MEASUREMENT AND MECHANISM OF CONTROL OF STEROID HORMONES.

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

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SUMMARY

Two methods of measuring peripheral plasma deoxycorticosterone (DOC) concentration have been developed using the techniques of gas-liquid chromatography with electron capture detection and radioimmunoassay. The reliability of these methods has been assessed and compared. A number of physiological studies have been carried out in order to further evaluate the techniques and also to investigate some factors controlling the peripheral plasma concentration of DOC.

Plasma DOC concentration was consistently raised by injection of synthetic β^{1-24} ACTH (adrenocorticotrophic hormone) or by increasing endogenous ACTH by insulin hypoglycaemia. Infusion of mildly pressor doses of angiotensin, dietary sodium depletion and change of posture did not affect plasma DOC concentration in normal subjects. However, in obese subjects, dietary sodium depletion was found to increase plasma DOC levels while total starvation caused a marked fall. Plasma DOC concentration was also elevated during normal pregnancy.

Plasma DOC concentrations were measured in a number of hypertensive subjects known to have low plasma concentration of renin and potassium and

normal or low aldosterone level. DOC levels were elevated in 6 out of 21 of these subjects. It is not known whether these levels maintained over a prolonged period of time would be sufficient to cause hypertension.

The concentration of DOC in whole blood was measured in rats given injections of DOC-pivalate or saline in order to investigate the suitability of the gas chromatographic technique for measurement of DOC in whole blood in the study of induced hypertension in the rat. Maximum DOC concentration in whole blood in rats receiving DOC pivalate was 10-12 times higher than the levels attained in saline injected rats and was achieved within 12 hours of injection.

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I. INTRODUCTION

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1.1. Biological Importance of Corticosteroids.

The control of electrolyte and water metabolism in the living organism is of fundamental importance to the function of animal tissues. Any factor which influences this metabolism is therefore of considerable interest. Early endocrinology revealed the importance of the adrenal cortices in this respect. In the mid nineteenth century the effects of clinical and experimental adrenal insufficiency were described (Addison 1855; Brown-Sequard, 1865) and their correction by administration of adrenal extracts has also long been known (Baumann and Kurland, 1927; Loeb, 1932).

Studies of the constituents of blood leaving the adrenal cortex and of the adrenocortical tissue itself revealed that a number of biologically active substances are synthesised by the adrenal cortex. These fall roughly into three categories, those which affect secondary sex characteristics (i.e. oestrogens and androgens), those which affect intermediary metabolism (i.e. glucocorticoids) and those which affect the metabolism of sodium and potassium (i.e. mineralocorticoids). This thesis is mainly concerned with the mineralocorticoids.

At least 3 compounds secreted by the adrenal cortex have significant mineralocorticoid activity. Of these, the last to be discovered and most potent is aldosterone and this has been studied in great detail, both physiologically and clinically, almost to the exclusion of the remaining compounds, corticosterone and 11-deoxycorticosterone (DOC). However, an examination of Figure 1 shows that, in addition to possessing considerable mineralocorticoid potency, DOC occupies a key position in adrenocortical biosynthesis. The aims of the work described in this thesis were to devise a technique of studying the changes of DOC concentration in the peripheral circulation in man and, using this technique, to study the factors which control these changes.

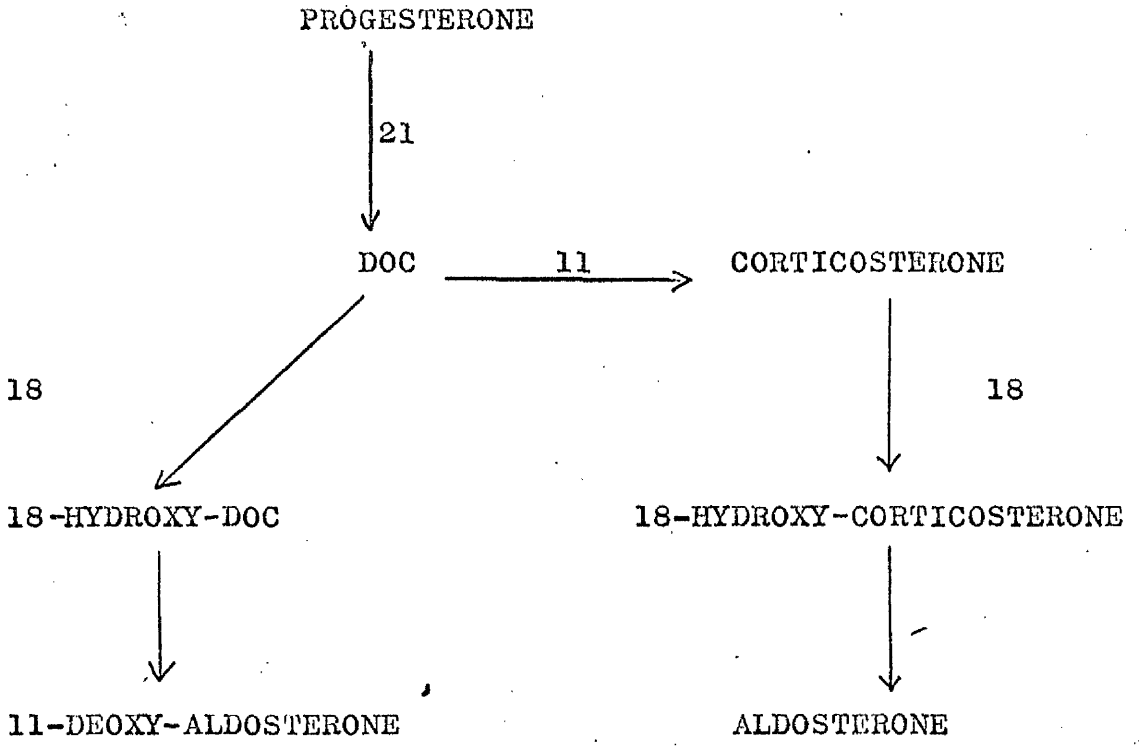

FIGURE 1.

Biosynthesis of mineralocorticoids: hydroxylation at positions 21, 18 and 11 on the steroid nucleus are indicated by numbers

1.2. NOMENCLATURE

The following trivial names have been used:

TABLE 1.

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
Deoxycortisol	17α, 21-dihydroxy-4-pregnene-3, 20-dione
Progesterone	4-pregnene-3, 20-dione
Testosterone	17α-hydroxy-4-androstene-3-one
Spironolactone	B-(7α-acetylthio-17α-hydroxy-3-oxandrost-4-en-17 yl) propionic acid lactone.
Dexamethasone	9α-fluoro-16α-methyl-11β, 17α, 21-trihydroxy-1, 4-pregnadiene-3, 20-dione
Deoxycorticosterone bisheptafluorobutyrate	3,21-bis (heptafluorobutoxy)-3,5-pregnadiene-20-one
Progesterone heptafluorobutyrate	3-heptafluorobutoxy-3, 5-pregnadiene- 20-one
Testosterone bisheptafluorobutyrate	3, 17-bis (heptafluorobutoxy)-3, 5-androstadiene
Metapyrone	1,2-di (3-pyridyl)-2-methyl-propanone.
Cortisol	11β, 17α, 21-trihydroxy-4-pregnene-3, 20-dione
18-hydroxy corticosterone	11β, 18,21-trihydroxy-4-pregnene-3, 20-dione
18-hydroxy DOC	18, 21-dihydroxy-4-pregnene-3, 20-dione
11-deoxy-Aldosterone	21-hydroxy-4-pregnene-3, 20-dione-18-al

2. REVIEW OF LITERATURE ON METHODS OF ASSESSING SECRETION OF DOC AND RELATED STEROIDS.

2. REVIEW OF LITERATURE ON METHODS OF ASSESSING SECRETION OF DOC AND RELATED STEROIDS.

Assessment of adrenocortical function requires both qualitative and quantitative analysis of the steroid products of this endocrine gland. However, as direct access to the adrenal venous effluent is rarely possible, particularly in subjects with normal adrenocortical function, indirect methods must be employed. These fall into two main categories, analysis of urine and analysis of peripheral plasma.

2.1. Analysis of urine.

Steroids secreted by the adrenal cortex are metabolised in the liver and kidneys and excreted in the urine. A small proportion of the total steroid is unchanged but most is in the form of water soluble sulphates and glucuronides (McKerns, 1969). The 24-hour urine collection thus contains a large quantity of steroids allowing the use of less sensitive techniques such as colorimetry (Vogt, 1955; Neher and Wettstein, 1956). However, the information derived from urine studies is limited since small or short term changes in adrenal secretion would not be detected due to the time lag between secretion and excretion of the steroid (Laidlaw et al, 1954). Two

different approaches to urinary steroid analysis have been adopted, measurement of the rate of excretion and the rate of secretion of steroids.

(a) Excretion rates

The total quantity of a particular compound in a 24 hour urine collection is termed the excretion rate. This type of measurement has been widely used in studies of steroid secreting endocrine glands (Biglieri, 1965). Unfortunately, most corticosteroids yield a variety of metabolities. The ratios of these may differ in situations of low and high adrenocortical activity and in the presence of renal or hepatic disease (Cope and Black 1958). Estimation of a single metabolite may therefore give an erroneous assessment of adrenocortical secretion.

Urinary excretion rates of TH-DOC have been reported (Harris et al, 1967; Biglieri, 1965, Nolten et al, 1968; New et al, 1969) and the normal range was found to be 20-30 ug/24 hours.

(b) Secretion rates.

The principle of isotope dilution has been employed in the measurement of steroid secretion rate. If a known quantity of radioactively-labelled steroid hormone of relatively high specific activity is administered at zero time, it will

mix with the steroid secreted endogenously and will be diluted in proportion to the amount secreted. Metabolism of exogenous and endogenous steroid is assumed to be identical. Collection of urine, usually over 24 hours and measurement of the specific activity of a metabolite unique to the parent compound under study gives, by a dilution factor, the secretion rate of that compound (Tait et al, 1961). Measurements of the secretion rate of DOC have been reported (Bledsoe et al, 1966; Crane and Harris, 1966; Biglieri et al, 1968) and the normal range was found to be 140 - 190 ug/24 hours.

2.2. Analysis of blood and plasma.

(a) Secretion rate studies.

As already mentioned, samples of adrenal venous blood are rarely available except at operation. As the adrenal cortex is extremely susceptible to surgical stress (Sayers, 1950) great care must be taken in the sampling of the adrenal venous effluent and in the interpretation of the results of analysis. DOC is particularly sensitive to stress (see section 4.2) and this approach is therefore unlikely to be of value.

(b) Peripheral plasma concentration

For estimation of the concentration of corticosteroids in the peripheral circulation whole blood or plasma may be used. When whole blood is allowed to stand after sampling corticosteroids adsorb on to the erythrocytes (Peterson et al, 1955; Bush, 1951; Sandberg et al, 1957) combining with the lipid membrane of the red cells (Willmer, 1961). Also, whole blood can metabolize steroids in vitro at room temperature (Werthessen et al, 1950; Lindner, 1961; Coyle and Romanoff, 1964). However, using heparin or oxalate as an anticoagulant whole blood can be stored up to 48 hours in the cold without altering the concentration of corticosteroids in the plasma phase. Deep-frozen plasma may be stored for several months without alteration of plasma corticosteroids (Fraser, 1967).

The concentration of corticosteroids in the peripheral circulation reflects more accurately than secretion rate or excretion rate studies the concentratiion of the hormones at tissue level. It is a measure of the equilibrium between secretion and catabolism and is therefore affected not only by kidney function but also by the function of the liver (Brown et al, 1957; Cohn and Bondy, 1958; Peterson, 1959) and thyroid (Eik-Nes and Brizzee, 1956; Hellman et al, 1961).

Unfortunately, the concentration of corticosteroids are much lower in the peripheral circulation than in adrenal venous effluent or urine. Consequently, very sensitive techniques are required to measure these low concentrations of corticosteroids.

2.3. Conclusion.

Measurement of peripheral plasma corticosteroid concentrations is a sensitive and convenient method of investigating the mechanisms of control of secretion of corticosteroids.

In the following section various methods of measuring corticosteroid levels are reviewed with particular reference to their applicability to the measurement of peripheral plasma concentrations.

3. PREVIOUS METHOD OF ASSESSING DOC SECRETION

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3.1. Extraction of corticosteroids

Corticosteroids are lipophilic compounds dissolved in plasma or bound to corticosteroid binding globulin (see later). Procedures for their extraction from plasma usually involve the use of organic solvents such as chloroform, dichloromethane, ethyl acetate, and petroleum ether. The choice of solvent depends on the compound under investigation (Johansson, 1969). Partial purification of the extract may be achieved by solvent partition which removes less polar substances such as cholesterol or by alkali washes which removes 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 should be removed by means of a dilute acid wash.

Purification of solvents before use may be essential. For example, ethyl acetate may form peroxide on standing while dichloromethane generates phosgene. Both of these contaminants destroy corticosteroids.

Removal of the solvent may be accomplished by distillation under reduced pressure at a low temperature or by evaporation under a stream of nitrogen. It may not be advisable to use a stream of air as this may oxidise the corticosteroids.

3.2. Chromatographic purification.

Utilising the small differences in polarity of the corticosteroid, further purification of individual compounds in plasma extracts may be achieved by chromatographic techniques such as thin layer chromatography (Adamec et al, 1962; Oertel and Ungar, 1964), paper chromatography (Neher, 1958; Bush, 1961; Zaffaroni, 1953; Eberlein and Bongiovanni, 1955), gas-liquid chromatography with fraction collectors (Weinstein et al, 1971), Sephadex (Murphy and Pattee, 1964) and more recently high pressure liquid - liquid chromatography (Thomas, 1972). Chromatographic purification of individual corticosteroid may be enhanced by changing the polarity of the compound under study by means of chemical modification. In order to achieve good purification all contamination in the chromatographic media must be removed by extensive washing (Holzbauer and Vogt, 1961). Paper chromatography is a particularly useful technique since paper is very easily washed and chromatograms may be conveniently run overnight.

3.3. Methods of measuring steroid mass.

Corticosteroids are present in plasma in relatively low concentrations together with large numbers of compounds of similar chemical and physical properties. Accurate assessment of their concentrations requires sensitive and specific techniques which can be conveniently applied to large numbers of samples. Methods of assay can be divided into two stages, purification and mass measurements. Specificity may be achieved either by the use of comprehensive purification techniques followed by non-specific assay or by the use of highly specific assays requiring little preliminary purification. Non-specific assays include fluorimetry, saturation analysis using plasma proteins; and double isotope derivative assays. Gas liquid chromatography and radioimmunoassay are specific techniques.

(a) Fluorimetry

A number of corticosteroids are known to fluoresce in acid (Reichstein and Shoppee, 1943; Sweat, 1954; Peterson, 1957; Goldzieher and Besch, 1958; Muelbaecher and Smith 1970) and using alkali, fluorescence can be developed and measured directly on paper (Tait and Tait, 1960).

The mechanism by which this fluorescence is produced has not been entirely elucidated (Linford, 1957; Kalant, 1958; Muelbaecher and Smith, 1970), although some features of the reactions have been investigated and reported (Sandee et al, 1971).

The first quantitative determination of cortisol and corticosterone by fluorimetry was first developed by Sweat (1954) using dilute sulphuric acid. Similar methods have now been developed by Peterson (1957), Ely et al (1958), Silber et al (1958), Braunsberg and James (1960), and Mattingly (1962). The original method of Mattingly (1962) for the measurement of 11-hydroxy steroids has since been modified by James et al (1967) by incorporating alkaline extraction of samples and the use of strong acid reagents and finally automated by Townsend and James (1968). The results obtained by this method were found to correlate well with true cortisol levels measured by double isotope derivative assay (Fraser and James, 1968).

In general, fluorimetric techniques are simple, rapid and permit the processing of large numbers of samples. Although for pure steroids, as little as 10 ng. of pure steroid can be detected, the practical limit of sensitivity of these methods is much higher when applied to biological samples due to non-specific interference which appears to derive from various

sources including thin layer and paper chromatograms (Braunsberg and James, 1960).

Muelbaecher and Smith (1970) reported details of fluorescence of 11-deoxycorticosteroids in perchloric acid using 11-deoxy-cortisol as a representative steroid. However, the sensitivity attained would not be adequate for the measurement of DOC in peripheral plasma again because of the limitations imposed by non specific fluorescence.

(b) Double isotope derivative assay

Double isotope derivative assays for steroids involve the use of a radioactively-labelled form of the steroid as a measure of recovery throughout the method. Mass measurement is accomplished by formation of a derivative of the steroid using a radioactively labelled reagent.

Double isotope derivative assays of steroids were first introduced by Peterson (1960) and Bojesen and Dean (1961) and have since found wide application to the measurement of steroids in biological samples.

The method of Peterson et al (1960) involved addition of (^{3}H)- aldosterone to the samples as an index of recovery and the formation of the (^{14}C)-acetate derivative of the endogenous steroid. The sensitivity of this method was limited by the specific activity of (^{14}C)-acetic anhydride. The sensitivity of the method was improved by inversion of the isotopes (Kliman and Peterson, 1960)

Peterson, 1960). This has formed the basis of a number of methods for measuring corticosteroids by double isotope derivative assay procedures (Koding et al 1962; James and Fraser, 1966; Coghlan and Scoggins, 1967; Fabre et al, 1969). However, due to the relatively low specific activity of (^{14}C)- corticosteroids, a significant quantity of radioactivity labelled steroid is required for estimation of recovery.

Bojesen and Begn (1961) introduced ^{131}I- p - iodosulphonates as recovery standards which were added after esterification with ^{35}S - p - iodo-sulphonylic anhydride. Incomplete esterification obviously could cause serious errors in this method. ^{35}S- p toluene sulphonates also form the basis of a sensitive method developed by Bojesen (1965). Thiosemicarbazones, which have been used for the measurement of progesterone (Riondel et al, 1965) and testosterone (Riondel et al, 1963), would also be a useful derivative for the estimation of corticosteroids in plasma.

