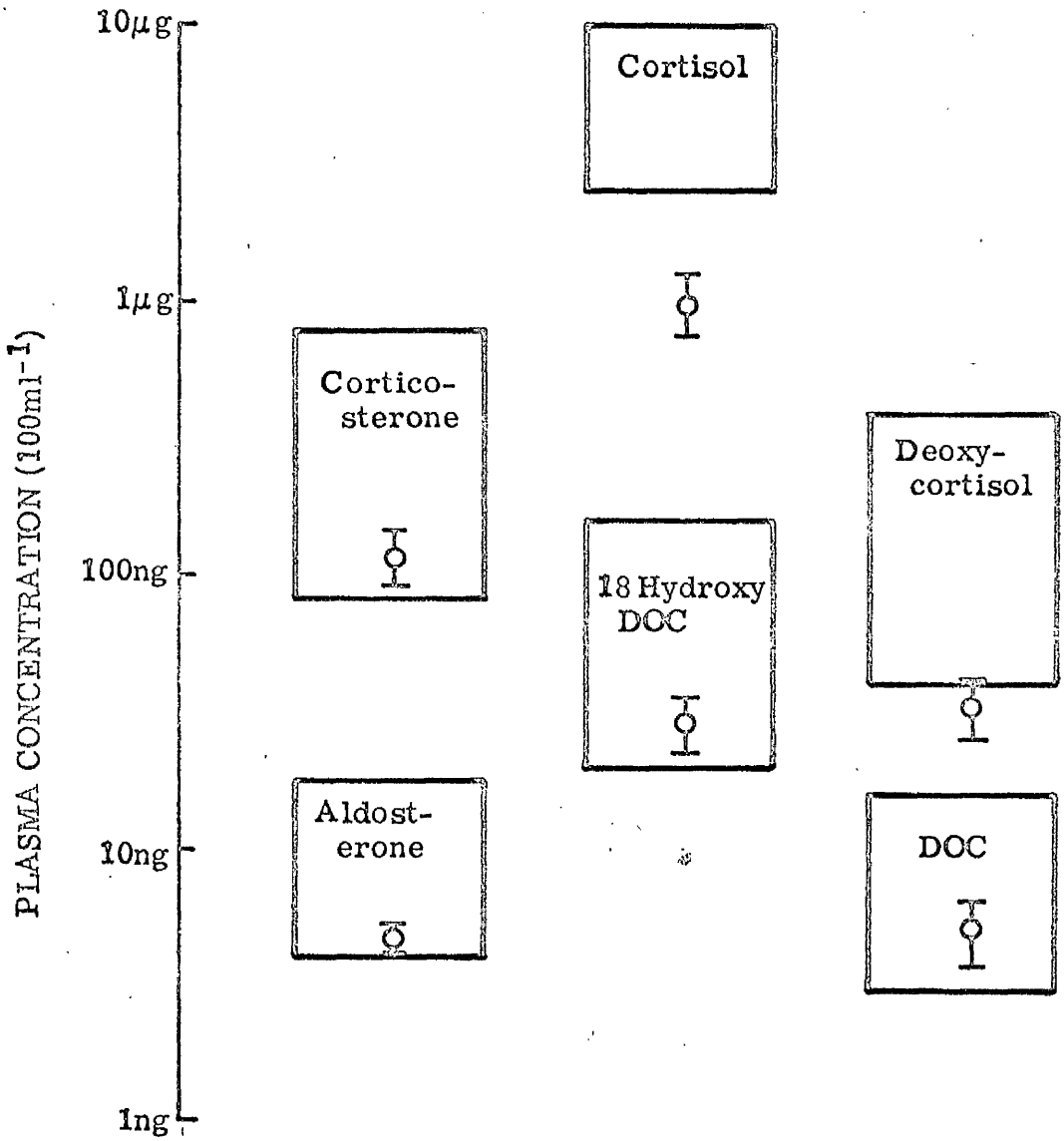
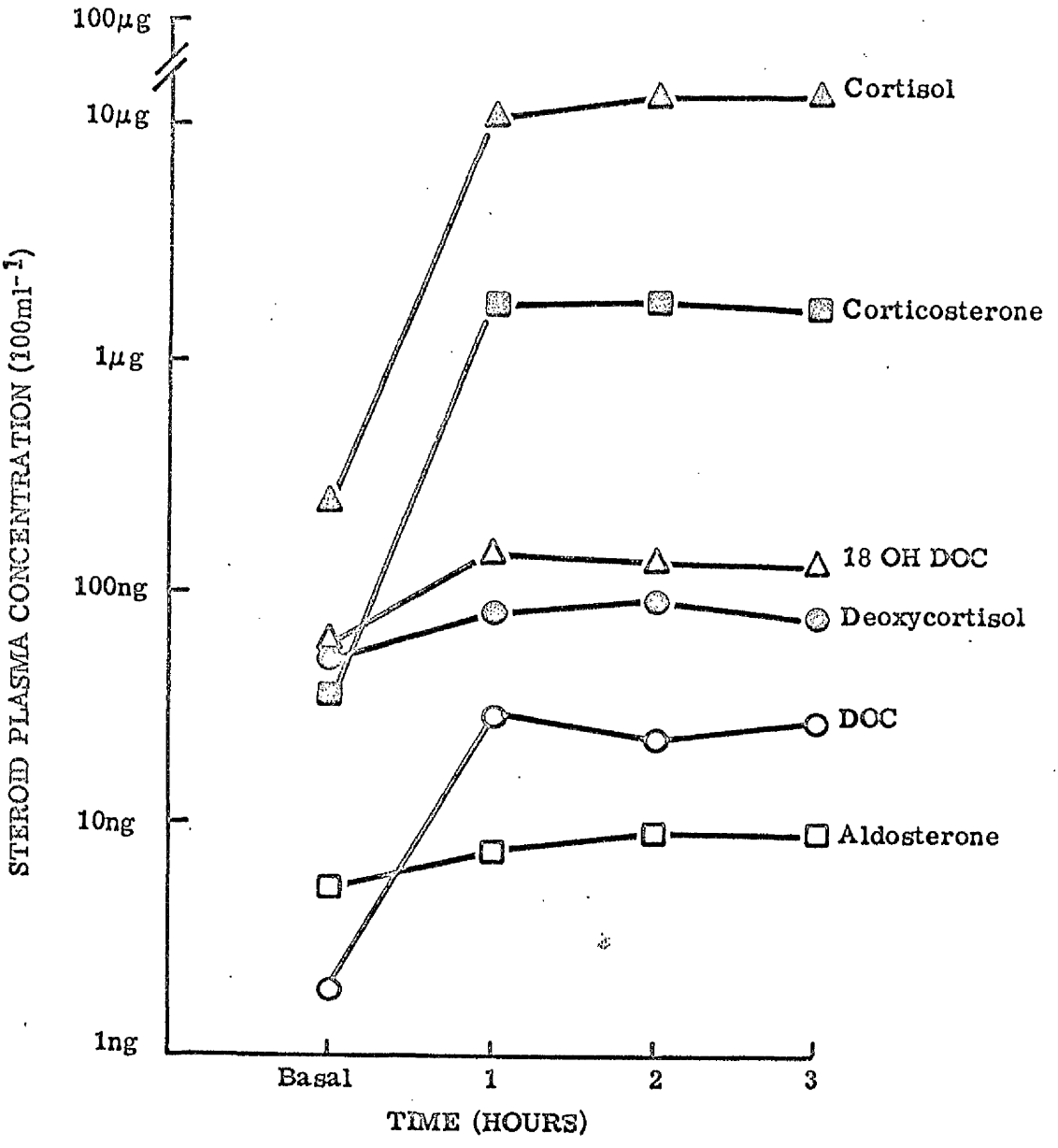


Table 7 Effect on steroid concentrations of infusion of 20 ml/hour 5% dextrose i.v.
(means of two subjects)

Time of infusion (hrs)	Aldosterone ng/100 ml	18-OH-DOC ng/100 ml	Corticosterone µg/100 ml	DOC ng/100ml	Cortisol µg/100 ml	Deoxycortisol ng/100 ml
0	3.9	30.1	0.149	2.9	0.47	19.0
1	4.1	28.7	0.152	3.4	0.68	8.7
2	4.3	29.4	0.121	3.3	0.47	6.9
3	2.6	33.2	0.138	4.1	0.40	8.9

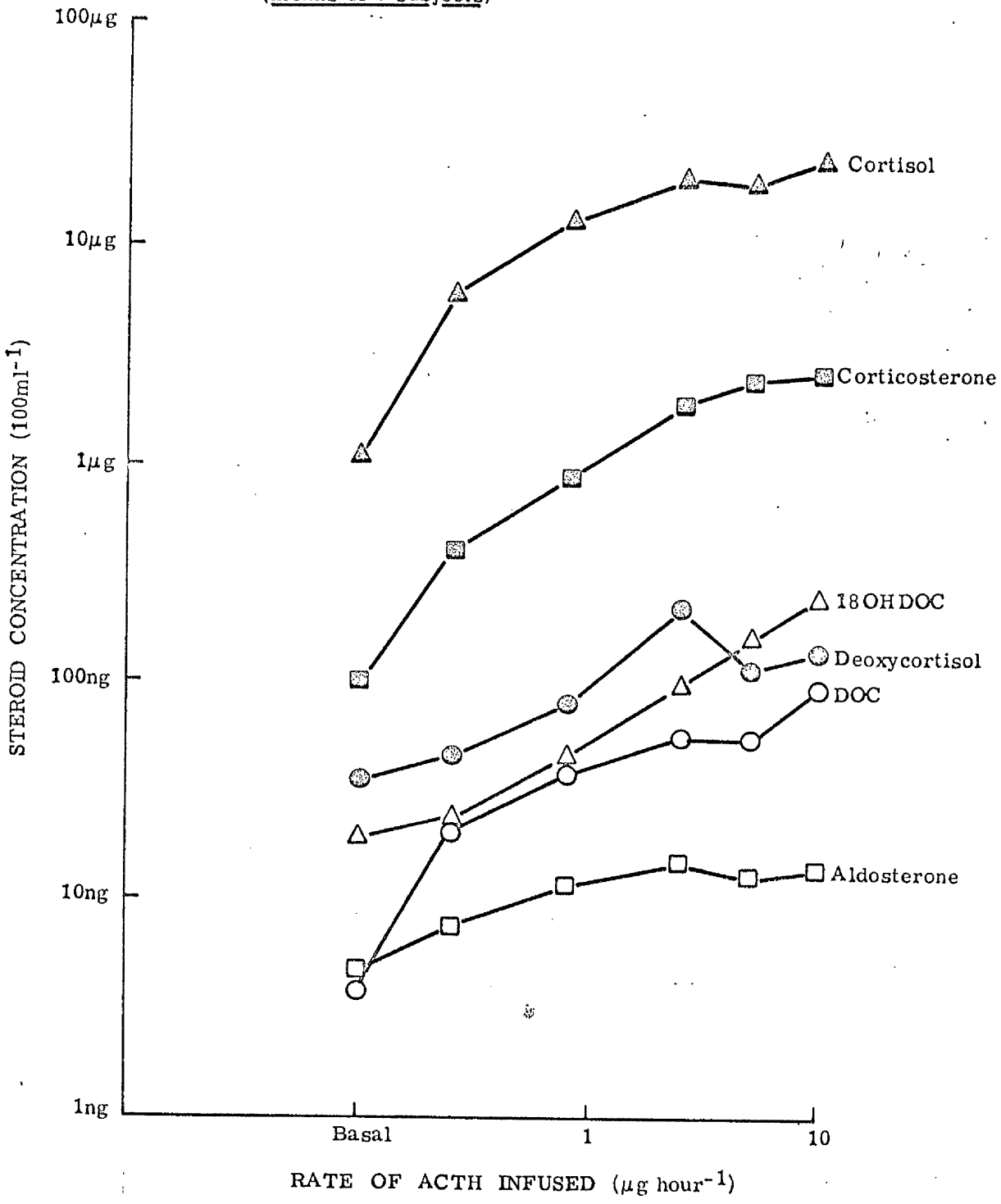
Fig. 10

EFFECT OF INFUSION OF ACTH (α^{1-24}) AT 5μg/hr.
(1 SUBJECT)