Double isotope derivative assays involve a non-specific mass determination; therefore specificity can only be attained by using extensive chromatographic procedures. However, the technique can be applied readily to the simultaneous analysis of several steroids in one sample. For example, James and

Fraser (1966) developed a double isotope derivative assay for the simultaneous determination of aldosterone, cortisol and corticosterone. This technique possesses adequate specificity and accuracy for the estimation of these steroids in peripheral plasma.

Recently, Oddie et al (1972) reported a method for the simultaneous determination of aldosterone, corticosterone, cortisol, deoxycorticosterone and deoxycortisol in peripheral plasma by double isotope derivative assay using 10 - 30 mls of plasma. The normal range of plasma DOC was reported to be 6.3 ± 1.0 ng/100 ml.

Although double isotope derivative assay is a very sensitive technique which permits estimation of corticosteroids like aldosterone and DOC in peripheral plasma, specificity can only be achieved by inclusion of several chromatographic stages. Consequently, this type of method is laborious and time consuming and was considered inconvenient for routine analysis of plasma samples.

(c) Saturation Analysis

Steroids are bound reversibly and with variable affinity to protein molecules. If a known quantity of steroid-binding protein is saturated with radioactivity labelled hormone and a quantity of unlabelled quantity is then added, this latter

compound will compete with the labelled hormone for binding sites. At equilibrium, the quantity of labelled hormone remaining bound to the protein will be inversely proportional to the quantity of unlabelled substance added. This principle is called saturation analysis. Two types of methods have developed depending on whether a specific antibody or a naturally occurring binding protein is used, namely protein binding and radioimmunoassay.

(I). Protein binding using plasma proteins.

Two steroid binding proteins have been found in plasma, namely, albumin and corticosteroid binding globulin (Eik-Nes et al 1954; Daughaday, 1958). Both of these proteins bind cortisol although the affinity varies from species to species (Seal and Doe, 1965; Murphy, 1967). For example, monkey CBG has a much higher affinity for corticosterone than does human CBG. CBG has a relatively high affinity for cortisol but a low capacity, whereas albumin has a low affinity but a high capacity for cortisol. The steroid binding properties of these proteins (Daughaday, 1958; Slaunwhite and Sandberg, 1959; Sandberg et al, 1957; Chen et al, 1961) have been exploited as a means of measuring plasma corticosteroids by saturation analysis techniques.

Most protein-binding methods are based on the early work of Murphy and colleagues (1963, 1964)

who reported a technique for plasma cortisol measurement. This involved extraction of plasma followed by chromatographic purification of cortisol. An aliquot of this extract was incubated with (^{14}C)-cortisol and corticosteroid binding globulin and then bound and free cortisol were separated by dialysis techniques. The method was later improved by the use of (^{3}H)- cortisol of a much higher specific activity than (^{14}C)- cortisol thus increasing sensitivity. Further improvement was achieved by the use of simpler methods of separating free and bound steroid such as gel filtration or adsorption onto an insoluble matrix, for example, dextran-coated charcoal, florisil or Lloyds reagent (Murphy, 1967).

Protein binding is a non-specific mass measurement, and as was indicated earlier, specificity can usually be attained by the inclusion of one chromatographic purification step. Beitins et al (1969) showed that cortisol levels measured by double isotope derivative assay (Kliman and Peterson, 1960), colorimetry (Porter and Silber, 1950) and also by protein binding compared well at low and normal cortisol levels. In such samples chromatographic purification prior to incubation was not necessary. However, in samples containing increased concentrations of cortisol a good correlation

between protein binding and the other two methods was obtained only when a paper chromatography step was included in the method prior to incubation.

Recently, Brown and Strott (1971) reported a competitive protein binding method for measurement of peripheral plasma DOC concentration. Specificity was achieved by inclusion of two chromatographic steps prior to incubation of samples with (^{3}H)-deoxycorticosterone and corticosteroid binding globulin. Normal range of plasma DOC was found to be 5 - 10 ng/100 ml. However, using a 10 ml. sample of plasma the method was not sensitive enough to measure less than 5 ng/100 ml. Consequently, the usefulness of the technique was restricted since in 40% of analyses DOC was undetectable.

Protein binding methods are generally simple, rapid and suitable for analysis of large numbers of samples. Again, specificity can be achieved by introducing chromatographic purification. Unfortunately, this type of method does not appear to possess adequate sensitivity to detect small changes in plasma concentration of steroids such as DOC or aldosterone and therefore would not be suitable for the present study.

(ii) Radioimmunoassay.

Radioimmunoassay is a form of saturation analysis involving competitive inhibition of binding between

antibodies and specific radioactive antigen, by unlabelled antigen. The use of antibodies raised to certain steroids confers on this type of method an inherent specificity which is not found in conventional protein binding methods using naturally occurring proteins (see preceding section), Raising and testing of antisera, development of a practical assay and assessment of its reliability pose a variety of problems which will be briefly reviewed.

A. Production of Antibodies.

Steroids are haptens which can be rendered antigenic by coupling them to large carrier molecules such as proteins (Erlanger et al, 1957; Erlanger et al, 1959; Goodfriend and Sehon, 1958). In order to achieve antisera of good titre, avidity or sensitivity and specificity, careful choice of antigen, carrier protein, injection programme, and experimental animal are essential. Some of these decisions are purely arbitrary, and the success or failure of different protocols is often difficult to explain.

1. Antigen synthesis.

The early work of Bieser et al (1959) indicated that antisera may be most specific for those parts of the steroid molecule which are remote from the point of attachment of the carrier protein. The stereospecific property of antisera which has been further confirmed

by the work of Midgley and Niswender (1970) shows that the unique chemical features of a given steroid should be prepared as intact as possible in formation of steroid-protein conjugates as antigens.

Conjugation of proteins to steroids. Most of the conjugation methods are based on the pioneer work of Erlanger et al (1957, 1959), Goodfriend and Sehon (1958, 1961) and Lieberman et al (1959) who developed methods of forming stable covalent linkages between proteins and steroids. Earlier attempts to induce antibody synthesis to androstenediol were unsuccessful, presumably due to the ready hydrolysis of the steroid protein linkage.

Lieberman et al (1959) developed a mixed anhydride reaction suitable for coupling oximes or hemisuccinates directly to amino groups of lysine residues in the protein molecule. This type of reaction is obviously suitable for any steroid which possesses a hydroxyl or carbonyl functional group. However, the reaction involves the use of strong alkaline conditions at which all steroids may not be stable. A similar condition applies to the Schotten-Baumann reaction used to link proteins to the acyl chloride derivatives of steroids.

Goodfriend and Sehan (1958, 1961) used a carbodiimide condensation reaction to link protein and steroid. This technique was further developed by Gross et al (1968) who coupled diazobenzoic acid to oestradiol - 17β at position 2 to yield an oestrogen azobenzoyl derivative which they linked to proteins through the azobenzoyl carboxyl by carbodiimide condensation.

Conjugation of the protein to the steroid at position 6 requires the formation of a suitable functional group.

In any steroid-protein conjugate used for antibody formation the steroid protein ratio may be important in the induction of antibody synthesis. For example, Midgeley and Niswender (1970) failed to induce antibody formation using corticosteroid - bovine serum albumin conjugates in which the steroid-protein ratio was 10:1, whereas, Erlanger in an otherwise similar experiment successfully raised antibodies using conjugate in which the steroid protein ratio was 20-30:1. This ratio may be estimated by including in the synthesis of the steroid derivative a quantity of radioactively labelled steroid which allows the quantity of steroid in the final conjugate to be calculated. Other methods include the dinitrophenylation technique of Sanger (1945, 1949) and quantitative uv absorption methods.

2. Other factors influencing production of specific antibodies.

Carrier Protein. A complete immune response requires the recognition and participation of the carrier protein (Landsteiner, 1945; Green et al, 1968). As the steroid itself is not immunogenic in the natural state, it requires to be linked to a larger molecule such as a protein. This stimulates antibody production by the macrophages. Serum albumin which itself is immunogenic generally gives good results (e.g. Goodfriend and Sehon, 1961). It has been postulated that direct exposure of lymphocytes to the antigen leads to tolerance whereas exposure to the immunogen via the macrophage leads to immunity (Dresser, 1962).

Choice of animal species. As immunity depends on the responsiveness of the individual animal to the antigen and is genetically determined, probably by a single gene, certain inbred strains may synthesize antibodies to a particular steroid antigen while others may not (Green et al, 1968). Random-bred strains are usually the safest choice.

The choice of species must also depend on the ease of handling the animal, the ease of production of antibodies, and the volume of blood which can be obtained. Rabbits have been used successfully for

the production of antibodies by many workers. They are easily handled and provide reasonable volumes of serum. Guinea pigs have also been widely used but provide smaller volumes of serum which must be obtained by cardiac puncture. Sheep are reported to yield very high antibody titres and also provide very much larger volumes of serum.

Immunisation programme. The antibody response to an injected immunogen is greatly enhanced by use of an adjuvant such as a mineral oil or aluminium hydroxide. The most common one is Freund's complete adjuvant (Freund, 1951) which consists of paraffin oil, a neutral detergent and killed mycobacteria. The antigen, injected in a stable emulsion in Freund's complete adjuvant, is slowly released over a period of weeks. The adjuvant also facilitates phagocytosis of the immunogen by macrophages and causes local granulomatous lesions which may act as a locus of antibody formation (Frei et al, 1965).

Few critical studies have been made of the route of administration of immunogen, among the commonest being subcutaneous and intradermal sites. Most workers have used 1 mg. or less of conjugate for the initial immunisation followed by 0.5 mg. or 0.25 mg. conjugate for subsequent immunisations. However, Vaitukaitis et al (1971) reported production of antisera to testosterone using a total of 100 ug. immunogen

for each immunisation which was given intradermally at 20 - 30 sites.

The timing of injections is also important. Due to the slow sustained release of antigens from the Freund's emulsion, antibody levels rise to a maximum 6 weeks after the first immunisation. Thereafter antibody levels attain a maximum 7 - 10 days after each booster injection when blood samples may be taken for testing of antisera.

B. Testing of Antisera

Titre. The presence of antibodies may be demonstrated by quantitative preciptin or haemaglutination techniques (Lieberman et al, 1959; Goodfriend and Sehon, 1961; Ferin et al, 1968; Gross et al, 1968). However, if the antiserum is to be used for radio-immunoassay, the presence and titre of antibodies is more conveniently demonstrated by incubating serial dilutions of the antiserum with radio-iodinated antigen (Midgley and Niswenden 1969; Niswender and Midgley 1970) or with tritium - labelled hapten or hapten which is protein bound. This is usually expressed as percentage bound or as the ratio of bound: free and plotted against the corresponding dilution of the antiserum.

Specificity. The specificity of the antiserum may be determined by incubating it in serial dilutions with

a series of tritium-labelled steroids and constructing a dilution curve as shown in section 4.2. Obviously, all steroids cannot be tested and the obvious choice would be those with similar functional groups and those of similar polarity which may interfere in the radioimmunoassay of the steroid under study. Steroids present in large concentrations must also be tested since a small proportional cross reactivity could yield a false positive result by virtue of the mass of steroid present.

Sensitivity. The effective avidity of an antiserum used for radioimmunoassay is obviously important when very low concentrations of steroids require to be measured. Berson and Yalow (1964) first suggested that the steepness of the descending part of the titration curve is an indication of the sensitivity of the antiserum. However, it has since been shown from a theoretical point of view and by practical means that it may not always be an accurate guide (Greenwood, 1968; Berson and Yalow, 1968). Assuming a bimolecular reaction and that the concentration of labelled antigen is small compared to that of antibodies present in all tested dilutions it can be shown that the ratio of bound : free must fall proportionately with dilution of antibodies regardless of the equilibrium constant of the antibody

antigen reactions. Therefore, on dilution of antisera, per cent bound steroid will decrease in the same way in all antisera and all curves will appear parallel under these conditions. A more accurate guide of sensitivity is to incubate the antiserum with varying amounts of radioactively labelled antigen. The minimum amount of labelled antigen required to give a separate curve indicates the quantity of labelled antigen required to give maximum sensitivity.

C. Use of Antisera.

Preparation of Samples . The extent to which samples must be purified depends on the specificity of the antiserum in use. With a highly specific antiserum little purification of sample may be required. However, most methods include a chromatographic step in order to achieve good specificity. For example, Abraham et al (1970), in the measurement of plasma oestradiol 17β concentrations in peripheral plasma, compared results obtained with and without a chromatographic step in the method. Results correlated well when levels were normal or low, but anomalously high results were often obtained in women during pregnancy when no chromatographic step was included.

Although the use of chromatographic purification will improve the specificity of radioimmunoassay by eliminating interference from most other steroids, it may also introduce non specific interference from the chromatographic medium in use thus causing measurable blank values. Careful purification of the chromatographic medium is essential to avoid this type of contamination. Furuyama and Nugent (1971) used small alumina columns which were conveniently cleaned by solvent washing and gave low blank values. Sephadex LH-20 columns have also been used and reported to give very low blank values (Mikhail et al, 1970; Wu and Lundy, 1971; Ito et al, 1972). Celite columns (Abraham et al, 1971; Tulchinsky and Abraham 1971), paper chromatography (Ito et al, 1972; James et al, 1971) and thin layer chromatography (Farmer et al, 1972) have also been employed.

Johanssen et al (1969) using petroleum ether of a precise boiling range, developed extraction systems which allow fairly specific and quantitative removal of individual steroids. Specificity can be achieved in this way without introducing problems of non-specific interference. Similarly, high pressure liquid - liquid chromatography may also offer convenient methods of purifying samples prior to assay.

Purification of antisera. Animals injected with steroid - protein conjugates develop antibodies not only to the steroid but also to the carrier protein. The non-specific binding of the latter may interfere with the assay. A convenient method of removal is to expose the antiserum to bovine serum albumin and then centrifuge, thus precipitating the interfering antibodies before using the serum for RIA (Furuyama et al, 1970).

Treatment of serum with Rivanol (2-ethoxy-6, 9-diamino-acridine lactate) was reported (Horejsi and Smetna, 1956) to precipitate all serum proteins except gamma globulins. Kaldor, Saifer and Vecsler (1961) demonstrated that the mechanism of action of Rivanol precipitation of bovine serum albumin is related to the number of carboxyl groups not interacting with amino groups. Rivanol forms an insoluble cationic complex with the protein. Rivanol treatment is a useful means of purifying antisera (Abraham, 1969).

Assay Systems. Mass measurement by radioimmunoassay involves incubation of samples with radioactively labelled antigen and antibodies followed by separation of antibody bound and free steroid. Procedures using various systems of incubation and separation of bound and free have been developed. Variables such as temperature and time of incubation,

form of the antibody and amount of protein present affect the equilibrium of the reaction and require consideration.

Conditions of incubation. The pH and ionic strength of buffer used for incubation can alter the antibody-antigen reaction (Pressman and Grossberg, 1968; Ganguly et al, 1967; Ganguly and Westphal, 1968). Therefore, investigation of the effect of alteration of pH on the extent of binding of the steroid may be necessary in order to obtain optimal assay conditions.

The rate of the first order reaction between antibodies and antigen is temperature dependent. Incubation at 37°C (Bayard et al, 1970) or at room temperature (Mikhail et al, 1970; Yoshizawa and Fishman, 1971) will achieve equilibrium more rapidly than at 4°C (Mayes and Nugent, 1970). However, a stable temperature, essential in order to maintain reproducibility may be achieved most conveniently at 4°C.

Addition of radioactively labelled antigen. The labelled antigen may be added to the plasma extract prior to incubation or may be included in the dilute buffered antiserum. Rodbard et al (1971) investigated this problem by kinetic analysis methods and using computer simulation studies. They

demonstrated that improved sensitivity can be achieved by delayed addition of radioactivity labelled antigen, thus providing a non equilibrium type assay.

Separation of bound and free steroids. Many radioimmunoassay methods involve separation of bound and free steroid by precipitation techniques.

Free steroid may be removed by adsorption on to an insoluble matrix such as florisil (Bayard et al, 1970; Ito et al, 1972) or dextran-coated charcoal (Abraham et al, 1971; Wu and Lundy, 1971; Yoshizawa and Fishman, 1971). Bound steroid may be removed by protein denaturation using half-saturated ammonium sulphate solution (Mayes and Nugent, 1970: Furuyama et al, 1970).

Midgley and Niswender (1969) developed novel methods of separating bound and free using a double antibody technique. Samples were incubated with excess bovine serum albumin and antibody specific to the steroid under study. After 24 hour radioiodinated steroid bovine serum albumin conjugate was added and incubated again before addition of sheep anti-rabbit γ globulin serum. After a further 3 days the samples were centrifuged and counted to obtain the per cent bound steroid. Unfortunately, this technique is not as convenient as the other simpler methods of separating bound and free. It is, however,

a very specific method suitable for radioimmunoassay of many haptens.

Solid phase methods. Abraham et al (1970) reported a solid phase radioimmunoassay of oestradiol - 17β using antibody coated tubes (Catt and Treager, 1967). The samples are incubated with the antibody after which the buffer containing the free steroid is quantitatively removed. This technique allows equilibration and separation of bound and free steroid in a single step. However preparation of the tubes requires larger quantities of antibodies initially in order to achieve adequate and reproducible coating.

Solvent partition. Banks et al (1971) developed a rapid radioimmunoassay of aldosterone in which toluene scintillator was used to separate bound and free steroid. The technique is simple and obviously useful for analysis of large numbers of samples.

Application to the measurement of plasma DOC concentration. Recently, James et al (1971) reported a radioimmunoassay of plasma DOC. Based on the initial techniques of Mayes et al (1969) antibodies were produced in the rabbit using DOC - 3 - carboxymethyloxime conjugated to bovine-serum albumin as antigen. The method involved purification of plasma extracts by paper chromatography prior to incubation with antiserum and tritium-labelled DOC

in borate buffer. Bound and free steroid were separated using ammonium sulphate precipitation.