EFFECT OF GRADED INFUSION OF ACTH (α^{1-24})

(means of 7 subjects)



The responses of the steroid concentrations to the graded infusion of Synacthen are shown in Fig. 11. An increase in the levels of all the steroids was seen, in response to the infusion, which tended to plateau at the highest rates, for all except DOC and 18.OH DOC. Compared with the normal range of concentration, cortisol, corticosterone and DOC exceed the upper limit, while 18.OH DOC reaches the upper limit. Aldosterone and deoxycortisol rise only into the centre of the normal range.

However, when percentage increase in concentration of steroid is plotted against rate of infusion (Fig. 12) a totally different picture is revealed. Deoxycortisol, 18.OH DOC and aldosterone show a small but steady increase throughout the infusion. Cortisol, although showing the greatest percent increase at low infusion rates is superceded by DOC and corticosterone at the higher rates.

If the steroids are then grouped together on a structural basis, those possessing an oxygen function at position 18, those possessing a 17α-hydroxyl group and those possessing neither, the picture is further clarified (Fig. 13). The 17 and 18 substituted steroids show a steady increase throughout the range of infusion with the 17-hydroxylated steroids showing the greatest initial rate of increase. The 'unsubstituted' steroids, however, show a biphasic response, increasing/

Fig. 12.

RELATIVE EFFECTS OF ACTH (α^{1-24}) ON PLASMA CORTICOSTEROID CONCENTRATIONS

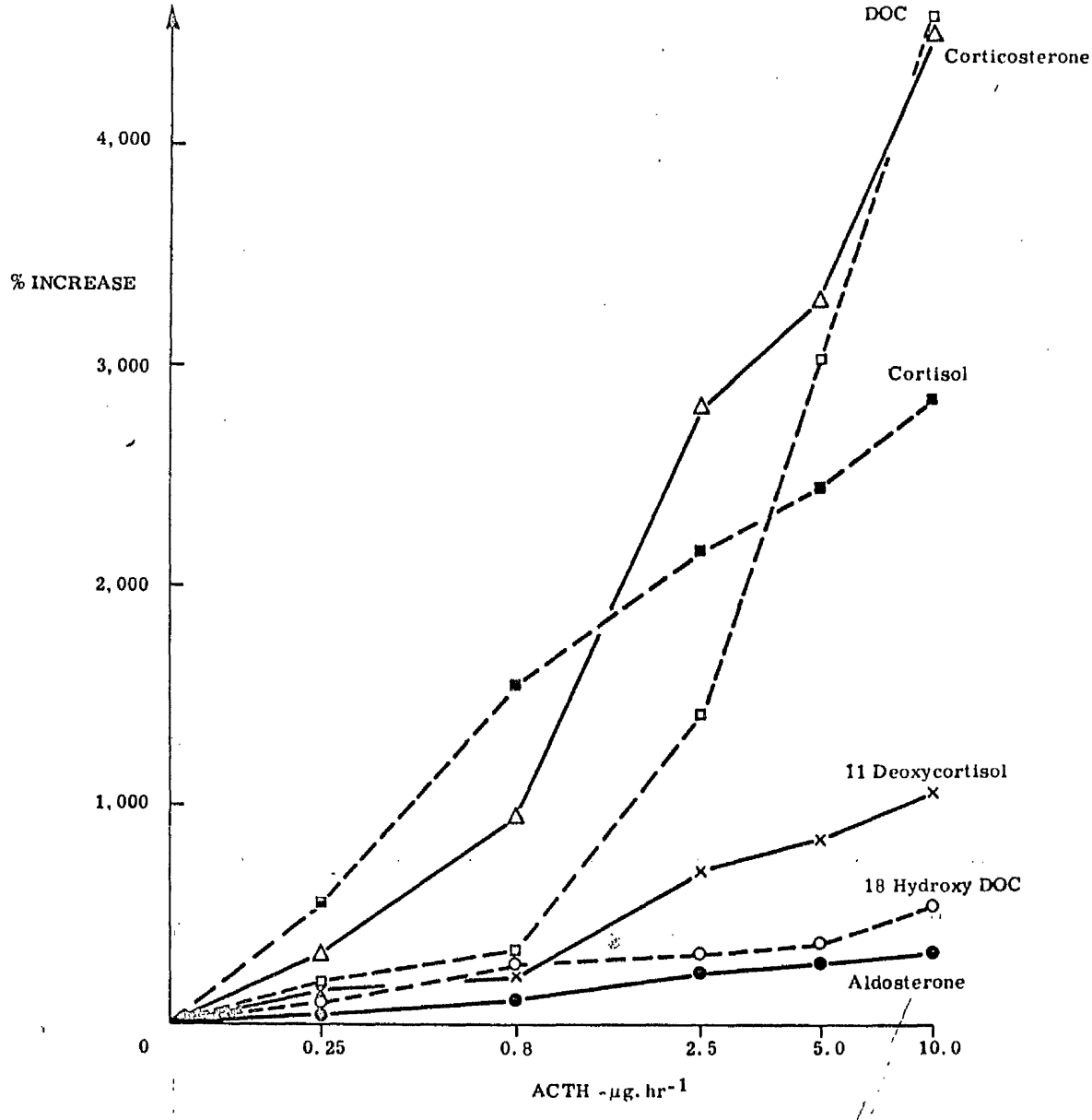
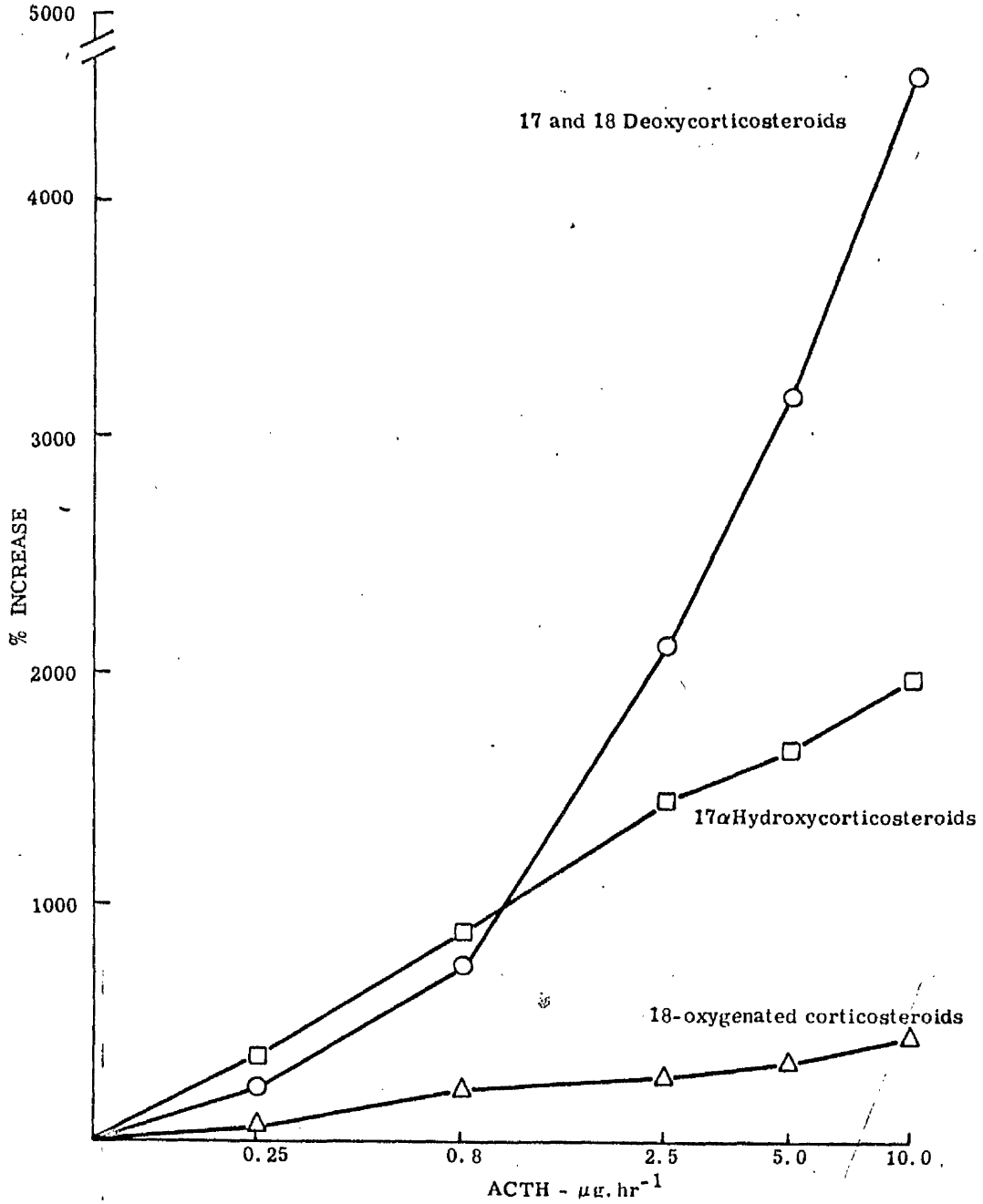


Fig. 13

RELATIVE EFFECTS OF ACTH (α^{1-24}) ON PLASMA CORTICOSTEROID CONCENTRATIONS : STEROID GROUPS



increasing at approximately the same rate as the 17-hydroxylated steroids up to a point between 0.8 and 2.5 µg/hour ACTH when the rate of increase accelerates to far above that of the 17-hydroxylated steroids.

(c) Effect of normal endogenous ACTH

(i) Effect of circadian variations in ACTH secretion.

A significant rise occurred in the levels of DOC, corticosterone, cortisol and 18.OH DOC between the night and early morning samples (Fig. 14). A significant fall was also noted in the same steroid levels between early and late morning samples. No change could be detected in 11-deoxycortisol levels. Aldosterone, however, showed a marked rise between early and late morning samples. This is most probably an effect of posture.

(ii) Effect of insulin-induced hypoglycaemia.

Thirty minutes after administration of insulin a marked rise occurred in levels of all steroids measured except aldosterone (Fig. 15). Forty-five minutes after administration of insulin the levels of DOC, 18.OH DOC and deoxycortisol had fallen and in fact DOC had returned to original levels. A further rise was seen in cortisol concentration, however, and a slight rise was also noted for aldoterone. Two hours after the insulin all the steroid concentrations had returned to pre-test levels.

DIURNAL VARIATION IN NORMAL HUMANS

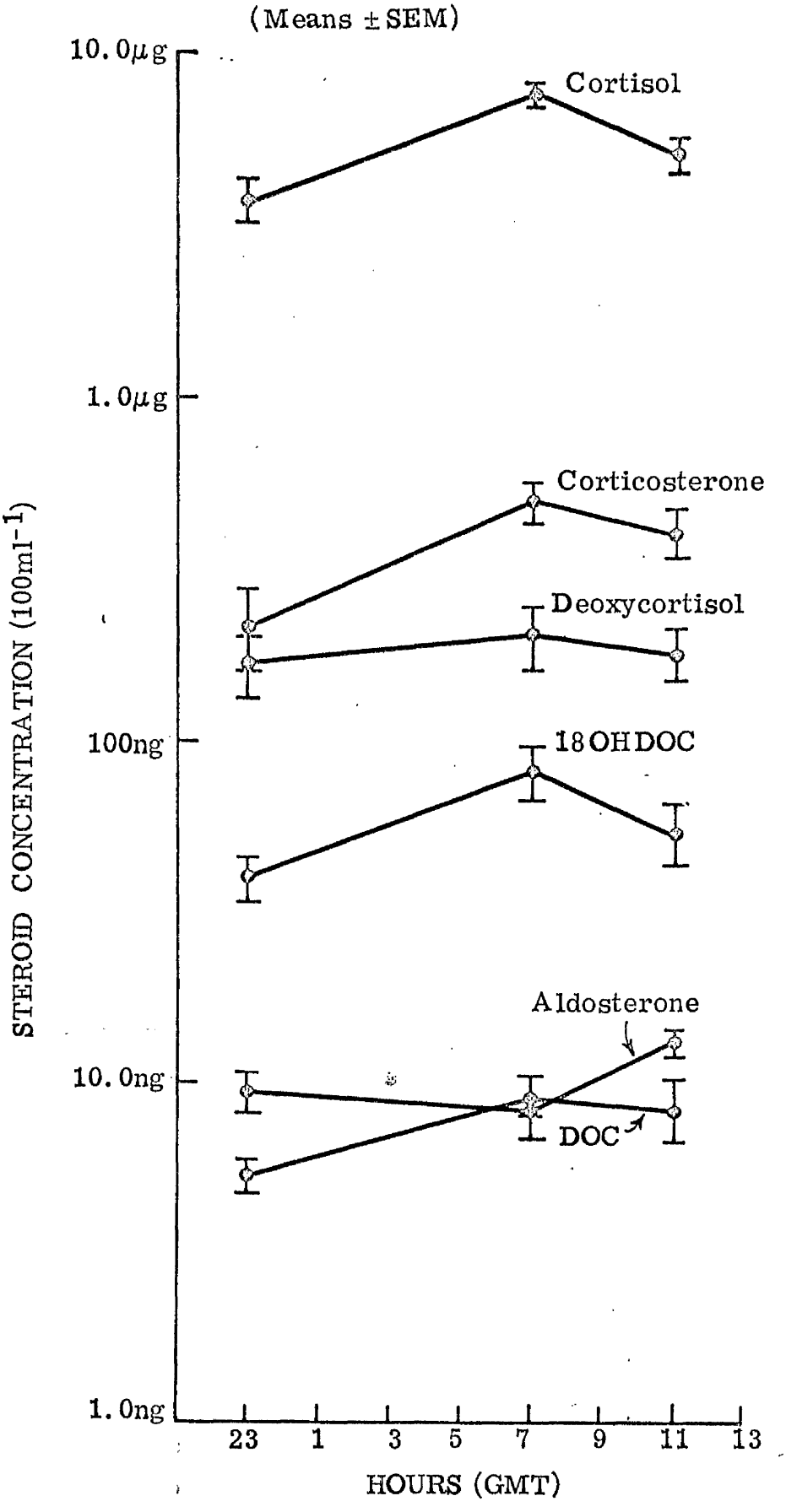
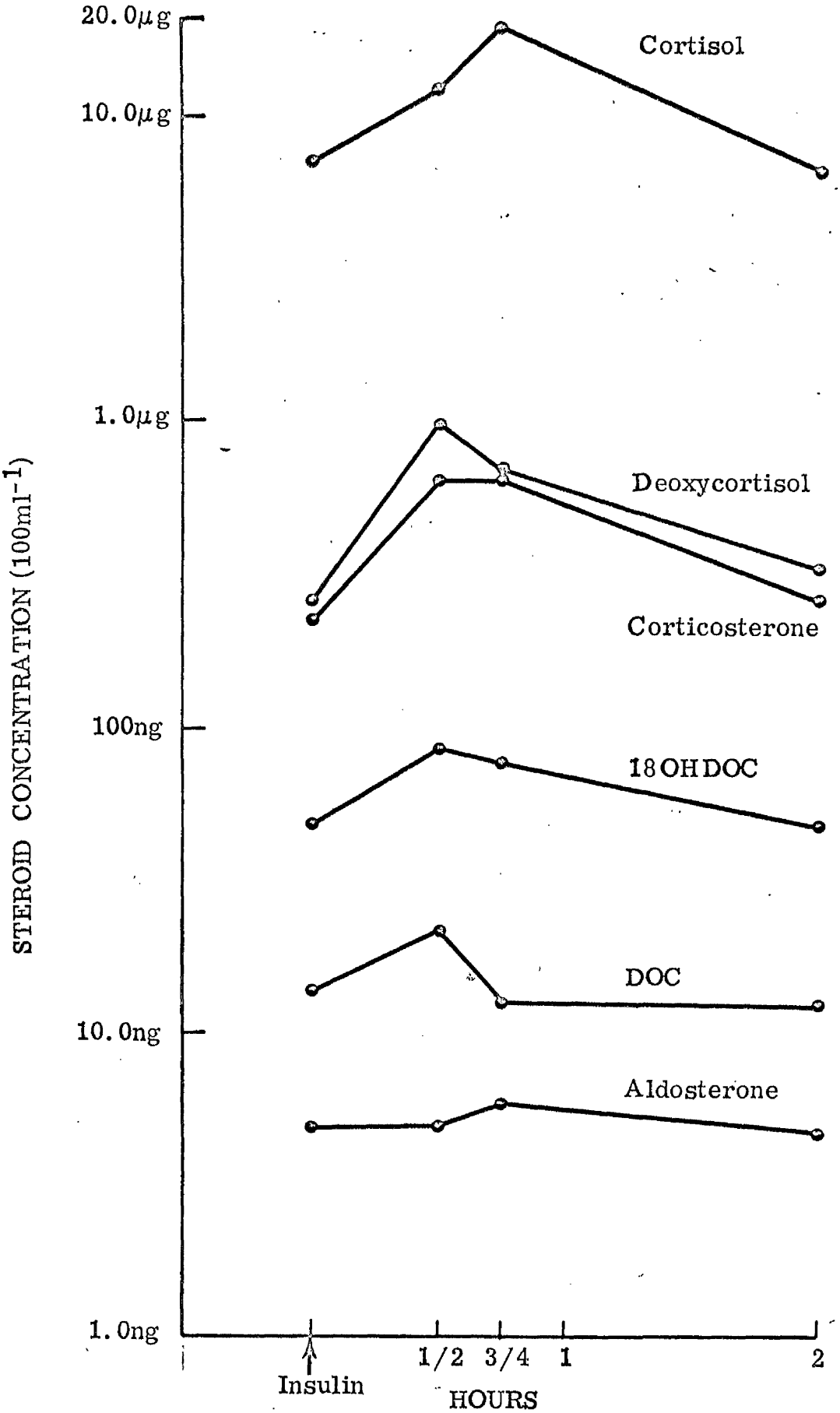


Fig. 15 INSULIN STRESS TEST (Means of 2 subjects)



(iii) Long-term stimulation by ACTH from a carcinoma.

Table 8

Plasma corticosteroid concentrations in two male subjects with ACTH-secreting bronchial carcinoma.

Compound	(TH)	(EH)	Upper normal limit
Cortisol	123	37	10 $\mu g\ 100\ ml^{-1}$
11-deoxycortisol	401	325	400 $ng\ 100\ ml^{-1}$
Corticosterone	3.6	0.8	0.8 $\mu g\ 100\ ml^{-1}$
DOC	106	84	18 $ng\ 100\ ml^{-1}$
18.OH DOC	322	311	160 $ng\ 100\ ml^{-1}$
Aldosterone	2	13	18 $ng\ 100\ ml^{-1}$

Table 8 shows the results obtained after analysis of plasma from two patients. In both patients plasma ACTH was grossly elevated (approximately 20 x normal) and plasma K^+ was 1.0mEq/l(T.H.) and 2.3 mEq/l(E.H.) where normal values are 3.7 mEq/l.

Patient/

Patient T.H. appears to be the more severe case both in terms of steroid levels and hypokalaemia. It is particularly interesting to note that in this case there is suppression of aldosterone in the face of very high ACTH levels.

3.2.2.3 DISCUSSION

Introduction

As can be inferred from the introductory survey to this section, there is already much information available concerning the effect of ACTH, administered in a number of ways, on the secretion rates and plasma concentrations of the corticosteroid hormones. The experiments just described can therefore be used to assess further the usefulness of the GLC methodology described in section 1. However, since few if any, workers have described simultaneous measurements of so many compounds, the results may have an additional value by allowing comparison of the ACTH influence on individual hormones.