Specificity was determined by comparison of results with those obtained by double isotope assay and gas chromatography (see section 4). Close correlation of results indicated good specificity. Within batch precision was $\pm$ 0.83 (S.D.) ng/100 ml and between batch precision ranged from 4.4 $\pm$ 0.97 (S.D.) to 7.8 $\pm$ 1.4 (S.D.) ng/100 ml. Normal range of plasma DOC concentration was reported to be 1 - 12 ng/100 ml with a mean of 5 ng/100 ml.

Reliability of radioimmunoassay.

Specificity. Radioimmunoassay is a specific mass measurement. Evidence of specificity can only be obtained by cross reaction studies and comparison of results with those of more physico-chemical techniques such as gas chromatography. However, when a chromatographic step is employed prior to assay specificity can be further improved.

Sensitivity. Radioimmunoassay is a highly sensitive technique which allows quantitation of steroid present in a few ng/100 ml plasma and requires only a few ml. of plasma. For example, several methods have been reported for radioimmunoassay of aldosterone (Mayes et al, 1970; Farmer et al, 1972; Ito et al, 1972) using between one and five millilitres of plasma.

Convenience. Radioimmunoassay is a rapid technique which is much less time-consuming than double isotope derivative assays and technically simpler than gas chromatographic techniques. However, production of antisera may be costly, laborious and time consuming.

Due to the simplicity of the techniques and the small size of sample needed, radioimmunoassay is suitable for duplicate analysis of samples which may improve accuracy and reliability of the method.

Conclusion. Radioimmunoassay techniques offer a specific and sensitive method which is suitable for measurement of peripheral plasma concentrations of steroids especially where small or short term changes require to be measured. As only a few millilitres of plasma should be required repeated sampling would be feasible.

From these criteria, it was decided to develop antisera to DOC and a radioimmunoassay for plasma DOC. Development and assessment of this method is described in section 4.

(d) Gas liquid chromatography (GLC) of corticosteroids.

GLC has found particularly useful application to the study of plasma steroids since it combines the features of excellent chromatographic separation and very sensitive detection.

Principle. The principle of GLC is basically similar to that used in all types of chromatography. A group of steroids under study is mixed in gaseous or vapour phase with a small volume of an inert mobile gas phase which then passes through a column packed with a solid support coated with stationary phase. The components of the steroid mixture interact to varying degrees with the stationary phase as they pass through the column, the affinity of each component for the stationary phase determining the extent of interaction and this produces a relative retardation on the column. Depending on the relative retardation of individual steroids chromatographic separation is achieved, The eluate from the column then passes through a detection system which responds to each component. The response is potentiometrically recorded.

The retention time of any steroid, that is, the time elapsing between injection of the sample and detection of the appropriate peak on the recorder, is characteristic of the steroid and may be used to identify the steroid.

Factors influencing retention time. The retention time of any particular compound on GLC depends upon a variety of factors including the characteristics of the stationary phase in the column, carrier gas flow rate and temperature.

Stationary phase. Selective and non-selective stationary phases are available. The separation of steroids on a selective phase depends primarily on the active steroid groups such as hydroxyl or carbonyl groups. Selective phases include OV-17, OV-22, OV-225 which are silicones with polar characteristics. Separation on non-selective columns such as OV1, XE-60 or SE-30 depends mainly on the molecular weight of the steroid. These phases are non polar.

The stationary phase is coated on an inert support such as diatomaceous earth and then packed in silanised glass columns.

Longer retention times and better chromatographic separation of steroids may be obtained by using long columns although this is also affected by the efficiency with which it is packed.

Temperature and carrier gas flow rate. Raising the column temperature or increasing the carrier gas flow rate decreases the retention time of a steroid. Optimal chromatographic separation can be achieved by manipulating these two variables.

Retention Index. There are various standard methods of denoting the retention values of a particular steroid on a particular column, for example Kovats Retention Index (Kovats, 1958) describes the retention time of a compound by comparison with those

of straight chain alkanes.

Quantitation by GLC. The height or area of a peak obtain on GLC for a particular steroid is a measure of the quantity injected on the column. Therefore, samples may be measured quantitatively by comparison of peak height or area with that of standard injections of the same steroid. An internal standard of similar chemical characteristics and similar but not identical retention time is added to each sample and standard in order to account for losses on the column due to decomposition or adsorption and losses on injection of samples.

Development of GLC for measurement of steroids. The first practical separation of steroids by GLC was achieved by Van den Heuvel et al in 1960. However, many steroids were subsequently found to be unstable when chromatographed on SE-30 at 220°C. For example, cleavage of the side chain of corticosteroids occurs, giving rise to the corresponding 17-ketosteroids. This was confirmed by Luetscher and Gould (1964) who compared the retention times with those of the corresponding androstenes. The problem of instability of corticosteroids on the GLC column, has been solved in a number of ways.

Oxidative cleavage. Oxidative cleavage of the side chain of corticosteroids was reported (Merits, 1962) to yield derivatives which were stable on gas chromatography. The corticosteroids were converted into the corresponding etio-cholenic acids which, on esterification with diazomethane gave stable methyl esters which were suitable for gas chromatography. However, aldosterone and 18-hydroxydeoxycorticosterone were oxidised to internal esters or lactones which were stable without esterification.

Kittinger (1964) exploited this oxidation reaction for the estimation of steroids produced by the rat adrenal gland and later for simultaneous estimation of several corticosteroids (Kittinger, 1967; Kittinger and Beamer, 1968).

Bailey (1963, 1964) used sodium bismuthate for oxidative cleavage of the side chain of cortisol and its metabolites to estimate these compounds in urine by gas chromatography.

Formation of stable derivatives. Acetates (Brooks, 1964, 1965) and trimethylsilyl ethers (Luukkainen et al, 1961; Rosenfeld, 1964; Brooks et al, 1967) were also introduced as stable corticosteroid derivatives suitable for GLC. Rosenfeld (1964) used the trimethylsilylether for the estimation of urinary

17-hydroxy steroids. Steroid methoxime - trimethylsilylethers have also been extensively used in the study of urinary steroid profiles (Horning and Gardiner, 1965).

Kirschner and Fales (1962) prepared the bismethylenedioxy derivatives of 17-hydroxy steroids. They demonstrated the use of this stable derivative in the estimation of cortisone in guinea pig urine by gas chromatography. Unfortunately, cortisol, 11-deoxycortisol and tetrahydrocortisone were found to be unstable during gas chromatography using a 1% SE-30 column.

Conclusion. Much of this early work in the development and validation of corticosteroid derivatives served to demonstrate the feasibility of the technique for the estimation of steroids in biological samples and also illustrate the inherent specificity of the method in that the steroid could be characterised on the basis of retention time.

Using conventional detector systems such as flame ionisation or β ionisation gas chromatographic techniques do not possess adequate sensitivity to detect and measure the levels of many corticosteroids present in peripheral plasma. A great improvement in the sensitivity of GLC analysis has been achieved by the introduction of the electron capture detector.

Principle of the electron capture detector. The electron-capture detector employes a source of low-energy free electrons such as tritium foil or ^{63}Ni isotope. As a molecule passes through the detector it will capture electrons by one of two processes, either

(a) $e + AB \rightarrow AB^- + \text{energy}$

or

(b) $e + AB \rightarrow A + B^- \pm \text{energy}.$

where AB is the molecule under study. The ion AB^- or B^-, being less mobile than the free electron, reaches the collector plate more slowly than the free electron. As a result, there is a decrease in current across the detector cell proportional to the number of electron-capturing molecules passing through the detector cell.

Electron capture detection of steroids. On the observation that some organic vapours capture electrons it was postulated that submicrogram quantities of steroids should be detectable by electron capture following gas chromatography (Lovelock and Lipsky, 1960; Lovelock, 1961; Wentworth and Becker, 1962; Lovelock et al, 1963; Landowne and Lipsky, 1963). Formation of derivatives capable of electron capture would

enhance the level of sensitivity of detection by gas chromatographic techniques. Obviously groups containing several halogen atoms which have a high electron affinity would be most electron capturing. Resonance stabilisation of the negative charge via conjugated double bonds may also enhance electron capturing power of a molecule.

Formation of electron capturing derivatives.

Halo-acetate derivatives. Landowne and Lipsky (1963) prepared a series of haloacetates of cholesterol and studied the relative molar response to electron capture detection for each derivative. Surprisingly, the order was found to be monochloroacetate > dichloroacetate > monobromoacetate > trichloroacetate > trifluoroacetate. The unexpected order of molar response to electron capture detection was thought to be due to attachment of electrons to the carbonyl carbon atom instead of the halogen atoms. Increased polarity of the double bond caused by the interspacial electron attraction of the halogens would be greatest for monochloroacetate.

Subsequently methods were developed for the estimation testosterone (Brownie et al, 1964), progesterone (van der Molen and Groen, 1965) and oestradiol (ÅÅkvaag et al, 1964) in human plasma. In other studies van der Molen et al (1965)

reported that steroid monochloroacetates were stable derivatives suitable for electron capture detection and which allowed precise measurement of 1 - 10 ng. amounts of steroid.

Silyl ethers. Thomas et al (1966) investigated the molar response to electron capture detection of various halogenated silyl ethers of testosterone. These derivatives were more responsive to electron capture detection than haloacetates. Using the iodomethyl - dimethyl silyl ether derivative they developed a sensitive method for the measurement of testosterone in plasma. However at this time these derivatives were not readily available in very pure form and were very easily hydrolysed.

Aldosterone γ-lactone. Rapp and Eik-Nes (1965) reported that aldosterone γ-lactone was electron capturing and used this derivative for the measurement of aldosterone in biological samples (Rapp and Eik-Nes, 1966). However, the method was not sensitive enough for the measurement of aldosterone in small volumes of plasma (Brodie et al, 1967) but was suitable for the measurement of aldosterone in urine.

Heptafluorobutyrates. Improved sensitivity was achieved by the introduction of heptafluorobutyrate derivatives (Clark and Wotiz, 1963; Nakagawa et al, 1966). Quantitative methods of preparing these derivatives for many steroid were developed by Exley and Chamberlain (1967) and molar responses to electron capture detection and retention data on GLC were tabulated for many corticosteroid-3-enyl heptafluorobutyrates (Exley and Chamberlain, 1967; Chamberlain, 1967). These derivatives were also prepared in micro-quantities and characterised physico-chemically by van der Molen & Groen (1967) and Dehennin and Scholler (1969). The bisheptafluorobutyrates (3-enyl-21) of most corticosteroids are stable to gas chromatography and are much more electron-capturing than the corresponding chloroacetates. Unfortunately, the bisheptafluorobutyrates are unstable on thin layer chromatography and sometimes on paper chromatography (Exley and Chamberlain, 1967) and so cannot be purified by these means.

Despite these problems, methods have been developed using these derivatives for the estimation of plasma concentrations of testosterone (Exley 1967, 1968; Vermeulen, 1967; van der Molen and Groen 1967), oestrogens (Wotiz et al, 1967; Charransol and Wotiz, 1966) and androstenedione (Kirschner

and Coffman 1968).

Kirschner and Coffman (1968) and Sarda et al (1968) prepared the monoheptofluorobutyrate of testosterone for the measurement of the steroid in plasma. This derivative is stable on thin layer chromatography and so can be purified more easily than the corresponding bisheptafluorobutyrate but is less responsive to electron capture detection. However, these methods are much more sensitive than the method of Brownie et al (1964) using the chloroacetate.

Other polyhalogenated alkyl derivatives. Kirschner and Taylor (1969) proposed two new electron capturing derivatives for hydroxy steroids, namely 9-H- hexadecafluoronanoate and 11-H-eicosafluoro-undecanoate which were more than twice as sensitive to electron capture detection than the corresponding monoheptafluorobutyrates. They reported methods for estimation of testosterone and androstenedione using these new derivatives in which the minimum amount of steroid which was readily measured was 100 pg.

Nakagawa et al (1966) prepared the 21-pentadecafluoro-octanoate of testosterone and found it to be almost as electron capturing as the 3 enyl, 21-bisheptafluorobutyrate.

Oestrogen derivatives. Gas liquid chromatography with electron capture detection has also been applied to the measurement of oestrogens which are present in low concentration in peripheral plasma. A number of electron capturing oestrogen derivatives have been prepared and used for the measurement of these steroids in plasma.

Wotiz et al (1967) prepared the 17-monoheptafluorobutyrate derivative and showed that, using this derivative, the practical limit of sensitivity of their GLC method for estimation of oestrogens in plasma was 2 ng/sample, which limits the usefulness of this technique.

Kirschner and Taylor (1969) compared this derivative with the 17-monopentadecafluorooctanoate, 17-mono-(monochloro)-acetate and 17-monoeicosa-fluoroundecanoate and found the latter to be the most sensitive derivative for electron capture detection.

Attal et al (1967) prepared the 3-methyl ether - 17 - pentafluorophenylhydrazone of estrone and found it to be more sensitive than the chloroacetate but less than the monoheptafluorobutyrate.

Exley and Dutton (1969) in an attempt to rationalise the criteria governing response of any derivative to electron capture detection, prepared a series of 27 derivatives of oestrone and

oestradiol and investigated their behaviour on GLC with electron capture detection and their stability on thin layer chromatography. The derivative of choice, namely 17β-oestradiol - 3-(2-iodomethyldimethylsiloxy) propyl ether) - 17β-iodomethylsilylether was prepared by hydroxy-etherification of the phenolic hydroxyl followed by iodomethyldimethylsilylation. It is stable on thin layer chromatography and during GLC and more responsive to electron capture detection than the monoheptafluorobutyrate. This derivative proved suitable for measurement of 17β -oestradiol in plasma. This study also confirmed the initial finding of Lovelock (1963) and confirmed a previous observation that addition of more than two heptafluorobutyrate groups may not further enhance the response to electron capture detection.

Conclusion. A vast number of steroid electron capturing derivatives have been prepared and most of the reagents have now become commercially available. A suitable derivative of a steroid for GLC with electron capture detection must obviously be easily prepared in a quantitative, reproducible manner. It must be stable during GLC and be very responsive to electron capture detection. Stability on thin layer and paper chromatography is

desirable in order that the derivative can be purified by these means. The choice of derivative must also be governed by the purpose for which the assay is being developed. For studies of urinary steroids a less sensitive, more stable derivative may be the rational choice because the steroid is present in larger amounts. Thus, in the estimation of urinary oestrogens, Knorr et al (1970) reported that the 3- methyl ether 17-hexadecafluoronanoate provided adequate sensitivity. For the measurement of very small concentrations of steroid in peripheral plasma as is required in this present study, maximum sensitivity is important. At the time this work began the most sensitive derivative available for corticosteroids was the 3-enyl, 21-bisheptafluorobutyrate. Unfortunately, as previously stated, this is unstable on thin layer chromatography and purification of the derivatives is not possible.

Disadvantages of the electron capture detector. The limit of sensitivity of electron capture detection may depend not only on the electron capture activity of steroid derivatives but also on other factors such as column adsorption and purity of extracts, solvents and glassware.

Biological extracts which have been purified by paper or thin layer chromatography are known to contain impurities which may 'poison' or 'contaminate' the detector and reduce sensitivity (Rapp and Eik-Nes, 1966). A serious drawback of the tritium foil detector is that the upper temperature limit is 220°C so that superheating to remove impurities is not permissible as with the ^{63}Ni detector. With both detectors, addition of an internal standard for gas chromatography is essential in order to correct for column adsorption and changes in detector sensitivity (Horning and Gardiner, 1965; Brownie et al, 1964). In this present study, the development of a detector by-pass valve was considered necessary in order to prevent contamination of the detector.

Measurement of DOC by GLC. Studies of DOC by GLC are few. Using the 21-acetate derivatives of DOC, which is electron capturing, Rapp and Eik-Nes (1966) measured venous plasma concentrations of this steroid in rabbits. The sensitivity of this method (20 ng per sample) was not adequate for detection and estimation of this steroid in human peripheral plasma.

The retention characteristics of deoxycorticosterone bisheptafluorobutyrate on a number of

stationary phases have been tabulated (Exley, 1967; Exley and Chamberlain, 1967; Chamberlain, 1967) and the molar response to electron capture detection indicates that this derivative would provide adequate sensitivity for estimation of this steroid in peripheral plasma.

Use of 3-enyl, 21-bisheptafluorobutyrate. The following section describes the development and assessment of a method for measurement of plasma DOC concentration by GLC with electron capture detection using the 3-enyl, 21-bisheptafluorobutyrate derivative of DOC.

4. METHODS USED IN THE PRESENT STUDY

4. METHODS USED IN THE PRESENT STUDY

4.1. Measurement of plasma DOC concentration by gas chromatography with electron capture detection.

This section describes the development and assessment of a method for measurement of peripheral plasma DOC concentration using gas liquid chromatography with electron capture detection of the bis HFB, derivative of DOC.

4.1.2. Materials

Solvents. Dichloromethane, ethanol and petroleum ether (80 - 100°) (B.D.H. Ltd.) were redistilled immediately prior to use. Toluene (B.D.H. Ltd.) was reagent grade. All other solvents (acetone, benzene, ethyl acetate, hexane, methanol) were Nanograde quality solvents (Mallinkrodt Chemical Works, St. Louis). Glass distilled water was used throughout.

Standard steroids. Aldosterone (Ciba Ltd.), 18-hydroxy-deoxycorticosterone (Searle, Mexico, Ltd.), corticosterone, cortisol, DOC, deoxycortisol, progesterone and testosterone (CIBA Ltd.) were examined for purity on thin layer chromatography on systems 1 and 2 (see below). (^{3}H)- DOC, (^{3}H)-

aldosterone (30 and 25 Ci/mM., New England Nuclear Corp.) and (^{14}C) - DOC (Radio-chemical Centre) were diluted in dry benzene and stored at 4°C. The (^{3}H)-steroids were purified on paper chromatography (Bush B3) immediately prior to use.

Reagents. Heptafluorobutyric anhydride (HFBA) was prepared as described by Exley and Chamberlain (1967) and stored at 4°C in glass ampoules each containing 0.5 ml. Acetic anhydride and pyridine (B.D.H. Ltd., Analar grade) were refluxed over calcium carbide and potassium hydroxide respectively and then redistilled before storage in glass ampoules at 4°C. Sodium hydroxide, hydrochloric acid, sodium bicarbonate (B.D.H. Ltd.) were reagent grade and sodium carbonate (B.D.H. Ltd.) was analytical grade.

Scintillation Mixtures. 1. Diphenyloxazol (PPO, 10 g.) and methanol (40 ml) in toluene (2 l.) was used for liquid scintillation counting of aqueous solutions.

2. p-Terphenyl (6 g.) and dimethyl - POPOP (1, 4 - bis- 2-(4-methyl-5-phenyloxazolyl) benzene, 80 mg.) in toluene (2 l.) was used for liquid scintillation counting of non-aqueous solutions.

These scintillation mixtures were stored in the dark at room temperature.

Chromatographic Media.

Thin layer chromatography.

System 1. Silica gel (MNG/UV 254; Macherey - Nagel, Ltd.) was used to prepare t.l.c. plates (250 μm) which were prerun in methanol before use. Steroids were chromatographed in a mixture of hexane and acetone (3:1, v/v). RF values for standard steroids are shown in Table 2.

System 2. Glass fibre paper impregnated with silica gel (20 x 20 cm: I.T.L.C. Type SAF; Gelman Instrument Co.) were also prerun in methanol. Steroids were chromatographed in the system described above. RF values for steroids are shown in Table 3.

Paper chromatography. Chromatography paper (no. 1 Whatman Ltd.) was washed for 24 hours with methanol in a Soxhlet reflux condenser before use. Steroids were chromatographed in Bush B3 system (petroleum ether(80 - 100°): benzene: methanol: water: 330: 170: 400: 100, v) for 6 hours. R_F values for standard steroids are shown in Table 4.

Glassware. All glassware was steeped in detergent (Decon 75, Pharmaceutical Developments Ltd.) for several hours and then rinsed with water

STEROID	R_F
DOCA	0.25
PROGESTERONE	0.40
TESTOSTERONE ACETATE	0.43

TABLE 2.

R_F values of standard steroids used in T.L.C. system 1.

STEROID	R_F
DOC	0,35
PROGESTERONE	0.80
TESTOSTERONE	0.40

TABLE 3.

R_F values of standard steroids used in chromatography system 2 (T.L.C.).

STEROID	R_F
CORTISOL	0.01
ALDOSTERONE	0.025
CORTICOSTERONE	0.09
DEOXYCORTISOL	0.125
TESTOSTERONE	0.48
DOC	0.59
PROGESTERONE	0.90
18-HYDROXY-DOC	0.06
18 HYDROXY CORTICOSTERONE	0.04

TABLE 4.

R_F values of standard corticosteroids on paper chromatography in Bush B3 system.

followed by methanol. Test tubes were silanised (Repelcote, Hopkin and Williams, Ltd.) and then rinsed with benzene immediately before use.

Gas-liquid chromatography. A Pye 104 model 84 gas-liquid chromatography (Pye Unicam, Ltd.) was used equipped with a ^{63}Ni electron source and a Rikadenki recorder (Model B24, Rikadenki Kogyo Co. Ltd.) Pyrex glass columns (1.5 m x 4 mm.) were silanised and packed with 100 - 120 Supelcoport coated with 1% OV1 (Supelco, Inc.) Conditions were as follows:-

Temperatures

Column	:	205°C
Flash heater	:	250°C
Detector	:	250°C

Nitrogen Flow

Carrier gas	:	40 ml/minute
Quench gas	:	20 ml/minute

Electron Capture Supply

Pulse space	:	150 us.
Pulse width	:	0.75 $\pm$ 0.25 us.
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 weekly.

Gas liquid chromatograph detector by-pass valve (Fig. 2). This valve was designed and constructed in our department for use with the Pye 104 chromatograph. The by -pass valve was constructed from polytetrafluoreothylene (P.T.F.E.). With this device it was possible to select column effluent fractions for passage through the detector. For the first 4 minutes after sample injection nitrogen from the reference column was passed through the detector while the sample column eluate, found in preliminary experiments to contaminate the detector, was passed to waste. The remainder of the sample column eluate was then passed through the detector by altering the position of the valve plunger. In this way, contamination of the detector and subsequent loss of sensitivity were avoided. Since the introduction of the initial valve, other models of valves have become commercially available and a rotary action valve also constructed of P.T.F.E. (Carle valves, Inc.) has now replaced the original cylindrical valve.

Gas-chromatograph - mass spectrometry (G.C.M.S.).

G.C.M.S. analysis was carried out using an L.K.B. 9000 spectrometer under the following conditions:-

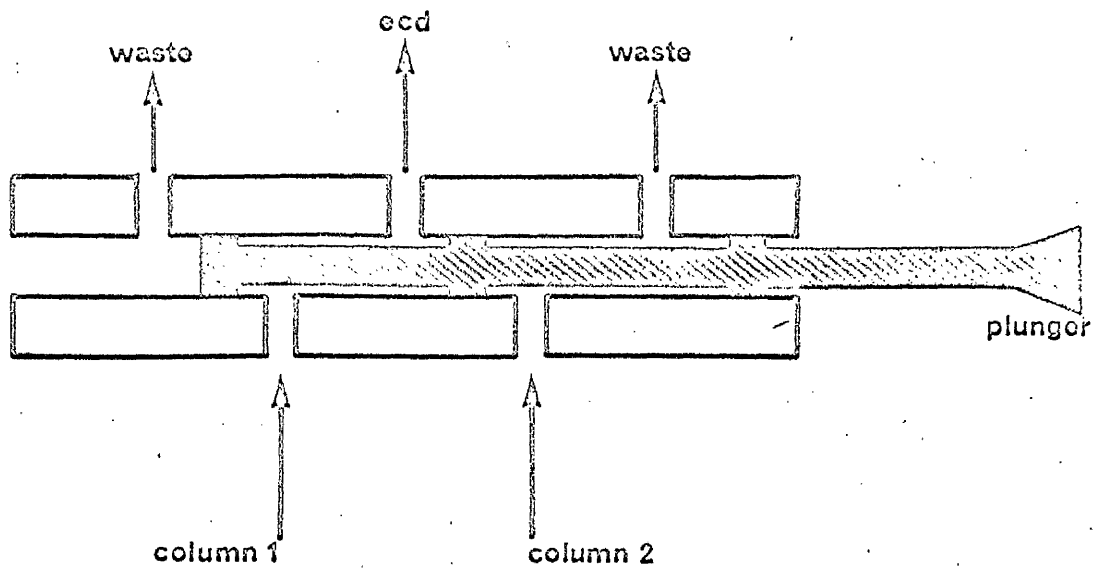

Figure 2.

Detector by-pass valve used as a means of preventing detector contamination. This valve was constructed in poly-tetro-fluoroethylene (PTFE).

Ionising voltage	:	70 e.V.
Ion source temperature	:	250°C
Molecule Separator temperature	:	250°C
Scan period	:	0.5 seconds
Oven temperature	:	200°C
Column (G.L.C.)	:	1% OV1

These analyses were performed by Dr. C.J,W. Brooks, Chemistry Department, Glasgow University.

4.1.2. PROCEDURE

Plasma. Blood samples were taken into lithium heparin (1000 i.u.) and the separated plasma (5- 25 ml.) was stored at -20°C until required for assay.

Extraction. Plasma (5 - 25 ml.) to which ^{3}H-deoxycorticosterone (5000 cpm) had been added was extracted with dichloromethane (10 vol.) and the extract was washed consecutively with sodium hydroxide (0.1M aqueous), acetic acid (0.1 M, aqueous), and water (1 vol. each.). The washed neutral extract was then evaporated to dryness in a rotary evaporator and transferred in dichloromethane (3 x 3 ml.) to a stoppered conical test-tube, the solvent being evaporated to dryness under a stream of nitrogen.

Purification. Two methods of measurement of plasma DOC concentration by gas chromatography were tested, the second being an attempt to simplify and improve the first.

Method 1. The dry residue was treated overnight with pyridine (0.05 ml) and acetic anhydride (0.05 ml.) at 35°C. The excess reagents were then removed in vacuo and the residue was chromatographed in system 1 with 20 µg. each of progesterone and the acetates of DOC and testosterone on adjacent

lanes separated by a trough. The standards were located in u.v. light (220 - 230 nm.).

The region of the plasma extract lane corresponding to the DOC acetate standard was removed and eluted with methanol (3 x 3 ml) which was then evaporated under a stream of nitrogen at approximately 35°C. To the residue was added 0.1 ml saturated methanolic sodium carbonate and the mixture left at room temperature overnight to hydrolyse the steroid acetate. The methanol was evaporated and the residue chromatographed in system 2. In this case, 20 ug. DOC was used as standard. The DOC region was located and eluted dropwise with methanol (1 ml.) and a sample (0.1 ml) was taken for counting of ^{3}H in a liquid scintillation spectrometer (54% efficiency) using scintillation mixture 2. The remainder was evaporated to dryness and dissolved in 100 ul. benzene preparatory to g.l.c.

Method 2. The dry residue from the neutral plasma extract was partitioned between 90% aqueous methanol (2 ml) and n-hexane (2 ml.) and the hexane layer was discarded. The methanol was evaporated to dryness under a stream of nitrogen and the residue was chromatographed on paper in Bush B3 system with standards of DOC, testosterone and progesterone (20 ug. each) in

adjacent separated lanes. The standard steroids were located in u.v. light (220 - 230 nm.) and the region corresponding to DOC in the sample lanes was eluted dropwise with methanol (1 ml). The methanol eluate was treated as described under method 1.

Gas-liquid chromatography

Preparation of standard heptafluorobutyrates. 10 ng. of steroid was dissolved in benzene (10 ul) heptafluorobutyric anhydride (10 ul) was added and the mixture was incubated at 60°C for 30 minutes. Excess reagents were removed at 60°C under a stream of nitrogen. Conversion was in excess of 98% (see later). The residue was dissolved in benzene and dilutions ranging from 25 - 500 pg/ul were made.

Plasma extracts. HFBA (10 ul) was added to the purified plasma extract and the mixture incubated at 60°C for 30 minutes. Excess reagents were removed as previously described and progesterone HFB (500 pg) in benzene (50 ul) was added to the sample. Between 1 and 25% of the total extract was applied to the g.l.c. column.

4.1.3. RESULTS

Characterisation of the plasma deoxycorticosterone derivative. Partial characterisation of the plasma deoxycorticosterone derivative was obtained by comparing some of its physical properties with those of the authentic derivative prepared according to the method of Dehennin and Scholler (1969) and recrystallised twice from methanol.

(a) Gas-liquid chromatography. In both cases a single major peak was obtained with g.l.c. together with two minor peaks accounting for less than 1% of the total peak area. Retention times of the authentic material and the plasma product were identical on different g.l.c. columns (1% OV1, 19 min; 1% OV17, 9.4 min; 1% QF1, 22 min; 1% OV 25, 6 min; 1% OV 225, 10 mins.). Gas liquid chromatography of mixtures of plasma product and authentic compound gave no indication of differences in chromatographic properties.

(b) Mass spectroscopic analysis. Results of mass spectroscopic analysis of the gl.c. effluent suggested that the plasma product was identical to the authentic deoxycorticosterone HFB (Fig. 3). Both preparations showed relatively little fragmentation. The ion of highest mass in both spectra was 722, corresponding to the molecular ion

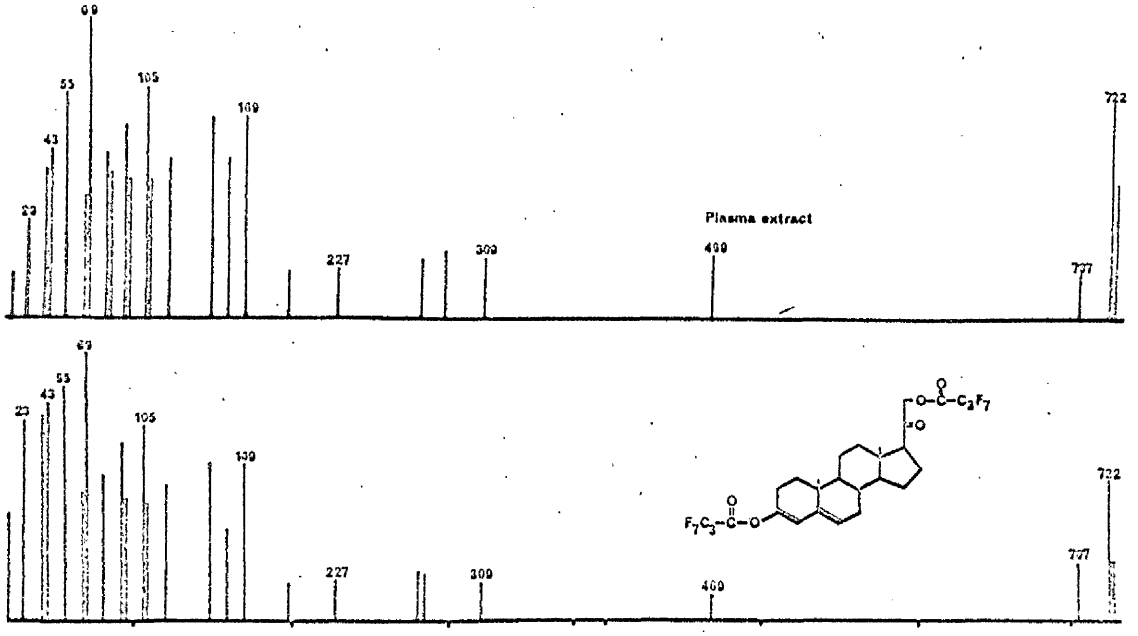

Figure 3.

Mass spectrograph of authentic DOC bis HFB and plasma product on esterification. The upper diagram shows the fragmentation pattern of the plasma extract and the lower the authentic steroid derivative.

of deoxycorticosterone HFB. The retention index value (Kovats 1958) was 2778 in each case.

The two minor peaks observed on g.l.c. showed apparent molecular ions at m/e 465 and 568.

Removal of endogenous plasma progesterone. Since progesterone HFB was used as an internal standard for g.l.c. it was necessary to show that all endogenous plasma progesterone was removed during chromatographic purification. On no occasion when random plasma extracts were analysed without addition of the internal standard was there a perceptible g.l.c. peak in the progesterone HFB region. Further confirmation was obtained by adding 50 ug. progesterone to each of five normal plasma samples before extraction and purification by methods 1 and 2. Again no evidence of progesterone contamination was present on g.l.c.

Efficiency of esterification with HFBA. Deoxycorticosterone (5 ug.) was treated with HFBA using the proportions and conditions employed in the method described previously for measuring deoxycorticosterone in plasma. The product was analysed using g.l.c. with flame ionisation detection in order to demonstrate the presence or absence of residual deoxycorticosterone. With the exception of two minor peaks mentioned above, neither of which corresponded to deoxycorticosterone, a single peak

corresponding to that of DOC HFB was obtained.

In a further experiment, deoxycorticosterone (5 ug.) containing ^{3}H-deoxycorticosterone (10,000 c.p.m.) was esterified as described previously and the product chromatographed in system 2. The region corresponding to deoxycorticosterone was eluted but contained no radioactivity, suggesting that esterification was quantitative. However, only 51% of the activity was found in the deoxycorticosterone region, the remainder occurring between deoxycorticosterone and deoxycorticosterone HFB. This was possibly due to partial decomposition of deoxycorticosterone HFB. Finally, three samples of deoxycorticosterone (1500 c.p.m.) were esterified and the products chromatographed on paper (cyclohexane: deoxan; methanol: water; 4:4: 2: 1, by vol.)

Recovery of ^{3}H from the DOC HFB region ranged from 98.5 to 99.3 (mean 98.9)and from the deoxycorticosterone region 0.7% to 2.5% (mean 1.1%).

Efficiency of the detector by-pass valve. Aliquots of esterified plasma extract were submitted to gl.c. with and without the valve in circuit. Traces obtained without the valve were unsuitable for measurement due to detector contamination, loss of

sensitivity and peak distortion whereas those obtained with the valve presented no such problems (Fig.4).

No broadening or tailing of peaks was observed when the valve was in use. Standard deoxycorticosterone HFB samples (5 to 200 pg) applied to the column with and without the valve in circuit gave identical peak areas indicating that no leakage or decomposition of samples was occurring in the valve.