Effect of absence of ACTH

Normal subjects were studied after a lengthy supine period and after ingestion of dexamethasone during the preceding 12 hours. This corticosteroid analogue suppresses the secretion of ACTH-dependent compounds (see section 3.2.1, d). The situation is somewhat complicated by the effect of supine posture on renin secretion and angiotensin II release (see section 3.3), which, together with alterations in metabolic clearance rates resulting from altered splanchnic blood flow, may affect the plasma concentrations of some steroids. This will be discussed more fully in the next section.

As/

As can be seen from the results of the experiments, dexamethasone led to a fall in the concentrations of all those compounds reported to be ACTH-dependent. Concentrations were at the lower limits of normal or subnormal. The low levels of 18.OH DOC in this situation are in good agreement with the urinary data of Melby et al (1972).

The concentration of aldosterone was also depressed although it is probable that a long period in the recumbent position may have been responsible for this. Nevertheless, as discussed in the literature survey, a role for ACTH in the control of aldosterone secretion cannot be excluded since both normal subjects and subjects with aldosterone-secreting adrenocortical adenomata exhibit patterns of aldosterone secretion akin to that of cortisol. Schalekamp (1975), has recently noted that lower, although still above normal, plasma aldosterone concentrations in patients with primary hyperaldosteronism can result from dexamethasone therapy. Thus, the low aldosterone concentrations in the normal subjects described here may have been partly due to dexamethasone administration.

An important consideration in the interpretation of the results of the ACTH infusion experiments is the effect of dexamethasone on the sensitivity of the adrenal cortex to ACTH. It/

It is possible that dexamethasone-suppressed subjects may respond less swiftly (i.e. may have a higher threshold response) or may produce a smaller increment of steroid secretion per increment of ACTH (i.e. be less sensitive) than untreated subjects. Williams, Rose, Dluhy, Dingman and Lauler (1971) state that cortisol response, but not that of aldosterone, may indeed be reduced in dexamethasone-treated subjects but this is disputed, at least where short-term treatment is concerned, by Kem et al (1971, 1975). These latter authors found that treatment with dexamethasone for 24 hours prior to study, a period twice as long as that used in the studies reported in this thesis, did not markedly alter the response of cortisol and aldosterone concentration to ACTH as compared with untreated subjects. Thus, in addition to indicating the importance of ACTH-support for adrenocortical secretion in man, pretreatment of subjects with dexamethasone is an ideal procedure for obtaining low, uniform resting levels of corticosteroids prior to ACTH infusion.

ACTH and corticosteroid secretion

Endogenous release of ACTH, induced by insulin administration, caused the expected increases in the plasma concentrations of the steroid hormones from the zona fasciculata but had little effect on aldosterone concentration.

No/

No ACTH concentration values are available but the level of hypoglycaemia attained satisfied the criteria of Greenwood, Landon and Stamp (1966), for assessment of adrenocortical function. It is clear from the results in Fig. 15 that a rise in the plasma concentrations of cortisol, deoxycortisol, corticosterone, deoxycorticosterone and 18.OH DOC occurred during the experiment and that this change was probably due to altering secretion, since posture, a major factor in metabolic clearance, was unaltered during the experiments. Although it would be unwise to attempt to draw valid conclusions from only two experiments it is interesting to note that the maximum concentrations of the 11-deoxycorticosteroids occurred earlier than that of cortisol. The position of the maximum concentration of corticosterone was equivocal. One possible interpretation of this is that the 11-hydroxylation step may be rate limiting. In agreement with previous reports, insulin hypoglycaemia in sodium replete subjects is a poor stimulus to aldosterone secretion, although a small (18%) rise occurred in both subjects.

Studies of endogenous ACTH release were also the aim of the experiment summarised in Figure 14. Samples of plasma from normal subjects were taken when ACTH release was high (i.e. early morning) and low (i.e. late evening and again towards midday). Again, highly significant changes occurred in/

in the concentrations of all steroids except aldosterone and deoxycortisol and these changes were positively correlated with expected changes in ACTH levels. The pattern of mean deoxycortisol levels also followed the rhythm of ACTH, but individual variation, particularly at 2300 hours, meant that statistical significance ($p < 0.05$) was not achieved. While no comparable studies of deoxycortisol are available, the behaviour of the remaining zona-fasciculata synthesised compounds are in keeping with previously reported studies (e.g. Wilson, 1973, Underwood and Williams, 1972, Newsome, Clements and Borum, 1972). The diurnal variation of 18.OH DOC concentration has not previously been reported.

The changes in plasma aldosterone concentration in this experiment (Fig. 14) may throw some light on the relative importance of ACTH in the control of aldosterone secretion. Plasma concentration fell across overnight recumbency in direct contrast to the changes in the other corticosteroids and ACTH itself. Concentration then rose markedly between early morning (recumbent) and midday (ambulant), again in contrast to other compounds. The controlling factor here is almost certainly posture which alters both metabolic clearance rate and also renin release (Love, Brown, Chinn, Johnson, Lever, Park and Robertson, 1971). It would seem therefore, despite the recent resurgence of interest in the anterior pituitary - aldosterone relationship, that ACTH

is/

is a rather minor factor in the control of aldosterone secretion at least when ACTH concentration is within the physiological range and when the subject is sodium replete.

The main experiments in this section were designed to compare the dose-response relationships of the various corticosteroid hormones and ACTH. Preliminary control experiments showed that dextrose infusion did not affect plasma steroid levels and that a state approximating to equilibrium was attained by infusing ACTH at a constant rate for 1 hour (Fig. 10). At a rate of infusion of 10 μg hr^{-1}, aldosterone levels tended to fall one hour after an initial rise and this may have been due to the rises in concentrations of DOC and corticosterone (see later). The results have been assessed in terms of concentration (Fig. 11) and change in concentration compared with resting levels (Figs. 12 and 13).

Figure 11 shows that, of the typically ACTH dependent compounds, only the cortisol response reaches a plateau within the range of ACTH doses used here. This range of infusion rates, according to Kem et al (1975) would cover the normal range of ACTH (rates up to 0.8 μg hr^{-1}) and above and the total range of cortisol response therefore occurs within the physiological range in these experiments. Response of deoxycortisol concentration also seems to be reaching/

reaching maximum levels. On the other hand, the gradient of the corticosterone, DOC and 18.OH DOC dose-response graphs remains steep even at the highest doses used here and pharmacological doses of ACTH may have to be administered before maximal response is achieved. This disparity of ACTH dose-response relationship between cortisol and corticosterone accounts for the falling cortisol: corticosterone ratios in stress and ACTH therapy previously reported (Krum and Glenn,1965; Fraser, 1967). From the data, it is obvious that a similar change in the ratio of cortisol and DOC concentrations must also occur.

The changes in aldosterone concentrations are smaller and more difficult to interpret. The failure to obtain a further increase in plasma concentration at the highest levels of infusion may have been due, either to attaining the maximum response at the lower rates or to the suppression of the response by the increasing concentrations of the minor mineralocorticoids. There is no doubt that elevated levels of DOC, for example, can suppress aldosterone secretion (Shade and Grim, 1975), although whether this can occur acutely in a period of 2 - 3 hours is not clear.

This also appears to be the explanation for the results of the analyses of plasma from subjects with bronchial carcinoma. Here, excess mineralocorticoid activity is reflected in the patients'/

patients' hypokalaemia (see section 4.1), but aldosterone is low or low normal. Presumably hypokalaemia is attributable to excess DOC and/or corticosterone, and aldosterone secretion is suppressed by these compounds.

When the proportional changes in steroid plasma concentrations are compared (Fig. 12), the remarkable change in the pattern of secretion is immediately apparent. The rate of increase of cortisol concentration within the lower range of ACTH levels was much steeper than those of DOC, 11-deoxycortisol and 18.OH DOC while that of corticosterone was intermediate. However, at infusion rates of 0.8 $\mu g\ hr^{-1}$ and above, while the rate of change in cortisol concentration was steady, there was a marked acceleration in the effects on DOC and corticosterone. The proportional effects on deoxycortisol, 18.OH DOC and aldosterone were much less dramatic.

The implications of this observation may be far-reaching if taken together with the following independent observations:

(a) Treatment of arthritis and related inflammatory diseases with ACTH instead of steroids such as prednisolone frequently leads to hypertension or oedema, presumably related to disturbances of electrolyte matabolism (Treadwell, Sever, Savage and Copeman, 1964; Irvine, Toft, Wilson, Fraser, Wilson, Young, Hunter, Ismail and Burger, 1974).

(b)/

(b) Frequent emotional stress is often blamed for the onset of hypertension.

(c) Rats develop hypertension when given periodic injections of DOCA, even though the plasma concentrations of DOC may be above the normal range for only a small fraction of the total time of the experiment (Hall and Hall, 1965; Beilin, Wade, Honour and Cole, 1970; Wilson, 1973).

Thus, although its effects on aldosterone secretion may not be marked, ACTH could possibly exert a profound influence on electrolyte metabolism and thus fluid balance and blood pressure by altering the secretion rates of the zona fasciculata-synthesised steroids. Moreover, such an effect may have a more profound effect than physiologically induced increases in aldosterone secretion because, whereas aldosterone secretion may be suppressed by the subsequent sodium retention, the secretion rates of corticosterone, DOC and possibly also 18.OH DOC do not respond to sodium status. Thus total mineralocorticoid status is more likely to become inappropriate to the prevailing sodium status.

In summary, from these experiments, ACTH is a major factor in the control of corticosteroid levels but its effect varies markedly from compound to compound, so that as the concentration of ACTH increases, the total mineralocorticoid component becomes more important.

3.3 ANGIOTENSIN II (AII)

3.3.1 Literature survey

Angiotensin II is an octapeptide found in small quantities in the plasma of normal human subjects. It has extremely powerful pressor properties, acting on arterial smooth muscle and it is also claimed to affect ADH and catecholamine secretion, renal function and, most relevant to this thesis, corticosteroid secretion.

(a) Angiotensin formation

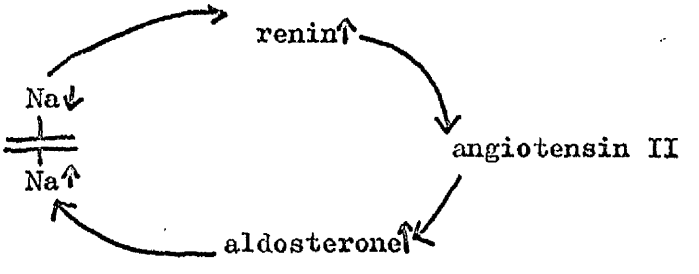
The peptidase, renin, is released from the juxtaglomerular region of the nephron in response to changes in sodium and water metabolism. The precise stimulus to release is a subject of some controversy, but seems likely to be either a change in the perfusion of the kidney, perceived by the afferent arteriolar stretch receptors or a change in the osmolality of the renal tubular fluid, influencing in some way the macula densa (Davis, 1973; Thurau, Dahlheim, Gruner, Mason and Granger, 1972). Renin, which exists at least partially in renal tissue as an inactive precursor, prorenin (Leckie, 1973), must be activated by removal of a peptide fragment either at the time of release or in the circulation (Leckie and McConnell, 1975). In the plasma, renin catalyses the hydrolysis of a leucyl-leucine bond in an α_2 globulin, manufactured/

manufactured in the liver, and thus releases an inactive decapeptide, angiotensin I.