Standard curve. The heptafluorobutyrates of deoxycorticosterone and progesterone had retention times of 19 and 11 minutes respectively, under the conditions employed. Calibration curves were obtained from duplicate estimations of deoxycorticosterone-HFB within the range 25-200 pg. The peak areas in mm^2 from 10 consecutive standard curves, expressed as means $\pm$ S.D. were 56.3 $\pm$ 2.5, 117.6 $\pm$ 7.7, 218.0 $\pm$ 8.2, 326, 1 $\pm$ 7.1, 438.1 $\pm$ 8.2 for 25, 50, 100, 150 and 200 pg of deoxycorticosterone respectively. For these data, a linear regression line, $y = 6.48 + 2.13 x$ was obtained and a correlation coefficient (r) of 1.00. The peak area of each sample was expressed relative to the peak area of the internal standard (progesterone-HFB). The calculated concentrations of DOC were

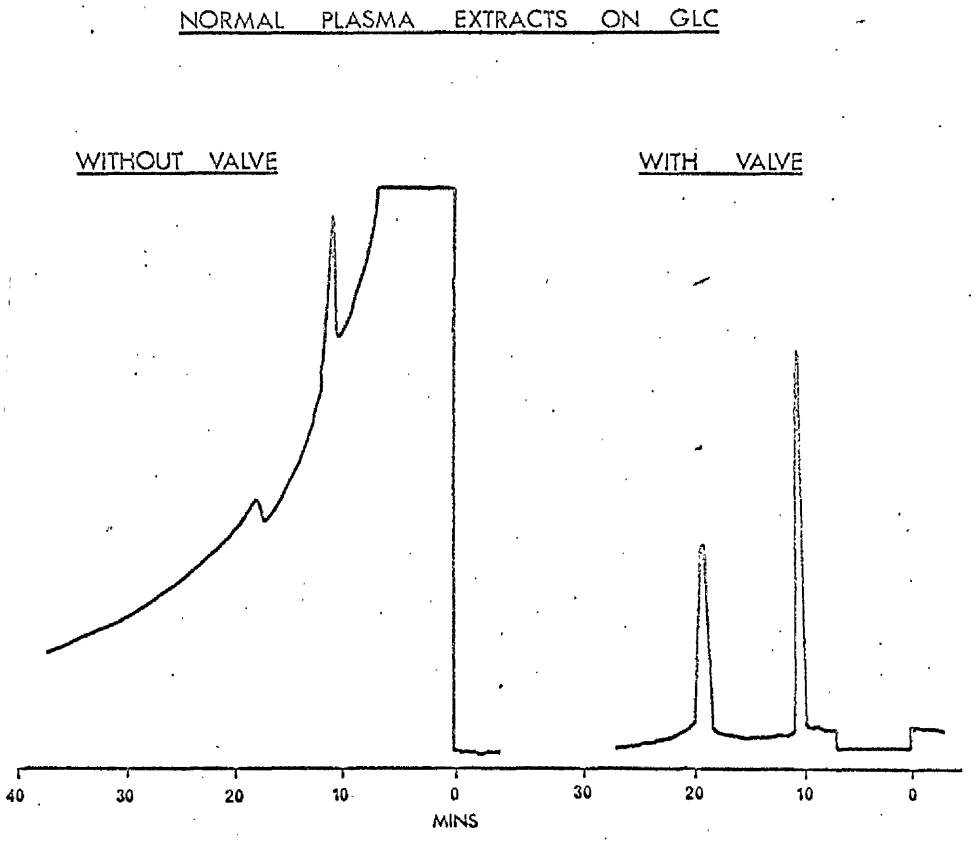

Figure 4.

GLC traces of normal plasma extract applied to column with and without valve in circuit.

corrected for losses from the recovery of ^{3}H-deoxycorticosterone.

Blanks.

Water. Two samples of water were assayed with each batch of plasma samples. On no occasion was any peak corresponding to DOC-HFB detected in these samples although peaks corresponding in position to testosterone HFB were found when the valve was not employed.

Plasma. No DOC-HFB could be detected in two 25 ml. samples of plasma from a bilaterally adrenalectomised patient maintained on cortisol and 9α-fluorocortisol.

Yield of ^{3}H deoxycorticosterone.

Method 1. The mean ($\pm$ S.D.) recovery of (^{3}H) deoxycorticosterone after purification (method 1) was 41.56 $\pm$ 16.04 (n = 226). The mean recovery of (^{3}H)-deoxycorticosterone after each stage of purification is shown in Table 5.

Method 2. The mean ($\pm$ S.D.) recovery of (^{3}H) deoxycorticosterone after purification (method 2) was 68.45 $\pm$ 14.62 (n = 200).

The mean recovery of ^{3}H - deoxycorticosterone after each stage of purification is shown in Table 6.

Stage of purification	Mean Recovery of (^{3}H) DOC	n
Extraction, washing	99.5%	8
Acelylation, t.l.c. 1, elution	85.5%	8
Hydrolysis, t.l.c. 2, elution	66.9%	8

TABLE 5.

Mean recovery of (^{3}H) deoxycorticosterone after each stage of purification method 1.

Stage of purification	Mean total Recovery (^{3}H) DOC	n
Extraction, washing	94.0%	10
Partition	90.6%	10
Paper chromatography, elution	75.4%	10

TABLE 6.

Mean recovery of (^{3}H) deoxycorticosterone after each stage of purification method 2.

Recovery of exogenous unlabelled DOC. After addition of 5, 10, 20 ng. deoxycorticosterone to sample (25 ml. each) of a normal plasma pool the means of duplicate recoveries (method 1) were 93%, 100%, 92% and 94% recpectively.

Precision and sensitivity.

Method 1. Replicate analysis was carried out on plasma (25 ml. each) from four different pools of normal plasma. These gave mean ($\pm$ S.D.) concentrations of DOC (in ng/100 ml) as follows:-

pool (i)	18.1 $\pm$ 2.32	(n = 7) ,	12.7 (CV)
pool (ii)	15.0 $\pm$ 3.16	(n = 8) ,	21.0 (CV)
pool (iii)	6.7 $\pm$ 1.08	(n =7) ,	16.1 (CV)
pool (iv)	10.2 $\pm$ 1.18	(n = 10) ,	1.0 (CV)

(CV - coefficient of variation)

Precision at lower concentration was determined using a plasma pool from a normal subject treated with dexamethasone. In this case, plasma concentration was 3.28 $\pm$ 0.03 ng/100 ml (n = 7). Using the standard deviation of the mean of this pool the sensitivity for single estimations calculated from the formula $\frac{10\ t\ .\ s}{\sqrt{n}}$ (see Braunsberg and James, 1961) was 0.5 ng/100 ml. (p = 0.05, max index of precision = 10% n = 1 where t is a critical value of students t,s an estimate of the standard deviation near the limit of sensitivity of

the method, and n the number of replicate determinations). Linearity of response as compared with the calibration curve was also tested by analysing in duplicate different volumes of plasma pool. The mean content in ng of 5, 10, 15 and 25 ml. of plasma were 0.24, 0.67, 0.90, 1.75. For these data a linear regression equation $y = 0.095x - 0.03$ was obtained with a correlation coefficient (r) of 0.93. Between batch variation was determined by duplicate analyses included in five different assays. These gave a mean concentration ($\pm$ S.D.) of 10.2 ± 1.8 ng/100 ml.

Method 2. Replicate analyses were carried out on plasma (10 ml. each) from three different pools of normal plasma. These gave mean ($\pm$ S.D.) concentrations of deoxycorticosterone (ng/100 ml) of 7.5 ± 0.44 ($n = 5$), 10.0 ± 1.21 ($n = 12$) and 11.3 ± 1.39 ($n = 5$). Linearity of response as compared to the calibration curve was tested by analysing in triplicate different volumes of plasma pool. The mean content of DOC in ng. of 5, 10 and 20 ml of plasma were 0.5, 0.98, 1.95 ($r = 1.0$) respectively. For these data a linear regression equation $y = 0.097x + 0.015$ was obtained. Between batch variation was determined by duplicate analyses included in twelve different assays. These gave a mean DOC concentration ($\pm$ S.D.) of 10.0 ± 1.54 ng/100 ml.

Eleven samples were analysed using both methods (1 and 2) of purification and the results were correlated (Fig. 5). For these data a linear equation $y = 0.068 + 0.91x$ was obtained and a correlation coefficient (r) of 0.976.

The specificity of the method was further examined by comparison of results obtained on the same sample analysed by gas-chromatography (method 1) and radioimmunoassay (James et al, 1971). For these data a linear regression equation $y = 2.7 + 0.816x$ was obtained with a correlation coefficient (r) of 0.872 (Fig. 6).

Normal range. Samples from 30 normal ambulant subjects aged between 20 and 40 years (25 male, 5 female) were taken between 10.00 h and 11.00 h. None of the females was pregnant or taking oral contraceptives. Concentrations range from 4.1 to 13.2 ng/100 ml with a mean of 8.7 ng/100 ml (see Table 20). Details of this method with studies of its reliability have been published (Wilson and Fraser, 1971).

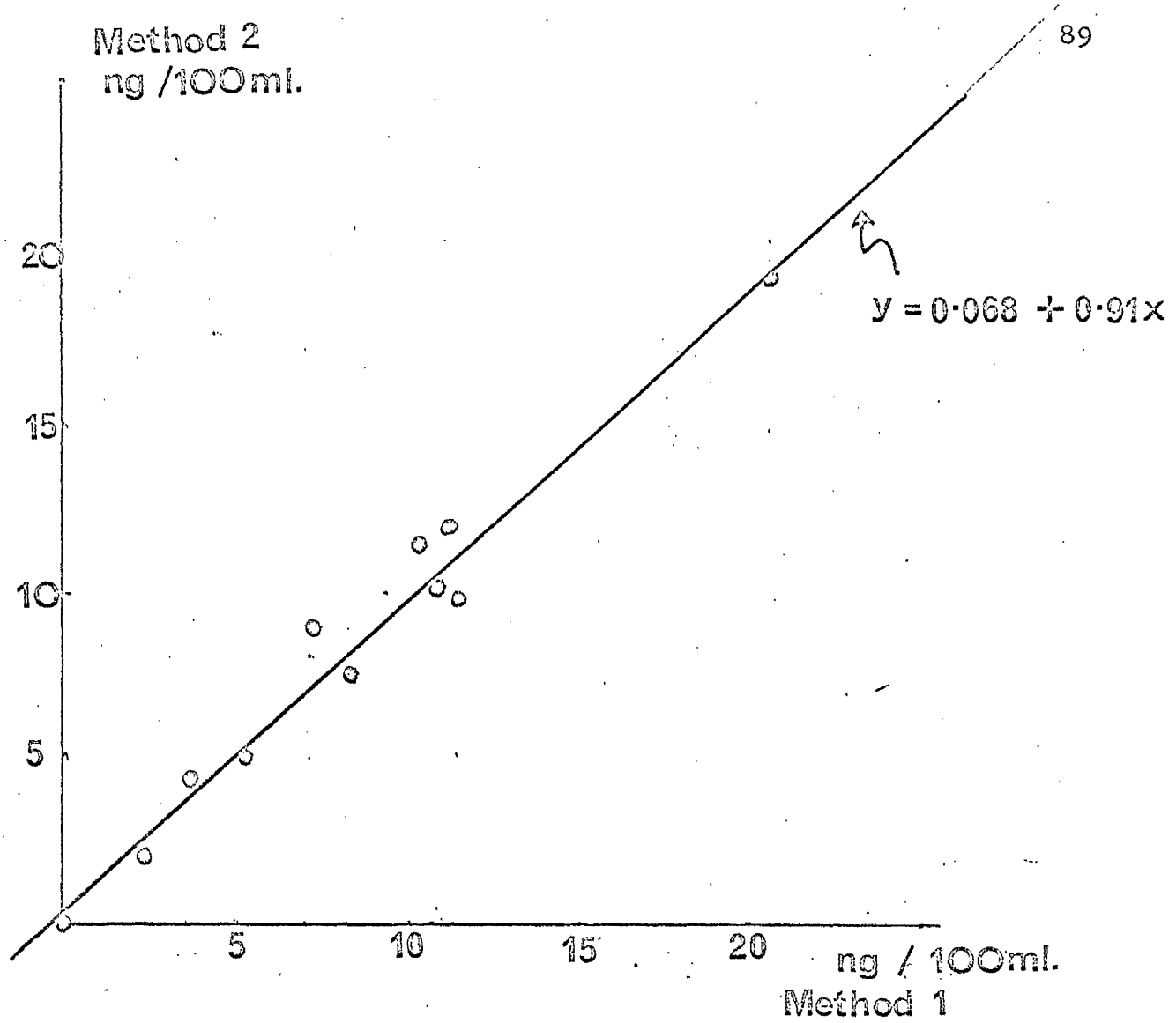

FIGURE 5.

Correlation of plasma DOC concentration measured by GLC methods 1 and 2. These data gave a regression $y = 0.068 + 0.91 x$ with a coefficient of correlation (r) of 0.976.

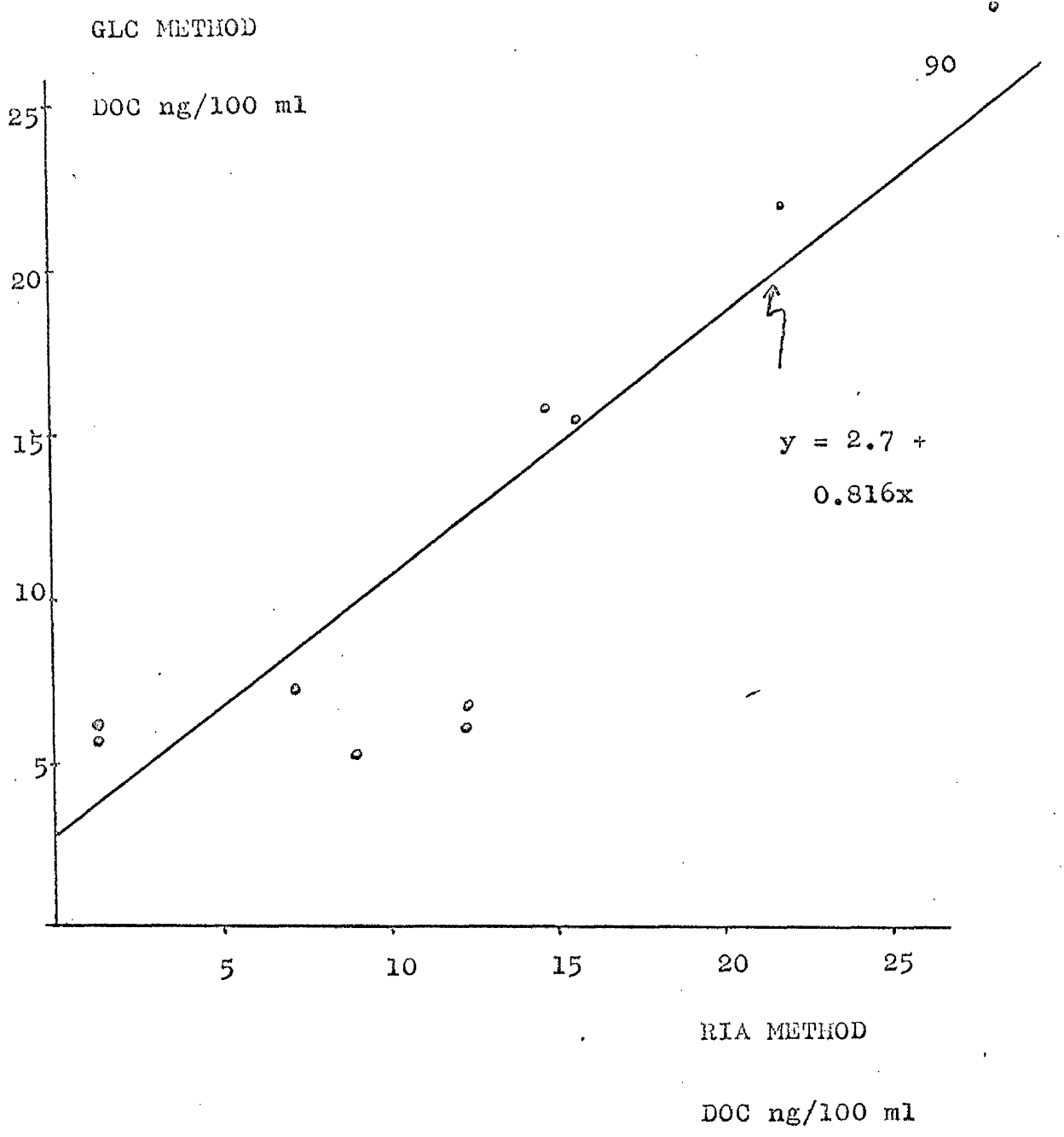

Figure 6. Correlation of plasma DOC concentrations measured by GLC (method 1) and radioimmunoassay (James et al, 1971). Radioimmunoassay was kindly carried out by Professor V.H.T. James of St. Mary's Medical School. For these data a regression equation $y = 2.7 + 0.816 x$ with a coefficient of correlation (r) of 0.872, was obtained.

4.2. Measurement of plasma DOC concentration by radioimmunoassay.

This section of the thesis describes the development and assessment of a method for measurement of plasma DOC concentration using antiserum raised to DOC. Methods of preparation of immunogens, immunisation programmes, testing of antisera, are described.

4.2.1. Additional Materials required for radioimmunoassay.

Solvents. Dioxan and ethylene glycol (B.D.H. Ltd) were reagent grade and analytical grade respectively.

Reagents. Boric acid, succinic anhydride, gelatin, tri-n-butylamine and isobutylchlorocarbonate (B.D.H., Ltd.) were reagent grade. Dextran T-80 (Pharmacia Ltd), o-carboxymethyloxime hemihydrochloride (Aldrich Chem. Co.) were used without prior purification. Charcoal (Norit A, activated, B.D.H.Ltd) was washed with methanol and dried in a vacuum oven prior to use. Tris buffer (Tris-hydroxymethyl-methylamine: Boehringer Corp.) was used without purification.

Biochemicals. Bovine serum albumin (Armour Pharmaceuticals, Ltd), γ-globulins (Cohn fraction II, rabbit and bovine) and poly-L-lysine (MW, 175,000) were obtained from Sigma, Ltd. and were used without purification.

Nicotinamide adenine dinucleotide (reduced, $NADH_2$) and 20β- hydroxysteroid dehydrogenase solution were obtained from Boehringer Corp. and were not further purified prior to use. Nicotinamide adenine dinucleotide (NAD, Grade III) and Freund's complete adjuvant were obtained from Sigma, Ltd. and were used without purification.

4.2.2. Preparation of steroid protein conjugate

(a) Preparation of

(i) Aldosterone-3 carboxymethyloxime

(ii) DOC- 3 carboxymethyloxime.