Two further amino acids are then removed during passage through the lungs and other peripheral tissues. The resulting active octapeptide is short-lived, being destroyed by various peptidases collectively called angiotensinases. Recently, interest has been shown in the activities of the larger fragments of angiotensin II, the heptapeptide (angiotensin III) and the hexa - and penta-peptides but the biological importance of these substances is as yet a subject for conjecture (Semple and Morton, 1975 (a) and (b)).

(b) Angiotensin II and aldosterone secretion

As discussed in section 2, ACTH, the major influence in the control of adrenocortical activity, has relatively little effect on aldosterone secretion. As the main target organ of aldosterone, the kidney was thought by several early workers in this field to be a logical source of control of secretion. Many comprehensive reviews of the evidence for a renal-adrenal relationship are available (Fraser et al, 1969; Bartter, Delea, Kawasaki and Gill, 1974; Gross, 1958; Brown, Fraser, Lever and Robertson, 1972), and the evidence is summarised below -



It/

It is suggested that during sodium depletion renin release is stimulated (Brown, Davies, Lever and Robertson, 1966), which raises angiotensin II concentration in plasma. This may cause increased aldosterone secretion, sodium retention and finally, inhibition of further renin release.

In favour of this, nephrectomy, removing the source of renin, leads to a dramatic fall in the secretion rate of plasma concentration of aldosterone in animals and man (Gleadle, Brown, Curtis, Fraser, Lawson, Lever, Linton, McVeigh, Robertson, de Wardener and Wing, 1969), but in the dog, levels can be restored by infusion of extracts of kidney containing renin (Davis, Carpenter, Ayers, Holman and Bahn, 1961). Further evidence of a renal humoral factor was obtained from classical cross-circulation experiments in the dog, where nephrectomised and intact dogs were connected (Davis, Urquhart and Higgins, 1963), If the above scheme is tenable, renin and angiotensin II concentrations should move in parallel. There is in fact a good correlation between renin and both angiotensin I and angiotensin II (Morton, Waite, Brown, Lever, Robertson and Semple, 1975). Again, according to the scheme, changes in plasma renin or plasma angiotensin II concentration should be accompanied by parallel changes in aldosterone secretion or plasma concentration. There/

There are many examples of studies of this type and some are listed below:-

(i) dietary sodium deprivation of normal subjects or acute natriuresis induced by diuretics such as frusemide lead to rises in both renin and aldosterone concentrations in plasma (see Fraser, James, Brown, Isaac, Lever and Robertson, 1965: Beevers, Brown, Fraser, Kremer, Lever, Morton, Robertson, Schalekamp, Semple and Wilson, 1975).

(ii) severe sodium depletion in a subject with sodium-losing renal disease caused massive increases in the plasma concentrations of both substances (Fraser, James, Brown, Davies, Lever and Robertson, 1966). Thus the changes in renin and aldosterone secretion appear to be in direct proportion.

(iii) a good correlation is obtained if aldosterone and renin concentrations in a wide variety of disease conditions, including renal disease but excluding primary hyperaldosteronism (see below) are compared (Fraser, Brown, Chinn, Lever and Robertson, 1969; Laragh, Sealey and Sommers, 1966).

(iv) in a case of hypertension due to a renin-secreting renal tumour, plasma aldosterone concentrations were high and fell when the tumour was removed (Brown, Fraser, Lever, Morton, Robertson, Tree, Bell, Davidson and Ruthven, 1973).

(v)/

(v) in a case of severe hyperkalaemia, undetectable levels of aldosterone in plasma were accompanied by very low levels of renin and angiotensin II. None of these substances were affected by sodium deprivation but plasma aldosterone concentration rose in response to angiotensin II infusion (Brown, Chinn, Fraser, Lever, Morton, Robertson, Tree, Waite and Park, 1973).

Finally, infusion of non-pressor or mildly pressor doses of angiotensin II into normal subjects causes a marked increase in secretion rate (Ames, Borkowski, Sicinski and Laragh, 1965) or plasma concentration of aldosterone (Fraser et al, 1965) whereas infusion of inhibitors of angiotensin II into subjects with hypertension and hyperaldosteronism secondary to high renin secretion leads to a fall not only in blood pressure but also in aldosterone secretion (Davis, 1975).

Again, examining the hypothesis summarised in the above diagram, it is logical to conclude that an increase in aldosterone levels as a result of exogenous administration or endogenously for reasons other than renal changes, renin and angiotensin II concentrations will be suppressed. An excellent example of this is the condition of primary hyperaldosteronism (Conn, 1955) in which a primary adrenocortical lesion, usually a benign adenoma, secreting aldosterone/

aldosterone autonomously, causes hypokalaemia, hypertension and suppression of renin and angiotensin II production.

It does not, therefore, seem unreasonable to conclude that angiotensin II and thus renin and the kidney, have an important role to play in the function of the zona glomerulosa although a number of reservations must be made. A part of the increase in plasma concentration following angiotensin II infusion may be due to a fall in metabolic clearance rate (Balikian, Brodie, Dale, Melby and Tait, 1968; McCaa, Read, Cowley, Bower, Smith and McCaa, 1973). However, this is unlikely to account for a major component of the rise, firstly as direct studies of secretion rate confirm an adrenal effect (Blair-West, Cain, Catt, Coghlan, Denton, Funder, Nelson, Scoggins, Wintour and Wright, 1968), and secondly because recent studies of live zona glomerulosa cells show that angiotensin II is capable of increasing aldosterone synthesis in vitro (Haning, Tait, Tait and Williams, 1971).

There are a number of situations in which the correlation between renin or angiotensin II and aldosterone is less clear cut or absent. For example, during total starvation therapy for obesity, renin is low but plasma aldosterone concentrations respond in a manner expected of a sodium-depleting situation (Chinn, Brown, Fraser, Heron, Lever, Murchison and Robertson, 1970). Obviously other factors must be responsible for this rise.

(c)/

(c) Sodium status and the effect of angiotensin II

At first sight, the pressor and aldosterone-stimulating effects of angiotensin II seem contradictory. Increases in blood pressure will tend to be natriuretic, by increasing GFR, while increased aldosterone secretion will mediate towards sodium retention. The answer to this paradox lies in the modification of the effects of angiotensin II by sodium status itself. Blood pressure in subjects replete or loaded with sodium is highly sensitive to angiotensin II, whereas subjects depleted of sodium are refractory to the polypeptide. The dose-response relationship between angiotensin II and plasma aldosterone is also dependent upon sodium status (Oelkers, Brown, Fraser, Lever, Morton and Robertson, 1974; Hollenberg, Chenitz, Adams and Williams, 1974). The slope is steeper and the threshold dose lower in sodium deplete subjects than in replete subjects.

Thus, sodium status appears to act as a 'switch' emphasising sodium retaining or excreting processes as required for homeostasis. The mechanism by which sodium status exerts its effect is by no means clear. One possible explanation is that during sodium depletion circulating levels of angiotensin II may be raised for an extended period and may cause hypertrophy of the zona glomerulosa. Thus more target organ tissue will exist to respond to the trophic substance and the dose-response curve/

curve will steepen. Evidence that such a mechanism may at least contribute to the sensitisation process has been obtained by Oelkers, Schöneshöfer, Schultze, Brown, Fraser, Morton, Lever and Robertson (1975) who replaced a sodium-deprivation regime with a lengthy period of angiotensin II infusion in sodium replete normal human subjects, maintaining sodium balance constant. When the dose response relationship was then tested, it was found to be steeper than that obtained in normal replete subjects but less steep than in subjects after dietary sodium deprivation.

Another possible locus for the intervention of sodium in aldosterone secretion is a direct effect on biosynthesis. As far as the author is aware, no direct studies of sodium status or osmolality have been carried out in vitro. The immediate precursor of aldosterone is thought to be 18-OH-corticosterone. This steroid exists in at least two (L & M) forms of which the L form can be more readily converted to aldosterone in vitro. There is some preliminary evidence that the position of the equilibrium between the isomers can be modified by pH and possibly also by osmolality (Lantos, 1975). If this is indeed true, then aldosterone secretion could be controlled by making more or less of the active/

active form of 18.OH-corticosterone available for oxidation to aldosterone. More simply, sodium depletion may affect 18-hydroxylation making a larger quantity of 18-hydroxy-corticosterone available for conversion to aldosterone.

(d) Effect of angiotensin II on other corticosteroids.

Studies of the locus of action of angiotensin II in the biosynthetic pathway seem to indicate, like ACTH (see section 3.2.1), the main effect occurs early in the conversion of cholesterol to pregnenolone (Müller, 1965, (a) and (b); Davis, Burwell, Kelley, Casper and Bartter, 1966; Baniukiewicz, Brodie, Flood, Motta, Okamoto, Tait, Tait, Blair-West, Coghlan, Denton, Goding, Scoggins, Wintour and Wright, 1967). Müller and Bauman (1973) also postulate a second locus of action in the series of reactions in which corticosterone is converted to aldosterone. However, if there is indeed an early effect then it would be expected that angiotensin II would have a rather non-specific effect on corticosteroid secretion. Although Slater, Barbour, Henderson, Casper and Bartter (1965) have shown that cortisol and corticosterone concentrations may, like aldosterone, increase in response to angiotensin II infusion, this does not seem to be the case in man. For example, cases of hyperaldosteronism secondary to hyper-reninism do not often show increases in cortisol levels.

Fraser/

Fraser et al (1965) found no increase in cortisol or corticosterone levels in angiotensin II-infused normal subjects. The possibility that any angiotensin-mediated rise in 11-hydroxycorticosteroid concentration in plasma would be counteracted by ACTH suppression via the negative feedback system was excluded by pretreating the experimental subjects with dexamethasone (Fraser, 1967). The case for a role of angiotensin II in the control of corticosteroid secretion other than that of aldosterone is not proven.

In the following experimental section the effects of angiotensin II have been examined in dogs, and the effects of posture in normal human subjects.

3.3.2 EXPERIMENTAL

3.3.2.1 Methods

(a) Effect of exogenous angiotensin II

(i) Two normal dogs on unrestricted diet with exteriorised carotid artery loops, were infused with 0.5% dextrose solution at a rate of 15 mls per hour for four hours. Arterial blood samples were taken hourly.