Aldosterone and deoxycorticosterone both possess 20 - carbonyl groups which may interfere in the preparation of the 3- carboxymethyloxime. In order to prepare specifically the 3-oxime and prevent the formation of any 3, 20-dioxime, the 20-carbonyl group was protected by enzymic reduction to the 20β- hydroxy group. After formation of the 3-oxime the 20β- hydroxy group was oxidised enzymatically to the 20 - carbonyl group. Using this 3-stage method (A), standard 3 - carboxymethyloximes of aldosterone and DOC were prepared and used to monitor the direct preparation of these oximes from the free steroids by method (B).

Method A.

(i) Reduction of 20 carbonyl group. The free steroid (20 x 200 ug.) containing (^{3}H)- labelled steroid (100,000 cpm) was dissolved in tris buffer (1.8 ml., 0.1M, pH 7.3) and $NADH_2$ solution (10 ul. 5 mg/ml. tris buffer pH 8.0) and 20β-hydroxysteroid dehydrogenase (5 ul., 1 mg/ml. aqueous) were added (Henning and Zander, 1962).

After incubation at 37°C for 48 hours, glass distilled water (1 ml.) was added and the mixture was extracted with ethyl acetate (3 x 3ml). The combined extracts were evaporated to dryness under a stream of nitrogen and then applied in dichloromethane to an I.T.L.C. plate with standard DOC and aldosterone in adjacent lanes. The plate was developed in a hexane/acetone mixture (3:1, v/v for 1 hour).

R_F values for DOC, aldosterone and their corresponding 20 β- hydroxy derivatives were 0.55, 0.20, 0.40, 0.08 respectively. The areas corresponding to the 20 β - hydroxy derivatives were dropwise eluted with methanol. The yields of aldosterone and DOC derivatives were 25% and 31% respectively.

(ii) Formation of 3-carboxymethyloxime derivative. '20 β -hydroxy steroid' (0.5 mg) was refluxed for 1 hour with o-carboxy-methylamine hemi-hydrochloride (4 mg.) in a solution of sodium hydroxide (0.4 ml., 5% aqueous) and ethanol (5 ml). On cooling the mixture was adjusted to pH 6 with hydrochloric acid (0.5 M) and the ethanol was then removed under a stream of nitrogen. After addition of water (10 ml.) the mixture was re-adjusted to pH 10 with sodium hydroxide solution (0.1M) and extracted with dichloromethane (20 ml) and the extract was discarded. The solution was adjusted finally to pH2 with

hydrochloric acid (0.5M) and extracted with dichloromethane (3 x 25 ml.).

The extract was evaporated to a small volume and applied to an ITLC plate which was developed in hexane-acetone mixture (3:1, v/v) for 1.5 hours R_F values were recorded as follows.

	R_F
DOC	0.58
'20-dihydro -DOC'	0.40
20-dihydro DOC'-3 carboxymethyloxime	0.05
aldosterone	0.30
20-dihydro aldosterone'	0.10
20-dihydroaldosterone' - 3 carboxymethyloxime	0.02

The area corresponding to the carboxymethyloxime derivatives were eluted with methanol (5 ml.). Yields of the DOC and aldosterone derivatives were 75% and 61% respectively.

(iii). Oxidation of 20β-hydroxy group. '20β-hydroxysteroid' - 3 carboxymethyloxime (10 x 30 ug) was dissolved in tris buffer (1.8 ml., 0.1M, pH. 7.3) and NAD solution (10 ul. 5 mg/ml. tris buffer pH 8.0) and 20β-hydroxysteroid dehydrogenase solution (5 ul., 1 mg/ml) were added. The solution was incubated at 37°C for 48 hours and was then extracted with dichloromethane and chromatographed as described in section 1 of method A. R_F values were

recorded as follows:-

	R_F
DOC	0.58
20-dihydro DOC' 3-carboxymethyloxime	0.05
DOC-3-carboxymethyloxime	0.2
Aldosterone	0.30
20-dihydroaldosterone-3-carboxymethyloxime	0.02
Aldosterone-3 carboxymethyloxime	0.10

Yields of DOC and aldosterone 3-carboxymethyloxime derivatives were 65% and 60% respectively. Overall yields of these two derivatives were 15.1% and 9.1% respectively.

Method B.

Direct preparation of (i) DOC-3-carboxymethyloxime
(ii) Aldosterone-3-carboxymethyloxime

Free steroid (100 ng.) containing (^{3}H)-labelled steroid (100,000 cpm) was refluxed with o-carboxymethyloxime hemihydrochloride (40 mg.) in sodium bicarbonate solution (1 ml., 0.2 M) and ethanol (20 ml) for 10 minutes. On cooling the carboxymethyloxime

derivative was extracted as described in method A, ii.

The extract was evaporated to a small volume and applied in methylene chloride to an ITLC. plate which was developed in hexane-acetone mixture (3:1, v/v) for 1.5 hours. By comparison with standards (prepared in method A) run in adjacent lanes the steroid 3-carboxymethyloxime derivatives were detected and eluted with methanol. Yields of the 3-carboxymethyloxime derivatives of DOC and aldosterone were 65% and 54% respectively.

Preparation of (a) DOC-3-albumin conjugate
(b) Aldosterone-3-albumin conjugate
(c) Aldosterone-3-poly-l-lysine conjugate.

Method. Steroid-3-carboxymethyloxime (100 mg) and tri-n-butylamine (0.05 ml) were dissolved in dioxane (4 ml) and after cooling to 10^{o}C isobutylchlorocarbonate (0.3 ml) was added. The reaction was allowed to proceed for 20 minutes after which the mixture was added in one portion to a well stirred, cooled solution of protein (bovine serum albumin, 0.4 g. or poly-L-lysine, 0.3 g) in water-dioxane mixture (1:1, v/v; 15 ml). containing sodium hydroxide (0.3 ml., IM aqueous).

A further 0.1 ml sodium hydroxide solution was added after 15 minutes in order to adjust the solution to pH 8.5. Stirring and cooling were continued for a further 4 hours and then the solution was dialysed overnight against distilled water (10 l.).

The mixture was adjusted to pH 4.5 with hydrochloric acid (0.1 M) to precipitate the conjugate which after storage overnight at 4^{o}C was collected by centrifugation.

The precipitate was redissolved in water (5 ml.) by adjusting to pH 5.5 with sodium hydroxide (0.1M) and then re-precipitated by addition of acetone and adjusting to pH 5.0 with hydrochloric acid (0.1M). The conjugate was again collected by centrifugation and finally lyophilised.

Preparation of deoxycorticosterone 21 hemisuccinate. A solution of deoxycorticosterone (100 mg.) containing 10,000 cpm (^{3}H) deoxycorticosterone and succinic anhydride (100 mg.) and dry pyridine (5 ml) was refluxed for 4 hours. The reaction mixture was evaporated to dryness under reduced pressure and the semi-solid residue was dissolved in chloroform, washed three times with water, dried over sodium sulphate and then evaporated to dryness under a stream of nitrogen. The residue was recrystallised

twice from acetone. The yield of deoxycorticosterone 21-hemisuccinate was 81% (103 mg.)

Preparation of deoxycorticosterone - 21 albumin conjugate.(d) Deoxycorticosterone-21 hemisuccinate (100 mg.) and tri-n-butylamine (0.05 ml.) were dissolved in dioxane (4 ml.) and after cooling the solution to 10°C isobutylchlorocarbonate (.03 ml.) was added. The reaction was allowed to proceed for 20 minutes at 4°C after which the solution was added in one portion to a well stirred cooled solution of bovine serum albumin (0.4 g.) in 1:1 water dioxane (15 ml.) and sodium hydroxide (0.3 ml., 1M). A further 0.1 ml. of sodium hydroxide was added after 15 mins. to adjust pH from 6.8 to 8.5. Stirring and cooling were continued for 4 hours. The solution was dialysed overnight against glass distilled water (10 l) after which the mixture was adjusted to pH 4.5 with hydrochloric acid (1M). The product precipitated and after storage at 4°C overnight was collected by centrifugation. The precipitate was redissolved in water (5 ml.) by adjusting to pH 5.5 with sodium hydroxide (0.1 M). The conjugate was reprecipitated by addition of acetone (4 ml.), adjusting to pH 5.0 with hydrochloric acid (0.1M), and collected by centrifugation subsequently to be lyophilised. The yield of the reactions are shown in

Table 7. The number of steroid residues per protein molecule was calculated from the yield of the reaction and the recovery of (^{3}H)-steroid and the results are shown in Table 7.

	a.	b.	c.	d.
% Yield (^{3}H)- steroid	48	51	31	45
Moles steroid in conjugate	109	137	55	105
Yield conjugate (mg)	370	391	250	310
Moles protein in conjugate x 10^{-6}	4.43	4.72	1.3	3.75
Steroid molecules per protein molecule	24.6	29.0	26.1	28.3

TABLE 7.

Preparation of steroid protein conjugates.

Yield of reaction and number of steroid residues per protein molecule.

4.2.3. Programme for immunisation and antibody titration.

Rabbits. 24 male New Zealand White rabbits (weight approximately 3 Kg) were used for this study.

1 mg. of steroid protein conjugate in aqueous suspension (0.5 ml.), was emulsified with Freund's complete Adjuvant (0.5 ml.) and injected subcutaneously in both thighs.

Booster injections were given in the same way, monthly, for a period of five months followed by a 3 month rest after which booster injections were continued monthly. One week after the third and all subsequent injections, a blood sample (5 ml.) was taken from the middle ear vein and the serum stored for antibody titration studies.

Guinea pigs. 12 male Duncan Hartley guinea pigs were used for this study.

0.5 mg. of steroid-protein conjugate in aqueous suspension (0.25 ml.) emulsified with Freunds complete Adjuvant was injected subcutaneously in both thighs. Booster injections were given after 1, 2 and 3 months, then the animals were exsanguinated 1 week later by cannulating the carotid artery. Serum was stored for antibody titration studies.

The immunogen used for each rabbit and guinea pig is shown in Table 8.

IMMUNOGEN	RABBIT NO.	GUINEA PIG NO.
DOC - 3- ALBUMIN	1 - 10	1 - 3
DOC- 20- ALBUMIN	11 - 15	4 - 6
ALDOSTERONE- 3- ALBUMIN	16 - 21	7 - 9
ALDOSTERONE - 3- POLY-L- LYSINE	22 - 24	10 - 12

TABLE 8

Immunogen used for each rabbit and guinea pig.

4.2.4. Assessment of Antisera.

Antiserum dilution curves. The presence of antibodies in each antiserum was demonstrated by preparing antiserum dilution curves.

Dilution and incubation. Doubling dilutions of each antiserum were prepared in 0.1M borate buffer pH 8.0, containing 5% methanol and 0.5% gelatin. 0.5 ml. of each antiserum dilution was added in duplicate to glass test-tubes (2.5 ml) containing (^{3}H)-steroid (2000 cpm). The tubes were well mixed then incubated at room temperature for 3 hours then at 4°C in an ice bath for 1 hour.

Separation of bound and free fractions. 0.5 ml. dextran coated charcoal suspension (1:6) was added to each sample which was well mixed and allowed to stand for 5 minutes before centrifuging at 4°C at 2,500 rpm. for 10 minutes. 0.5ml. of the supernatant liquid which contained the bound (^{3}H)-steroid was removed for estimation of ^{3}H by liquid scintillation counting. Per cent bound (^{3}H)-steroid was plotted against antiserum dilution (see Table 9).

Standard Curves. Standard curves were prepared for antiserum at dilutions at which 50 - 60% of ^{3}H-steroid was bound.

RABBIT NO.	IMMUNOGEN	DILUTION AT WHICH 50% (^{3}H) - DOC WAS BOUND
1	DOC - 3-	1:850
2		1:10
3	Albumin	1:700
4		1:800
5		1:5
6		1:5
7		-
8		-
9		1:5
10		1:6
11	DOC - 21-	1:50
12		1:5
13	albumin	1:8
14		-
15		-
16		1:30
17	Aldosterone -	1:5
18	3 - albumin	-
19		-
20		1:60
21		1:200
22	Aldosterone -	-
23	3-poly-l-	-
24	lysine	-
	Normal Rabbit Serum.	1:9

TABLE 9

Dilution of antisera required for 50% binding of (^{3}H) - steroid. Rabbit - 3 immunisations.

Incubation. 0.5 ml. antiserum, appropriately diluted in borate buffer (0.1M, pH 8, 5% methanol, 0.5% gelatin), was added in triplicate to glass test-tubes (2.5 ml.) containing (^{3}H)-steroid and steroid (0, 6.25, 12.5, 25, 50, 100, 200 pg). The tubes were well mixed and then incubated at room temperature for 2 hours followed by incubation at 4^{o}C in an ice bath for 1 hour.

Separation of bound and free steroid. Bound and free steroid were separated as previously described using dextran-coated charcoal. Bound steroid was estimated by liquid scintillation counting.

Per cent bound (^{3}H)-steroid was plotted against pg added steroid in each sample (see Table 11).

Results of assessment of antisera. Dilution curves obtained with normal rabbit and guinea pig sera are shown in Tables 9 and 12.

Rabbit antisera 1 - 10. (DOC). The antibody titration data obtained after three immunisations are shown in Table 9. The improvement in antibody titre in rabbits 1, 3 and 4 is shown in Table 10. The titre in the remaining rabbits did not improve after subsequent immunisations and were not

Month of Injections	Dilution for 50% bound (^{3}H) - steroid for each antiserum 1 : x					
	1	3	4	16	20	21
3	850	700	800	300	60	200
4	1000	750	1000	1000	200	1000
5	5000	2000	2500	6000	500	1500
6	8000	3000	7500	10000	1000	2000
7	10000	7500	10000	25000	2500	2000

TABLE 10

Improvement in antibody titre in individual rabbits during several months of immunisation.

Antiserum	Dilution 1 : x	% bound(^{3}H)-steroid			% Displacement (^{3}H)-steroid	
		0 pg	50pg	100 pg	0-10 pg	0 - 100 pg
1	8,000	75	47	38	7.5	37
	10,000	75	40	30	10	45
	12,000	63	38	29	6	34
3	5,000	76	57	47	8	29
	7,500	56	36	28	6	28
4	5,000	63	43	31	5	32
	7,500	52	36	26	4	26
	10,000	40	26	18	4	22
16	1,500	85	68	59	5	26
	2,500	64	47	37	5	27
20	2,000	80	62	51	6	29
	2,500	62	45	35	5	27
	3,000	57	41	31	5	26
21	2,500	82	57	42	8	40
	3,000	64	47	38	5	26

TABLE 11.

Sensitivity data for steroid antisera showing dilution of each antiserum and corresponding per cent bound (^{3}H) steroid. Incubation conditions and separation of bound and free steroid are described in section 4.2.

GUINEA PIG NO.	IMMUNOGEN	DILUTION AT 50% BOUND (^3H)-STEROID
1	DOC - 3 - albumin	1:8
2		1:30
3		1:7
4	DOC - 21- albumin	1:9
5		1:30
6		1:6
7	Aldosterone - 3- albumin	1:8
8		-
9		1:50
10	Aldosterone - 3- poly-l-lysine	1:9
11		1:9
12		1:5
	Normal Guinea Pig Serum	1:40

TABLE 12.

Antibody production by guinea pigs.

used for standard curves. Standard curves were prepared with antisera 1, 3 and 4 after 7 immunisations and are shown in Table 11.

Rabbit antisera 11-15 (DOC). The antibody titration data obtained after 3 immunisations are shown in Table 9. No improvement was obtained on subsequent immunisation. No standard curves were prepared.

Rabbit antisera 16-21 (Aldosterone). The antibody titration data obtained after 3 immunisations are shown in Table 9. The improvement in antibody titre in rabbits 16, 20 and 21 after subsequent immunisation is shown in Table 10. No improvement in antibody titre was demonstrated with the remaining rabbits which were not used for standard curves. Standard curves were prepared with antisera 16, 20 and 21 after 7 immunisations and are shown in Table 11.

Rabbit antiserum 22-24. Very low antibody titres were obtained after 3 immunisations (Table 9) and no improvement was demonstrated with subsequent immunisation. No standard curves were prepared.

Guinea pigs. Very low antibody titration curves were obtained with all guinea pig antisera (Table 12).

No standard curves were prepared.

4.2.5. MEASUREMENT OF PERIPHERAL PLASMA DOC CONCENTRATION BY RADIOIMMUNOASSAY

Method

Plasma. Plasma samples were collected as described in section 4.1. Two ml. was used for each assay and each batch of assays contained duplicate water blanks (2 ml.) and control plasma samples.

Extraction and Purification. The sample to which approximately 25,000 cpm (^{3}H)-DOC had been added was extracted with 10 volumes of dichloromethane which was then washed consecutively with one volume each of sodium hydroxide (0.1N, aqueous), acetic acid (0.1N) and water before evaporating to dryness. The final residue was chromatographed on methanol-washed paper (Whatman 2) in system 1 for seven hours. The DOC region was located by radioscanning (Panax, Ltd.) and eluted with methanol. An aliquot of the eluate was assessed for (^{3}H) recovery and, on this information, duplicate aliquots containing 5000 cpm (^{3}H)-DOC were taken for radioimmunoassay.

Radioimmunoassay. Samples and duplicate (0. - 300 pg) containing 5000 cpm (^{3}H)-DOC and 1% ethylene glycol were evaporated to dryness in 2 ml. glass tubes in a vacuum oven at 35°C. Antiserum solution of appropriate dilution (DOC 1, 1:10,000;

0.5 ml. containing 2% methanol and 0.5% bovine gamma globulin in borate buffer, pH 7.5).was added to each tube and mixed with a vortex mixer for 10 seconds. They were then left at room temperature for 1 hour and at $4^{o}C$ for a further hour before absorbing the free steroid component with dextran-coated charcoal (0.5 ml, 0.25% w/v dextran and 0.25% w/v Norit A charcoal in borate buffer, pH 7.5). If convenient, the $4^{o}C$ incubation was extended overnight (see later).