(ii) A group of four similar normal dogs, were infused with $Ileu^5$ angiotensin II (hypertensin, Ciba) in 0.5% dextrose solution at rates of 3, 6, 12 and 24 ng/kg/min, for one hour at each rate. As above, arterial blood samples were taken initially and after each hour of infusion.

(b) Effect of endogenously released angiotensin II: posture

The effect of posture on plasma corticosteroid levels was studied in five normal human subjects on unrestricted diet. An indwelling cannula was inserted in a peripheral arm vein. The subjects remained supine on a tilt table for 2 hours and then were tilted to an upright position for a further two hours. This was followed by a further two hours in a supine position. Blood samples were taken at the end of each hour.

3.3.2.2 RESULTS

(a) Effect of exogenous angiotensin II (Figure 16)

During infusion of angiotensin II, the only steroid concentration to change significantly ($p < 0.001$) was that of aldosterone. This rise can be seen more clearly in figure 17 where a percentage change in steroid concentration is plotted against rate of angiotensin II infused. The blank infusion had no appreciable effect on the plasma concentractions of any of the steroids measured (Table 9).

(b) Effect of endogenously released angiotensin II (Fig. 18)

Plasma levels of aldosterone rose on assumption of the erect posture ($p < 0.02$). The small rise seen in DOC levels was insignificant at the 95% level.

Once again the rise in steroid concentration is plotted against rate of infusion (Figs. 18 and 19).

Table 9 Effect of infusion of 0.5% dextrose solution i.v. (10 ml/hour) on steroid concentrations (means of two animals).

Time (hrs)	DOC ng/100 ml	Corticosterone µg/100 ml	Deoxycortisol ng/100 ml	Cortisol µg/100 ml	Aldosterone ng/100 ml	18-OH-DOC ng/100 ml
0	14.6	0.113	133.2	0.96	5.2	34.3
1	13.9	0.127	174.7	1.6	5.6	40.5
2	14.7	0.102	196.5	1.31	6.0	52.6
3	14.8	0.087	180.0	1.3	7.0	54.7
4	15.6	0.110	187.5	1.83	7.0	48.1

Fig. 16

EFFECT OF INFUSION OF ANGIOTENSIN II (1-Asp(NH_2)-5-Val AII) (means of 4 values)

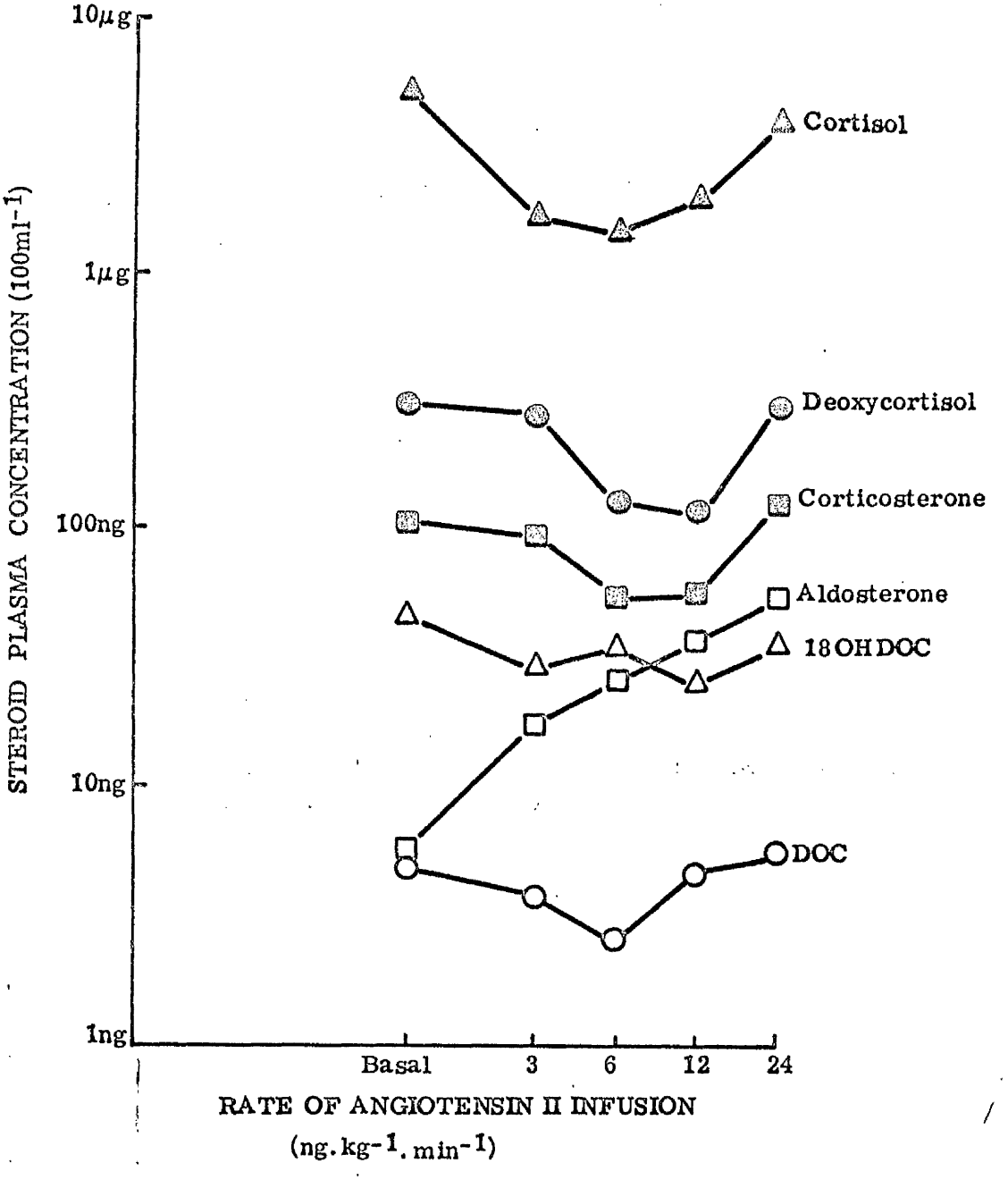
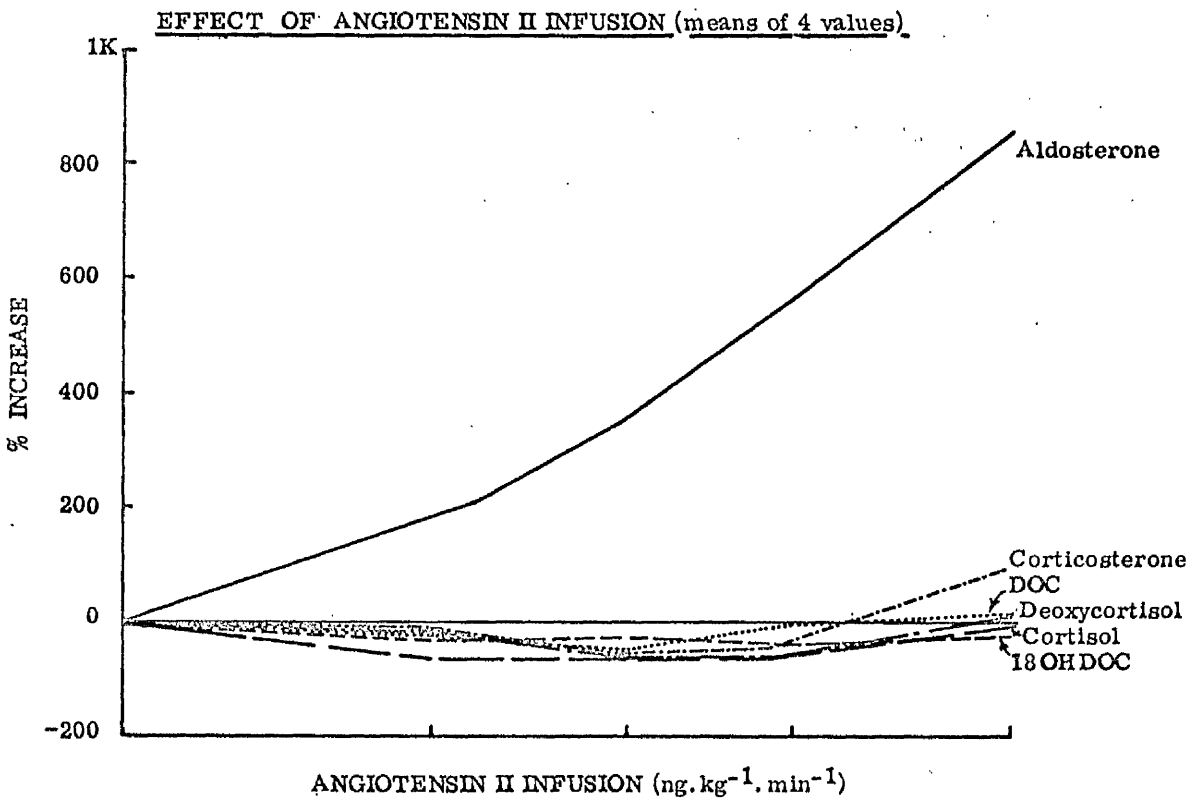


Fig. 17


EFFECT OF ANGIOTENSIN II INFUSION (means of 4 values)
1K
800
600
400
200
0
-200
% INCREASE
Aldosterone
Corticosterone
DOC
Deoxycortisol
Cortisol
18 OH DOC
ANGIOTENSIN II INFUSION (ng.kg^{-1}.min^{-1})

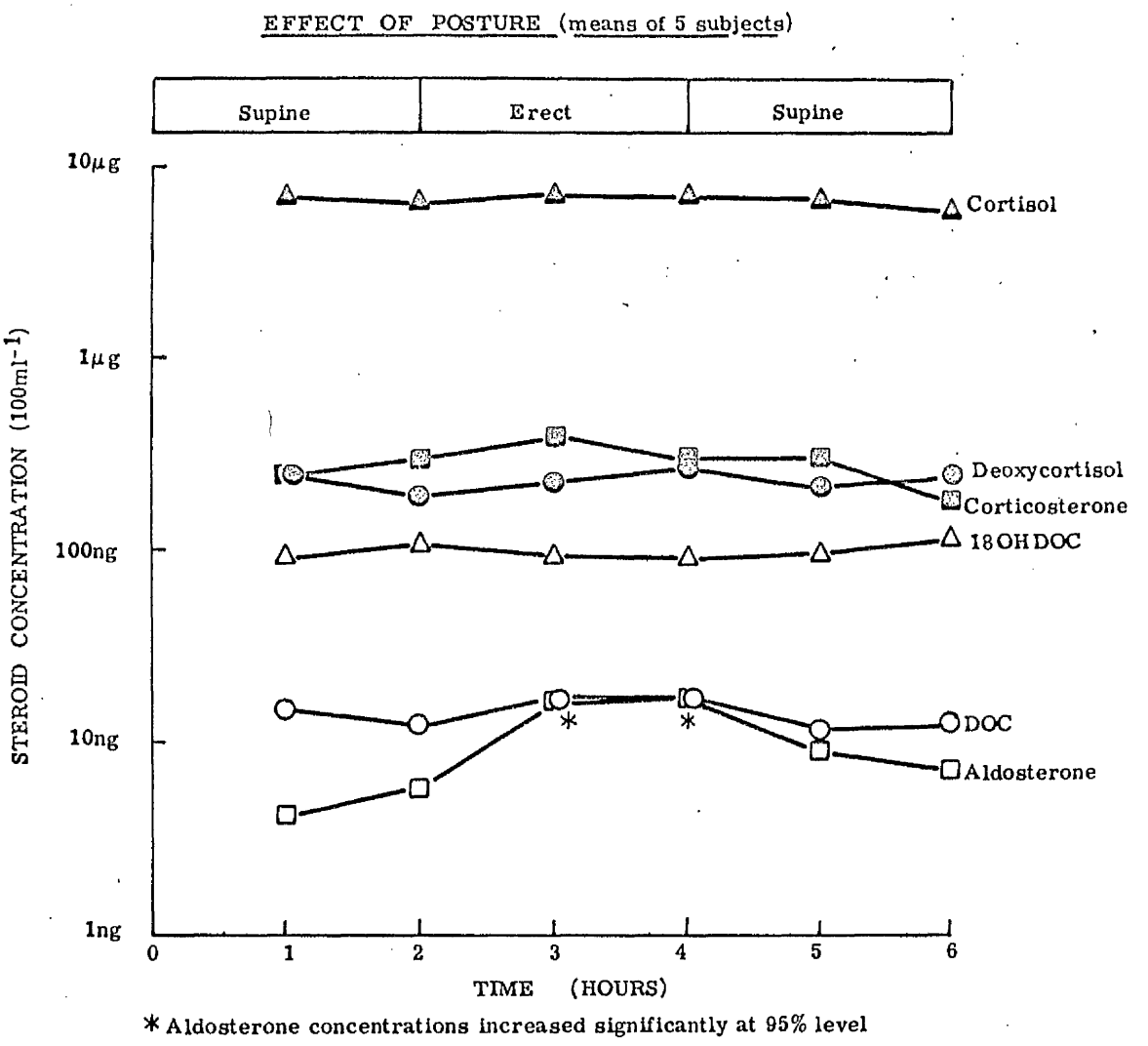
Effect of Angiotensin II infusion

Means* ± SEM

Compound	DOC	Corticosterone	Deoxycortisol	Cortisol	Aldosterone	18 OH DOC
Rate of infusion of AII ($ng\ Kg^{-1}\ mm^{-1}$)						
3	-21±11	-9±12	-18 ± 12	-63±19	209±23	-37±12
6	-48±17	-57±13	-63±13	-70±18	351±25	-28±12
12	-7±12	-48±20	-64±13	-64±16	559±27	-43±11
24	13±9	92±11	9±9	-10±17	857±34	-24±12

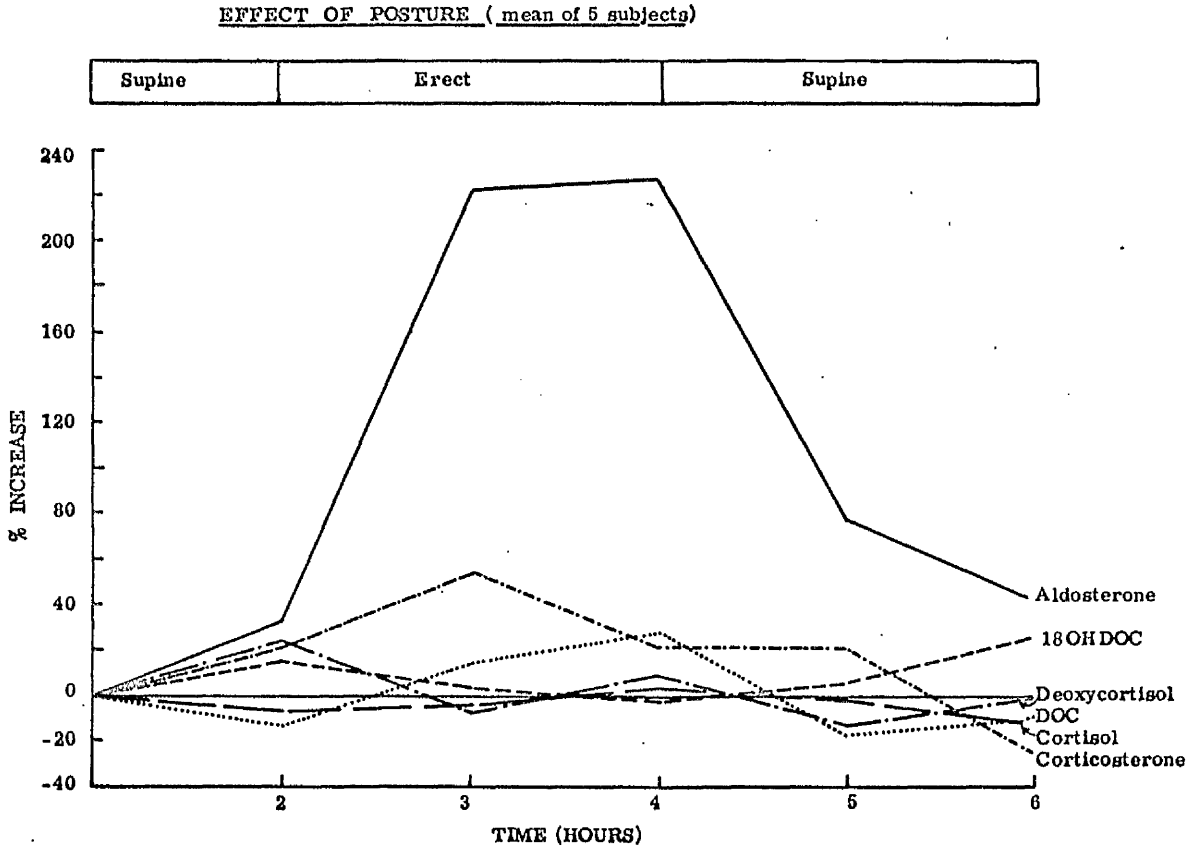
* Means of percentage changes in plasma concentrations are compared to basal (1 hr) values.

Fig. 18



* Aldosterone concentrations increased significantly at 95% level

Fig. 19


EFFECT OF POSTURE (mean of 5 subjects)
Supine
Erect
Supine
240
200
160
120
80
40
0
-20
-40
% INCREASE
2
3
4
5
6
TIME (HOURS)
Aldosterone
18OH DOC
Deoxycortisol
DOC
Cortisol
Corticosterone

Effect of Posture

Means* ± SEM

Compound	DOC	Corticosterone	Deoxycortisol	Cortisol	Aldosterone	18 OH DOC
Time (hrs)						
2	-12±13	22±31	24±23	-6±9	35±8	15±31
3	17±11	54±11	-7±24	-4±11	223±11	3±28
4	28±20	21±21	9±19	3±8	228±15	-3±19
5	-18±12	21±15	-14±11	2±8	78±16	5±12
6	-12±9	26±10	-2±16	-13±9	42±19	26±29

* Means of percentage changes in plasma concentrations are compared to basal (1 hr) values.

3.3.2.3 DISCUSSION

Angiotensin II caused an increase in plasma aldosterone concentration but failed to alter the concentrations of the remaining corticosteroids. The effect on aldosterone is in agreement with previously published work on secretion (Genest, Koiw, Nowaczynski and Sandor, 1960; Laragh, Angers, Kelly and Lieberman, 1960; Ames et al, 1965) and plasma concentration (Fraser, James, Isaac, Lever and Robertson, 1965). The lack of response of cortisol and corticosterone concentration contrasts with the results of Slater et al (1965) in the dog but confirms the work in human subjects of Fraser et al (1965).

Angiotensin II may alter plasma concentration of aldosterone in two distinct ways; by directly influencing biosynthesis at the cellular level and by changing the rate of catabolism by lowering metabolic clearance rate (MCR) (Tait, Little and Laumus, 1961). If changes in MCR due to changes in hepatic blood flow were the major factor, then rises in the plasma concentration of all corticosteroids, which are largely metabolised by the liver, would be expected although the concentration of those dependent upon ACTH secretion may then be readjusted by means of the negative feedback mechanism (see section 3.2.1). That this is probably not the case in man is shown by the fact that angiotensin II/

angiotensin II fails to increase the plasma concentration of cortisol and corticosterone in dexamethasone-suppressed normal subjects (Fraser, 1967).

It is also difficult to explain the apparent specificity of the action of angiotensin II by studies of its biosynthetic effect. An influence early in the biosynthetic pathway, similar to that of ACTH, seems generally agreed (Ganong, Boryczka and Shackleford, 1967) although a second point of control, between corticosterone and aldosterone has also been suggested (Müller and Baumann, 1973). In vitro, in rat adrenal zona glomerulosa cell preparations, angiotensin II is indeed a non-specific trophin, causing increases in corticosterone production as well as that of aldosterone (Tait, Tait, Gould and Mee, 1974; Williams, McDonnell, Raux and Hollenberg, 1974).

A possible solution to this paradox lies in the relationship of sodium status of the subject and the response of aldosterone secretion to angiotensin II infusion. Plasma concentration of aldosterone rises more markedly to a given dose of angiotensin II in sodium deplete than in sodium replete subjects. This apparent sensitisation of aldosterone secretion by sodium deprivation also occurs when/

when ACTH stimulation is used instead of angiotensin II (Ganong et al, 1966; Csanky, van der Wal and de Wied, 1968; James et al, 1968). Both ACTH and angiotensin II increase aldosterone production in vitro. It seems possible that the relative lack of sodium may potentiate the conversion of late precursors such as corticosterone or 18-hydroxycorticosterone to aldosterone and that these late precursors may increase as a direct result of an angiotensin II effect earlier in the biosynthetic pathway. Sodium may act directly in this way or by altering intracellular potassium status, a parameter said to influence aldosterone biosynthesis (Cannon, Ames and Laragh, 1966; Baumber, Davis, Johnson and Witty, 1971; Denton, Goding and Wright, 1959; Davis et al, 1963; Sharma, Nerenberg and Dorfman, 1967).

However, in man when changes in plasma concentration are considered, it seems safe to conclude that within the physiological range, angiotensin II has a specific effect on aldosterone.