After centrifugation at $4^{o}C$ (5 - 10 minutes, 2500 cpm) a 0.5 ml aliquot was taken for measurement of (^{3}H) content.

Results.

Effect of pH on sensitivity of standard curve. In order to determine optimal pH at which to incubate samples, standard curves (0, 50, 100 pg. DOC) were prepared at pH 6.5, 7.0, 7.5, 8.0 and pH 8.5 using borate buffer (0.1M). Results are shown in Table 13.

Maximum binding and the most sensitive standard curve was obtained at pH 7.5.

Temperature and time of incubation. The effect of variation in temperature and in duration of incubation on the sensitivity of the standard curve was studied by preparing replicate standard samples containing 0, 50 and 100 pg deoxycorticosterone and incubating them at room temperature for 5, 3, 2 and 1 hour

Incubation pH	% Binding (^{3}H) - DOC		
	0 pg	50 pg	100 pg
6.5	52	37	28
7.0	60	38	29
7.5	75	42	30
8.0	70	41	29
8.5.	63	39	29

TABLE 13.

Effect of variation of pH of incubation buffer on sensitivity of standard curve DOC 1 (1:10,000).

respectively, followed by 1 hour incubation at 4°C. Another set of standard samples were incubated at 4°overnight. Similar standard curves were obtained with all incubation schedules.

Quantity of ^{3}H deoxycorticosterone. To show the effect of variation of the amount of (^{3}H)-DOC used on the sensitivity of the method, standard curves were set up using 1500, 2000, 2,500 and 5,000 cpm (^{3}H)-DOC. Results shown in Table 14 suggest that the assay is most sensitive when 5000 cpm (^{3}H)-DOC was used.

Separation of bound and free. In order to assess the efficiency of the separation of bound and free steroid and to determine the optimum ratio of dextran/charcoal, the standard curves were prepared and samples were incubated for 2 hours at room temperature and 1 hour at 4°C. Bound and free steroid were separated using half saturated ammonium sulphate or dextran coated charcoal (ratio dextran/charcoal 1:1, 1:2, 1:4, 1:6). Similar standard curves were obtained using both methods

Carrier protein. In order to determine the optimum amount of protein in the incubation mixture for maximum sensitivity, standard curves were set up using bovine γ globulin (0.2 - 1%)

VARIATION OF QUANTITY OF (^{3}H)-DOC

Quantity (^{3}H)- DOC		% binding of ^{3}H-DOC in presence of DOC.		
		0 pg	50 pg	100 pg
1500 cpm		60	45	30
2000 cpm		60	45	25
2500 cpm		60	45	25
5000 cpm		65	40	20

TABLE 14.

The effect of variation of the quantity of (^{3}H)-DOC on the sensitivity of the standard curve obtained with antiserum DOC 1 at a dilution of 1:10,000 in borate buffer.

bovine serum albumin (0.2 - 1%) and gelatin (0.2 - 1%) as carrier protein (see Table 15). Maximum sensitivity was attained using 0.4% -γ globulin. Sensitivity of the assay using gelatin was consistently lower than corresponding standard curves obtained using globulin as carrier protein.

Solubility studies. On several occasions standard curves prepared without ethylene glycol did not produce smooth curves. In samples without DOC binding of (^{3}H)-DOC was anomalously low. This was presumably due to adsorption of the (^{3}H)-DOC to the walls of the tube despite adequate silanisation of tubes or even due to breakdown of the (^{3}H)-DOC when heated at high specific activity in absence of solvent. The problem was solved by addition of ethylene glycol to all samples. This prevented adsorption of DOC to the walls of the tube.

Standard curves. A typical standard curve is shown in Fig. 7.

Quality control data. The reliability of the method was investigated by replicate analysis of two pools of normal plasma and by analysis of blank samples (water, and plasma obtained from an adrenalectomised subject maintained on cortisol and 9α-fluorocortisol). The results shown in Table 16 were satisfactory in that blank activity was not

Concentration of carrier protein	% Binding of (^{3}H) - DOC		
	0 pg	50 pg	100 pg.
ɣ globulin			
0.2%	53	36	27
0.4%	76	42	32
0.6%	65	39	30
0.8%	63	39	31
1.0%	60	43	37
Gelatin			
0.2%	51	34	26
0.4%	71	41	30
0.6%	60	39	27
0.8%	45	31	25
1.0%	40	28	25
Bovine serum albumen			
0.2%	54	32	24
0.4%	62	34	25
0.6%	75	37	27
0.8%	65	46	32
1.0%	65	48	36

Table 15. Effect of variation of protein and protein concentration in incubation buffer on the sensitivity of standard curve DOC 1 (1:10,000).

POOL	MEAN (ng/100) ± SD	COEFFICIENT OF VARIATION.
J	10.07 ± 0.46	3.8
K	8.1 ± 1.05	5.8
Water	0	
Plasma (Adrenalectomised subject)	0	

TABLE 16

Results of replicate analysis of 3 plasma pools and water.

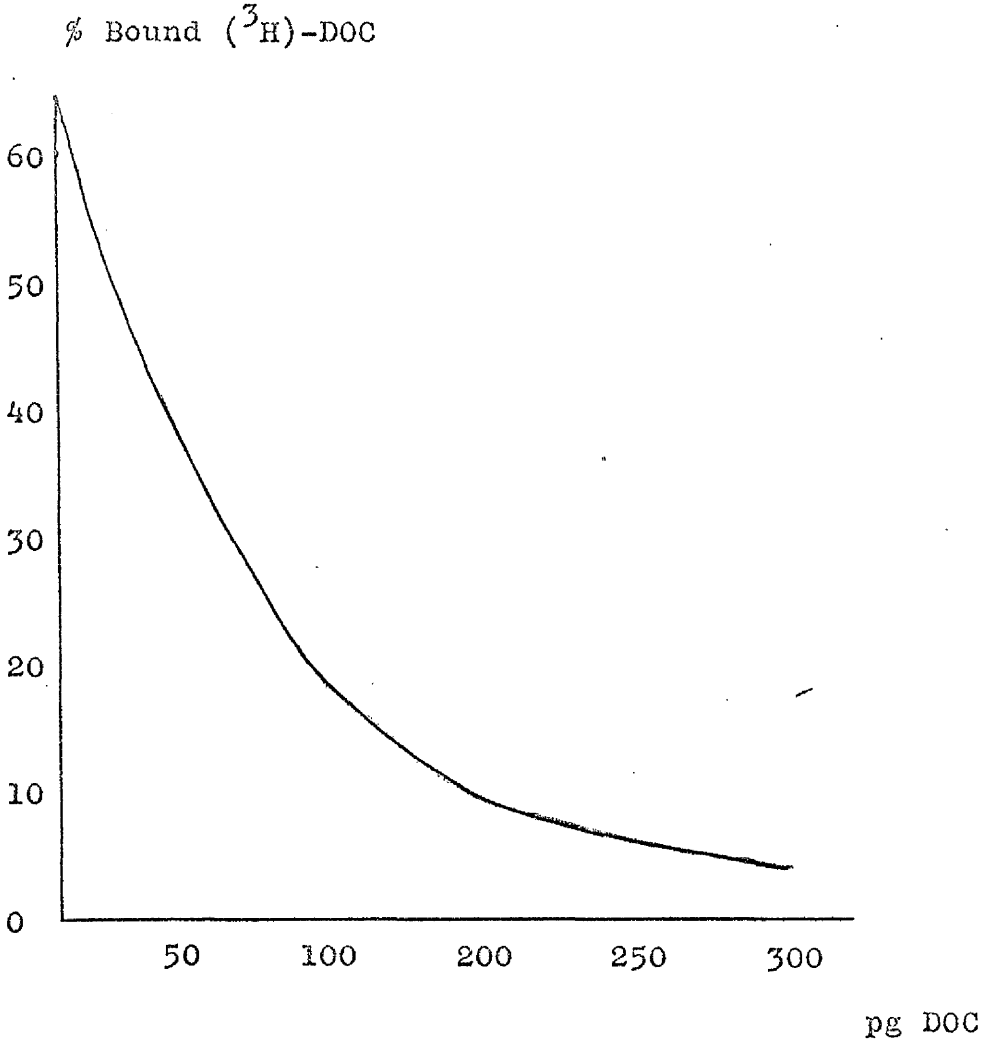

Figure 7 . Antiserum DOC 1. standard curve (dilution 1:10,000) using 5000 cpm (^{3}H)- DOC and dextran coated charcoal (ratio 1:1)

detectable and replicates showed coefficients of variation of 3.8% and 5.8% respectively

Specificity. This was assessed firstly by the comparison of the cross reaction of the antiserum to DOC with that to a selection of compounds likely to be present in normal human plasma and, secondly, by comparing the results of assay with those from gas-liquid chromatography estimation (see below).

Cross reaction studies. Cross reaction of other steroids to antiserum DOC 1 are shown in Table 17. The percentage cross reaction was calculated for each steroid (Abraham, 1969) from the formula $\frac{x}{y} \times 100$, where x is the amount of DOC required to displace 50% of the (^{3}H)-DOC bound to the antibody and y is the amount of a foreign steroid (pg) required to displace 50% of the ^{3}H-DOC from the antibody.

The specificity of the method was further examined by comparison of concentration of deoxycorticosterone measured in the same sample by g.l.c. and by radioimmunoassay. For these data a linear regression equation, $y = 0.83 x + 0.05$ was obtained and a correlation coefficient (r) of 0.89 (Fig 8).

STEROID	% CROSS REACTION
ALDOSTERONE	0.3%
CORTISOL	0.6%
CORTICOSTERONE	0.8%
18 HYDROXY DEOXY-CORTICOSTERONE	0.6%
DEOXYCORTISOL	0.4%
DEOXYCORTICOSTERONE	100%
TESTOSTERONE	0.1%
PROGESTERONE	1.0%

TABLE 17

Cross reaction of major steroids with antiserum DOC 1.

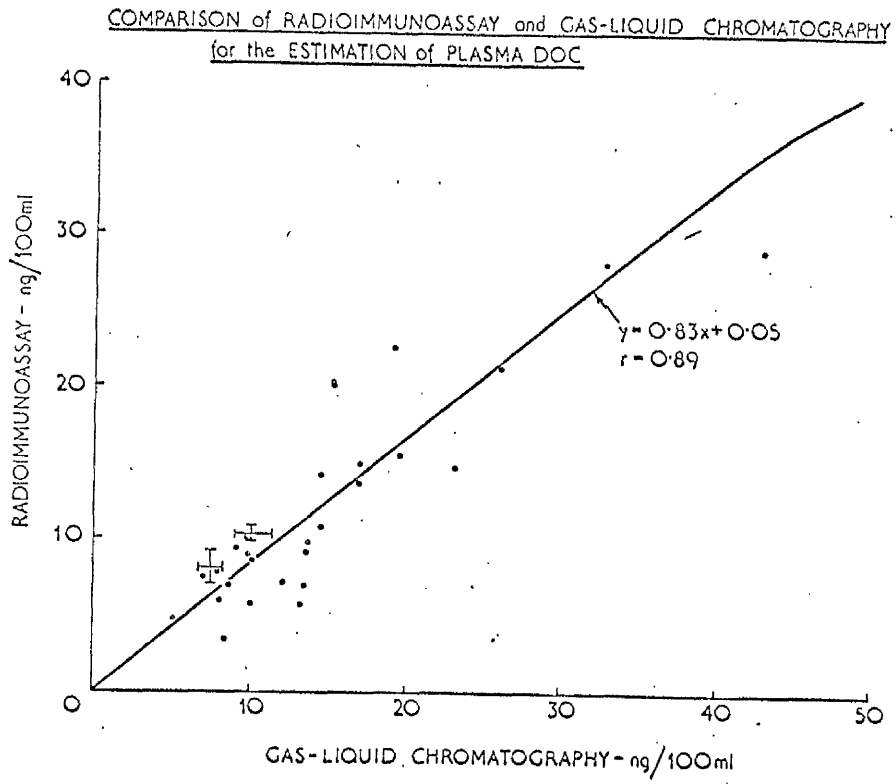

Figure 8. Correlation of concentrations of DOC in plasma sample measured by radioimmunoassay and GLC. For these data a regression equation y = 0.83 x +0.05 with a correlation coefficient (r) of 0.89 was obtained.

4.3. DISCUSSION

In the following discussion, the two techniques developed are examined individually. This is followed by a short comparison of their advantagesand disadvantages.

4.3.1. Gas liquid chromatography with electron capture detection. As previously mentioned in section 3 , gas-liquid chromatography with electron capture detection possesses a high degree of sensitivity providing suitable stable derivatives can be prepared. In the past, this advantage has been somewhat offset by the susceptibility of the detector to massive, irreversible contamination or poisoning with prolonged loss of sensitivity. Removal of the contamination requires extensive chromatographic purification of biological extracts prior to GLC. Unfortunately, chromatographic media such as silica gel or paper themselves contain trace contaminants. Even after acid washing and multiple solvent extraction, silica gel extracts, when esterified with HFBA, still produce g.l.c. peaks indistinguishable from that of testosterone HFB. A large proportion of the contamination is of short retention time and produces a massive detector response which may persist over a long period. The detector returns

very slowly to basal response and sensitivity of the detector may be greatly reduced for several days. Due to this contamination and subsequent loss of sensitivity, the response of the detector to steroid derivatives may be obscured or obliterated.

Use of detector by-pass valve as a means of avoiding detector contamination. (Cranmer, 1968). As stated above, the major fraction of the impurities responsible for detector contamination is of short retention time on gas-liquid chromatography. Diversion of this fraction from the detector by means of a by-pass valve avoids the early contamination, maintains high sensitivity and produces a trace which is suitable for quantitative analysis in the sub-nanogramme range (see Fig. 3). This also allows large fractions of the final purified extracts to be applied to the column without loss of sensitivity, thus improving the sensitivity and accuracy of the assay. A further advantage is that larger numbers of samples can be processed per day without contaminating the detector.

Valve characteristics. Construction of detector by-pass valves in the laboratory poses a number of problems.

The material from which the valve is constructed must be inert. Metals such as brass will catalyse decomposition of steroid derivatives at high temperature. Teflon (P.T.F.E.) proved a suitable material and was found to be chemically inert at temperatures less than 250°C. Unfortunately, certain grades of Teflon lose their inherent rigidity at high temperatures which may cause leaks in the system.

The internal volume of the valve must be low in order to prevent broadening of peaks with loss of chromatographic separation.

Valves constructed in the laboratory remained leak-proof for several weeks after which distortion of the barrel caused leakage and increase in volume. A more efficient commercial valve has since been installed in the gas-chromatograph oven. This rotary valve (Carle, Inc.) is constructed of pure teflon in a rigid metal case and has a very small internal volume. It causes no broadening of peaks or contamination of the detector and has remained completely efficient during several months of continuous use.

Steroid derivatives for gas chromatography with electron capture detection. DOC has been estimated in adrenal venous plasma and adrenal tissue incubates by g.l.c. with electron capture

detection of the acetate derivative (Rapp and Eik Nes, 1965, Seth, 1968). However, the affinity of the acetate derivative is low in comparison to polyhalogenated derivatives. Among the most effective of these is the bisheptafluorobutyrate derivative formed by enolisation of the unsaturated carbonyl group in ring A (Clark and Wotiz, 1963; Exley, 1967; Exley and Chamberlain, 1967; Horning et al, 1968; Horning and Maume, 1969).

Heptafluorobutyrates are used in methods for measuring the plasma concentrations of testosterone, androstenedione (van der Molen and Groen, 1967; Exley 1967; Kirschner and Coffman, 1968), oestrogens (Wotiz et al, 1967; Munson et al, 1970) and catecholamines (Vessman et al, 1969).

The volatility of the bisheptafluorobutyrates allows gas chromatography to be carried out at relatively low temperatures (180° - 220°C), thus reducing the risk of thermal decomposition.

Unfortunately, this advantage is somewhat offset by the instability of these derivatives on conventional chromatographic media such as silica gel (Exley and Chamberlain,1967), although they are slightly more stable on paper chromatography. For this reason, recoveries must be measured before esterification with HFBA. The present results

confirm the findings of Exley and Chamberlain (1967) that the esterification reaction is virtually quantitative. Dehennin and Scholler (1969), however, found 5-15% by products on esterification of a number of steroids with HFBA in acetone solution. It is therefore important to check the yield of the esterification reaction at frequent intervals.

Progesterone HFB was found suitable as an internal standard because of its similar molar response to electron capture detection and comparable retention time. Particular care was therefore taken to ensure removal of all endogenous progesterone in samples prior to gas chromatography.

Dehennin and Scholler (1969) studied in detail the mechanism of formation and the physico-chemical properties of steroid heptafluorobutyrate derivatives including DOC bis HFB. Results from gas chromatography - mass spectrometry and Kovats retention index reported are compatible with the findings of Dehennin and Scholler.

Other derivatives such as hexadecafluoro-octanoates reported by Kirschner and Taylor (1969), Exley and Dutton (1969), also have very high responses to electron capture detection and greater stability on thin layer chromatography and have also proved to be useful for measuring plasma concentrations of steroids.

However, at the time this work was begun the necessary reagents were not available in pure form.

Specificity and accuracy. Gas chromatographic mass spectrometric analysis of the purified and then esterified material from plasma indicated that the DOC HFB peak was pure. The absence of detectable DOC in the plasma from an adrenalectomised patient is additional evidence for the high degree of specificity achieved. Recovery of exogenous DOC and studies of replicate variation using purification methods (1) and (2) suggest that the technique is adequately accurate. This is further verified by the high correlation between plasma DOC concentrations obtained for the same samples using purification methods (1) and (2) and between method 1 and the radioimmunoassay of James et al (1971).