4. ADRENAL AND PERIPHERAL VENOUS CORTICOSTEROIDS IN PRIMARY HYPERALDOSTERONISM

4.1 INTRODUCTION

A small proportion of subjects with hypertension have associated hyperkalaemia, increased total exchangeable sodium and a metabolic alkalosis. These symptoms are frequently found to be due to a benign adrenocortical adenoma (Conn, 1955) which secretes aldosterone. In these subjects therefore peripheral plasma levels (Brown, Chinn, Davies, Düsterdieck, Fraser, Lever, Robertson, Tree and Wiseman, 1968; Cope, 1964) of aldosterone are raised but no abnormalities in the behaviour of other corticosteroids has been demonstrated (e.g. Conn, 1955). However, in vitro incubation of tumour tissue from these subjects shows that it is capable of synthesising a wide spectrum of corticosteroids (Bailey, Slade, Lieberman and Luetscher, 1960; Conn and Conn, 1961; Louis and Conn, 1961; Brode, Grant and Symington, 1962; Luetscher, 1962; Fazekas, Webb and Symington, 1966), and, while ability to synthesise is not synonymous with secretion, it is possible that the tumour may contribute to the circulating levels of steroids other than aldosterone. During the clinical procedures for diagnosis and localisation of an adrenocortical adenoma, it is necessary to obtain bilateral adrenal vein blood samples for corticosteroid analysis (Kahn, 1967; Melby, Spark, Dale, Egdahl and Kahn, 1967). The opportunity was therefore taken to compare the concentrations of/

of the major corticosteroids in the venous effluent blood from affected and unaffected adrenal glands.

4.2 EXPERIMENTAL

4.2.1 Method

1) Peripheral venous blood samples and samples of adrenal venous blood were collected from patients diagnosed as being likely to have primary hyperaldosteronism. After confirmation of the diagnosis by histology after operation, samples have been analysed and the results are shown in Tables 10 and 11.

4.2.2 Results

(a) Peripheral plasma steroid concentrations

The results obtained upon analysis of samples of peripheral blood from patients with primary hyperaldosteronism are shown in Table 10. The most striking abnormality upon comparison of these steroid concentrations with those of normal subjects is that in most cases the aldosterone values are elevated above the upper limit of the normal range. In fact the mean aldosterone concentration is approximately double this figure and is highly significantly elevated ($p < 0.001$).

The only other abnormal steroid concentration is that of DOC. This again is significantly raised ($p < 0.02$), when compared to the values obtained in a group of normal subjects under similar conditions.

Individual patients have some other steroid levels grossly elevated but the mean values for all patients are not significantly raised.

(b) Adrenal venous steroid concentrations

Analysis of adrenal venous blood from patients with primary hyperaldosteronism produced the results shown in Table 11. The difference between "normal" and tumourous adrenal glands is clearly seen. The steroid levels from the affected glands are on average at least five times as high/

Table 10

Peripheral plasma steroid concentrations in 12 subjects suffering from primary hyperaldosteronism.

	DOC ng/100ml	Corticosterone µg/100ml	Deoxycortisol ng/100ml	Cortisol µg/100ml	Aldosterone ng/100ml	18.OH.DOC ng/100 ml
1	15.1	0.46	65.4	14.2	18.8	72.7
2	10.8	0.31	143.2	11.2	23.5	100.3
3	-	0.44	89.3	10.1	73.0	41.5
4	20.1	0.12	48.1	8.0	58.9	41.9
5	36.8	0.02	79.6	5.7	59.6	27.7
6	17.5	0.09	32.2	7.1	7.1	34.6
7	17.7	0.16	114.2	8.6	7.8	33.9
8	20.0	0.05	55.7	3.2	43.6	48.4
9	16.5	0.12	63.0	4.0	24.3	69.2
10	51.2	0.71	725.2	13.3	11.1	70.3
11	4.3	0.27	58.2	15.1	67.2	39.3
12	24.1	0.05	21.0	10.3	41.3	40.6
Means	21.3*	0.23	124.6	9.2	36.3*	51.7

* Significant at 95% level when compared with results from 12 normal subjects (samples taken at same time of day)

Table 11

Concentrations of steroids in adrenal venous plasma of patients suffering from primary hyperaldosteronism.

	Adr. vein	DOC ng/100ml	Corticosterone µg/100 ml	Deoxy-cortisol ng/100ml	Cortisol µg/100ml	Aldosterone ng/100 ml	18.OH.DOC ng/100ml
13	L *	-	4.77	2865	32.1	3087	4602
	R	-	0.31	229	10.9	148	228
8	L *	222	0.61	119	13.8	1159	202
	R	32	0.06	92.4	9.3	83	66
9	L *	545	1.55	740.4	12.1	639	550
	R	139	0.81	330	13.5	62.8	96.9
4	L *	13127	196.0	19743	225.9	884	4844
	R	1921	24.4	2598	71.1	47	823
11	L*	399	3.30	1048	42.0	1834	253
	R	28	0.06	204	6.5	61	51.9
12	L	92	1.9	301	34.0	62	58.8
	R*	898	9.0	996	46.0	2740	253
10	L*	6250	25.6	3308	33.9	621	768
Mean (tumour)		3574	36.2	4117	57.6	1567	1640
Mean (non-tumour)		442	4.27	626	24.2	77.3	221
Mean difference tumour/non-tumour		8.3	8.05	5.6	2.72	23.1	7.3

* Site of lesion

high as from the other gland. In the case of aldosterone, the mean levels are approximately twenty times higher from the affected gland. However, with the other steroids there is much individual variation between the amounts secreted, as is the case with peripheral blood.

4.2.3 DISCUSSION

In the early work on estimation of steroid levels in adrenal blood from patients with primary hyperaldosteronism, Melby et al (1967) found that aldosterone levels were consistently very much higher from the tumour-affected than the normal gland. They also noted that cortisol levels were higher in 60% of cases on the tumour side. The present study has extended this work to another four steroids and it was found that in all cases studied, all steroid levels were higher on the tumour side than the unaffected side. However, the aldosterone levels were very much the highest of all those studied. Thus it seems that this type of lesion secretes predominantly aldosterone and while there is considerable oversecretion of the other steroids, this is reflected only in marginally increased DOC levels in the peripheral plasma.

The autonomous nature of the lesion may be part of the reason for the disparate steroid levels in the adrenal veins as excess glucocorticoid secreted by one gland will result in pituitary feedback (see section 3.2.1) and hence inhibition of ACTH release. Consequently the normal adrenal gland will show suppressed levels of those steroids whose production is controlled mainly by ACTH.

Similarly/

Similarly for aldosterone, the suppressed levels of renin and angiotensin II seen in the plasma of patients with primary hyperaldosteronism will obviously have little stimulatory effect upon the aldosterone producing cells of a normal adrenal gland.

Hence the steroid levels seen in the adrenal veins from unaffected glands of people suffering from this disease may well be suppressed far below those in normal human subjects. It is impossible however, on ethical grounds, to carry out the procedure of adrenal venous catheterisation in normal subjects.

One aspect of the study is that the site of the lesion can, in theory, be predicted with high accuracy. However, adrenal venous catheterisation requires extreme skill and on some occasions may not be possible at all, particularly with the right adrenal vein. Also, the adrenal venous blood may be diluted with blood from the inferior vena cava and so a false negative result could be obtained. In spite of these difficulties the results have proved useful clinically, particularly when the tumour is fairly small and difficult to visualise by radiography.

The technique may be of greater importance in allowing differentiation of adrenal hyperplasia from primary hyperaldosteronism/

hyperaldosteronism due to a tumour, since in the former case it is unlikely that aldosterone is over-secreted at a rate greater than the other steroids under study. However, cases of hyperplasia are much less common than adenomata and, as yet, none have become available for study in this way.

5. GENERAL CONCLUSIONS.

5. Gas-liquid chromatography as a technique for plasma analysis

The value of an analytical technique is usually assessed on a number of criteria, among which are specificity, sensitivity, accuracy and precision. These have been fully discussed in the thesis and it suffices to reiterate that GLC with electron capture detection performs as well as other ultra-sensitive techniques such as radioimmunoassay. Like these, however, it is certain that the full potential of GLC as a semi-routine technique in a clinical laboratory has not yet been realised. Much has to be done for example, in making maximum use of the very high degree of sensitivity offered by the method, lower limits well into the sub-picogramme range, by improving methods of protecting the electron-capture detector from non-specific contamination. A larger proportion of the sample extract could thereby be injected into the machine allowing smaller blood samples to be used. While, at the present state of the methods described in this thesis, blood sample volume has not presented any difficulties, studies have mainly been restricted to man. Smaller samples would enable the method to be used in small animal experiments, even allowing multiple sampling. Other ways in which technical improvements might be made is by investigating the use of derivatives of higher sensitivity and possibly also introducing a routine peak trapping procedure or direct

peak/

peak assessment for content of radioactivity so that (^{3}H) yield could be assessed after instead of before esterification.

An important factor in clinical laboratory methods is simplicity. It cannot be claimed that GLC with electron capture is a simple technique, because the chromatograph itself is a complex and often temperamental item of apparatus. Column preparation may also be unpredictable although in recent years the availability of prepared column phases or even packed columns, has simplified this aspect of the technique. However, much could be done to simplify the sample preparation prior to GLC and to automate the injection procedure. Some preliminary steps have already been taken in this direction and two examples may illustrate approaches to simplification. Severe oxidation of plasma neutral extracts converts some corticosteroids into other stable steroids, amplifying their polarity differences, destroys other corticosteroids and probably also destroys other contaminating molecules. It has been possible to reduce the purification stages for the 18-oxygenated corticosteroids aldosterone, 18-OH-DOC and 18-hydroxycorticosterone (Wilson, Mason and Fraser, 1976) by one paper chromatography using this approach. Many clinical laboratories require a rapid assay/

assay for plasma cortisol concentration based on competitive protein binding. GLC can offer an alternative to this, particularly as automatic, programmable injection modifications are now available for most gas-chromatographs. Small ($<$1 ml) plasma samples can be extracted, oxidised, esterified with HFB and automatically analysed by GLC. The capacity of such a method approaches that of competitive protein binding and the sensitivity is probably greater (Mason, Tremear and Fraser, 1976).

Another important criterion with which to assess the practicability of a technique is the ease with which a method for a new compound can be set up. Here the power of GLC is best assessed by comparison with radioimmunoassay. For this latter technique, the main requisite is a good antibody and production of this can be expensive of time and materials and the outcome can be unpredictable to say the least. In a multisteroid method such as described here, but using radioimmunoassay, such a procedure must be gone through for each compound. In contrast, for a group of similar compounds such as the corticosteroids, the same substituent will often suffice for each compound so that, once the initial problems have been overcome, the method is readily adaptable to other similar compounds.

In/

In the opinion of the author, therefore, in the almost religious fervour for the technique of radioimmunoassay, there is a danger that the advantages of the physico-chemical techniques in general and GLC in particular, may be forgotten.

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