Sensitivity. High sensitivity is required for analysis of corticosteroids in peripheral plasma. The sensitivity of the present method (0.5 ng/100 ml) is similar to those of the more sensitive g.l.c. techniques and double isotope techniques for estimating aldosterone, testosterone and oestrogens whose plasma concentrations are of the same order as that of DOC. However, while it is theoretically correct to calculate sensitivity from

replicate variation of estimates of plasma concentration near the limit of sensitivity of the method (Braunsberg and James, 1961) the replicate variation of the pool used for the purpose was consistently lower than that of the normal plasma pools and may have resulted in a fortuitiously high estimate of sensitivity.

Convenience. Using the shorter purification procedure (method 2) the analyses of a batch of plasma samples by the gas-chromatography method requires a period of 2-3 days compared with 7-10 days for a similar batch by double isotope derivative assays. Also, the small number of purification steps required (Hyde and Daigneault, 1968; James et al, 1971), results in a higher mean isotope yield which may increase sensitivity. The use of (^{3}H)- labelled steroids as monitors of recovery in place of relatively low specific activity (^{14}C)-steroids further allows accurate counting in shorter time without the disadvantage of increasing the gross blank.

Normal range. The concentration of DOC in plasma in normal subjects as measured by gas chromatography with electron capture detection compareswell with the results reported by other workers (see Table 18).

AUTHORS	NORMAL RANGE (PLASMA)	METHOD
BROWN AND STROTT (1971)	Less than 5 ng - 10 ng/100 ml (n=23)	Protein Binding
ODDIE et al (1972)	Mean 6.2 ± 4.0 ng/ 100ml (n = 22)	Double isotope derivative assay
JAMES et al (1971)	4 - 18 ng/100 ml (n = 24)	Double isotope derivative assay
JAMES et al (1971)	1 - 12 ng/100 ml (n = 20)	Radioimmunoassay
WILSON AND FRASER (1971) (present method)	4 - 14 ng/100 ml (n = 30)	Gas chromatography

TABLE 18.

Methods for measurement of plasma DOC concentration showing normal range and method used.

4.3.2. Radioimmunoassay

During the period covered by the work in this thesis (1969 - 1972) techniques of radioimmunoassay were rapidly established for assays of plasma-borne steroids and presented obvious possibilities of adaption to plasma DOC assay. It was therefore decided to compare its performance with the gas chromatography method. In order to do this, antisera were raised and tested and a simple purification and radioimmunoassay procedure was developed and evaluated.

Preparation of steroid protein conjugates. Using the original method of Erlanger et al (1957) for the preparation of carboxymethyloxime derivatives of DOC and aldosterone the main product in both cases appeared to be the 3, 20-dioxime which on conjugation with bovine serum albumin gave an insoluble precipitate. In order to prepare selectively the 3 carboxymethyloxime, the 20-carbonyl group was selectively reduced to the 20α-hydroxy group using the 20α - hydroxy-steroid dehydrogenase. The 3-oxime of this reduced steroid was then prepared according to the method of Erlanger et al (1957) after which the 20α-hydroxy group was reoxidised to give the required 3-carboxymethyloxime of DOC and aldosterone. Although the overall yield was

very low, this procedure was suitable for preparation of small quantities of standard 3-carboxymethyloxime derivatives suitable for thin layer chromatography standards (Section 4.2.2.). This allowed the continuous monitoring of a simpler direct procedure for preparation of larger quantities of these derivatives. The method of Erlanger et al (1957) involves the use of moderately strong alkaline conditions which are probably too severe for the selective preparation of the 3- oxime. It was found that a much higher yield of this compound was obtained by using mildly alkaline solution, lower temperature and a shorter reaction time.

Formation of antibodies. Steroids may be rendered antigenic by attachment to carrier proteins (Erlanger et al, 1957; Erlanger et al, 1959; Goodfriend and Sehon, 1958). Based on the early work of Lieberman et al (1959), methods have been developed for linking hydroxyl functions on steroids to lysine groups on proteins using Schotten-Baumann or mixed anhydride reactions. However, as hydroxyl groups of mineralocorticoids are on rings C and D and are usually necessary for the production of the most specific antibodies, other methods such as oxime formation have been employed to link the protein to the 3- position of the steroid nucleus.

Antibodies produced by immunising animals with steroid protein conjugates exhibit greatest specificity to those parts of the molecule which are remote from the point of attachment of the protein to the steroid (Bieser et al, 1959; Midgley and Niswender, 1970). Lieberman and colleagues (1959) developed antibodies to deoxycorticosterone - 21 - BSA and investigated the specificity of the antibodies using an hapten inhibition test. He showed that the dominant features in the steroid which determine antibody specificity were the nature of the oxygen function at position 11, the nature of the side chain function at position 17, and the α, β-unsaturated carbonyl group in Ring A. In the present study antibodies have been developed to DOC and aldosterone using the 3-carboxymethyloxime- BSA conjugate. Both show good specificity with maximum cross reaction by other major steroids of 1%. Antisera to DOC developed using the 21 - hemisuccinate - BSA conjugate are less specific cross reacting to a greater extent with deoxycortisol. Mayes and Nugent (1970) and James et al (1971) have both used the carboxymethyloxime derivative linked to BSA to develop antibodies to aldosterone and deoxycorticosterone respectively. The latter antisera cross reacted completely with progesterone

and to a lesser extent with corticosterone. Attempts to produce antibodies in the rabbit to aldosterone - 3- carboxymethyloxime - poly-l-lysine were not successful. This may be attributable to the possibility that induction of antibody response must include not only recognition of the hapten but also of the carrier protein. Levine et al (1963) in their study of poly-L-lysine conjugates in guinea pigs suggested that the ability to produce antibodies to poly-L-lysine conjugates depends on the capacity to metabolise the conjugate in the precise way necessary to induce an immunogenic response.

In the studies with guinea pigs, no circulating antibodies were detected after immunisation with steroid protein conjugates (Table 14). This failure to raise antibodies to steroids may be due failure to recognise either antigen or carrier protein and consequently the process of antibody synthesis would not be induced. It is also possible that induction of antibody response and development of a high titre of antibodies in these guinea pigs require a considerably longer immunisation schedule than was possible in this experiment. However, antisera obtained after long immunisation programmes may differ markedly in sensitivity, specificity and titre from those obtained by short immunisation programmes (Hurn

and Landon (1970).

Radioimmunoassay of DOC in plasma.

Specificity. Specificity studies with antiserum DOC 1 indicate that cross reaction with other major steroids is less than 1%. However, steroids such as cortisol are present in plasma in very much larger concentrations than is DOC and hence 1% cross reaction with such steroids would obviously constitute a significant interference in the radioimmunoassay of plasma DOC. An adequate separation of DOC from these steroids is achieved on paper chromatography in Bush B3 system. R_F values for various corticosteroids in this system are shown in Table 4. Further confirmation of the specificity of the method was obtained by comparison of results with those obtained by gas-chromatography (see below).

Sensitivity and accuracy. The sensitivity of the method is adequate for the measurement of the plasma concentration of DOC in 2 ml. of plasma. Like gas-chromatography, this method would be suitable for duplicate assay of samples without requirement of large volumes of plasma thus increasing the accuracy of this method.

Blank samples. There was no evidence of any non-specific interference in the assay as blank values using water in place of plasma remained effectively zero. However, in order to avoid the problem of dealing with measurable blank values, scrupulous cleaning of glassware and solvents and thorough washing of chromatography paper were necessary.

Convenience. The analysis of a group of plasma samples requires a period of 2-3 days which is similar to the gas chromatographic method.

4.3.3. Comparison of gas liquid chromatography and radioimmunoassay for measurement of plasma DOC concentration.

Comparison of results of analysis of plasma. The results of analysis of 29 samples by both methods (Fig. 8) showed a close comparison with no obvious systematic difference.

Specificity. In the gas chromatographic method, it is possible by physico-chemical means to prove that the compound extracted from plasma and measured by electron capture detection is identical to standard DOC - bis HFB. Gas-chromatography - mass spectrometric analysis is a convenient and efficient

method of identifying the plasma product which is being measured. However, with radioimmunoassay, evidence of specificity is less conclusive. Obviously one cannot measure the cross reactivity of every single steroid in plasma. Indirect evidence of specificity can be obtained by comparison of results with those obtained by other methods and by analysis of water and plasma from adrenalectomised subjects. These data confirm the specificity of this method in this department. Further, in patients receiving steroid drugs, the metabolites of which may not all be known, problems of specificity obviously occur. In such situations the gas chromatographic method offers the more reliable analysis.

Convenience. Both methods required a period of up to 3 days for analysis of plasma samples. However, the gas-chromatographic method requires much care and attention to maintain maximum sensitivity. For example, the columns require extensive 'conditioning' at temperatures higher than normal operating temperature for 36 hours prior to use. Also, in order to avoid column adsorption of the small amounts of steroid present in samples, frequent injection of standard steroids and occasionally of silylating solution is required. In practice,

it has been found that columns require replacing every few months. Gas supply to the electron capture detector and columns also requires purification by use of molecular sieves which need periodic reactivation or replacement.

In these respects, much time is spent in maintaining the apparatus required for smooth functioning of the gas chromatographic method. In contrast, the radioimmunoassay method requires little 'maintenance' work except absolute cleanliness of solvents, apparatus and chromatography paper and preparation of solutions.

The major problem with radioimmunoassay techniques is development of specific and sensitive antisera. From the results of this study it would appear that there is no guarantee that antibodies will definitely be produced in sufficient titre to be useful in radioimmunoassay. More studies are required to determine more reliable methods of raising antisera.

Range. The radioimmunoassay method has a limited range of accurate measurement between 6 and 300 pg. per sample which with a 2ml sample is equivalent to 75 ng/100 ml although this can be extended by doubling dilutions. The gas chromatographic method has no upper limit of detection as dilution of final solution allows quantitation of a wide range of concentration of DOC.

PART II

STUDIES OF THE PHYSIOLOGY AND PHARMACOLOGY OF DOC.

1. INTRODUCTION

In the absence of suitable methods of measuring plasma DOC concentration little is known about the mechanisms which control its plasma level although much can be inferred from studies of urinary secretion rate (see later).

The remainder of this thesis is concerned with the study of these control mechanisms. The results of some of these methods also serve as further validation of the gas chromatographic method (2), used exclusively in these studies.

A brief outline of the biosynthesis and metabolism of DOC precedes the experimental section.

2. REVIEW OF LITERATURE ON BIOSYNTHESIS AND METABOLISM OF DOC.

2. REVIEW OF LITERATURE ON BIOSYNTHESIS AND METABOLISM OF DOC.

A large number of steroids have been isolated and identified from extracts of adrenal glands (Hechter et al, 1951, 1953; Pincus and Romanoff, 1955; Wettstein et al, 1955). Although these may all be synthesised by the adrenal gland only those steroids which are found in adrenal effluent blood in significantly higher concentration than in the peripheral circulation can properly be termed secretory products.

Very little storage of steroid hormones occurs in the adrenal gland. Most of the liquid present consists of cholesterol or its esters which together with plasma cholesterol are thought to provide the substrate for adrenal steroid biosynthesis (Conn et al, 1950). From studies of ^{14}C-acetate and ^{14}C-cholesterol metabolism, present evidence indicates that the adrenal steroids are synthesised from a small discreet pool respresenting only a fraction of adrenal cholesterol (Hechter et al, 1953; Caspi et al, 1956, 1957, 1962).

Cholesterol to DOC. Cleavage of the cholesterol side chain by mitochondrial enzymes (Constantopoulas and Tchen, 1961) to form 3β-hydroxy-Δ^5-pregnenolone occurs in at least two stages via 20α-hydroxy-cholesterol (Solomon et al, 1956)

and 20α, 22-dihydroxycholesterol (Shimizu et al, 1961 a, 1961 b; Constantopoulas et al, 1962; Levitan and Lieberman, 1956).

This is followed by conversion of the Δ^5 - pregnenalone structure to the Δ^4 - 3 carbonyl structure typical of progesterone and the corticosteroids. This conversion is effected by the enzyme 3β-hydroxysteroid dehydrogenase, whose presence has been demonstrated in the endoplasmic reticulum of the outer cells of zona fasciculata (Dawson et al, 1961) and by an isomerase which occurs in the microsomes of the same region (Ewald et al, 1964; Kruskemper et al, 1964). Further hydroxylation at position 21 by microsomal enzymes yields deoxycorticosterone. Alternatively, 17α-hydroxylation gives 17α-hydroxyprogesterone which is a precursor of cortisol.

Steroid Hydroxylation. The normal order of hydroxylation of the steroid nucleus is 17α, 21 then 11β respectively. For instance, 17α-hydroxylase enzyme system will not act on 21-hydroxylated substrate (Dorfman, 1962) due to enzyme stereospecificity. These hydroxylation systems are mixed function oxidases which require NADPH as cofactor. The sequence of electron transfer is thought to involve flavoprotein, non-haem iron protein, cytochrome P450 and also requires oxygen

(Nakamura et al, 1966). The hydroxylases are probably genetically determined and familial enzyme deficiencies have been described (Cox, 1960; Rosenbloom and Smith, 1966). For example, virilising congenital adrenal hyperplasia (Bongiovanni and Root, 1963) is found in genetically and clinically different forms: 3β - dehydrogenase defect (Bongiovanni, 1961) and 21-hydroxylase defect (Jailer, 1951; Bongiovanni and Clayton, 1954). The 11β-hydroxylation defect causes marked reduction of cortisol and aldosterone secretion and consequent elevation of secretion of DOC and 11-deoxycortisol.

Zonation of adrenal cortex. Based on the work of Symington (1961) much is known about the morphology of the adrenal cortex. The human adrenal cortex consists of 3 main zones. Zona glomerulosa, which is in the outer zone, is not clearly divided from the zona fasciculata, but merges with it. Where zona glomerulosa is absent, zona fasciculata extends to the capsule. The cells of the zona fasciculata are filled with lipid globules giving them a clear appearance and are clearly divided from the compact cells of the inner zone of the zona reticularis.

In contrast to the human adrenal cortex the rat adrenal cortex zones are more distinct zones. The

outer zone, the zona glomerulosa lies under the capsule, is rich in lipid and separated from zona fasciculata by a thin layer of lipid-free cells. The zona fasciculata, which constitutes about half of the cortex, is rich in lipid and may or may not pass abruptly into zona reticularis.

This morphological zonation of the adrenal cortex is accompanied by a functional zonation of the enzymes involved in corticosteroid biosynthesis (Grant and Griffiths, 1962). However, deoxycorticosterone is of dual origin, occurring in zona glomerulosa and zona fasciculata. In the rat adrenal cortex the zona glomerulosa may be removed by stripping the capsule, from the remainder of the cortex. Capsular and decapsulated portions of rat adrenals produce equal amounts of DOC (Vinson and Whitehouse, 1969; Muller, 1970).

Further metabolism of DOC. DOC is further metabolised by two main routes, the biosynthesis of other active corticosteroids and its own biodegradation to a water soluble, excretable conjugate.

(a) Biosynthesis of other corticosteroids.

As previously stated, DOC occupies a unique position in the biosynthetic pathways of the corticosteroids as it is a precursor of all the known mineralocorticoids. The biosynthesis of aldosterone from DOC is not fully clarified but there is

evidence that corticosterone (Giroud et al, 1956, 1958; Mulrow and Cohn, 1959), 18-hydroxy-corticosterone (Vescei and Kessler, 1970) and possibly 18-hydroxy-DOC (Nicolis and Ulick, 1965; Kahnt and Neher, 1965) are intermediaries of aldosterone biosynthesis.

(b) Degradation of DOC

Metabolism of DOC occurs mainly in the liver and kidney and involves reduction of ring A with resultant loss of biological activity and finally excretion, mainly in the urine (Biglieri, 1965).

This product, tetrahydro-DOC glucuronide was first identified in the urine or normal subjects and later of subjects with Addison's disease after oral administration of DOC. More recently it has been identified and its excretion rate found to be in the range 50-250 ug/day (Biglieri, 1965).

3. DOC IN PLASMA.

3. DOC IN PLASMA.

The methods described in this thesis measure total plasma DOC concentration. However, some corticosteroids occur only partly in free solution in plasma the remainder being bound with variable tenacity to one or more fractions of plasma proteins (Eik-Nes et al, 1954). This protein-binding phenomenon has been studied mainly in relation to cortisol (see Mills, 1965).

Corticosteroids bind to at least two different protein fractions, namely albumin and corticosteroid-binding globulin (CBG or transcortin). While CBG has the highest affinity for corticosteroids, albumin has the higher binding capacity by virtue of its higher concentration in the blood (see section 3.3. on competitive protein binding methods).

The biological purpose of protein binding, whether for transport, slow release or inactivation of steroids, is not entirely clear but is obviously of considerable importance since most methods measure total steroid concentration which may not accurately reflect the activity of the steroid *in vivo*. A similar problem exists with cortisol and studies of free cortisol, using equilibrium dialysis techniques, have been published (Burke 1969). As far as the author is aware no similar studies have been made of plasma DOC.

The binding capacity of plasma proteins is known to be elevated during pregnancy (Gemzell 1953; Martin and Mills, 1958) and oestrogen therapy (Taliaferro et al, 1956; Wallace et al, 1957). This is thought to be due to a general increase in concentration of the plasma proteins.

Both albumin and CGB are capable of binding DOC and from the metabolic clearance rate and biological half life (Schambelan and Biglieri, 1972) it would appear that DOC is to some extent protein-bound. The nature and extent of this binding remain to be examined. The ability of plasma proteins to bind DOC has been exploited in an attempt to estimate plasma DOC concentration (see section 3.3.).

4. REVIEW OF LITERATURE ON FACTORS AFFECTING DOC SECRETION AND PLASMA CONCENTRATION.

4. REVIEW OF LITERATURE ON FACTORS AFFECTING DOC SECRETION AND PLASMA CONCENTRATION.

4.1. INTRODUCTION.

Steroid hormone secretion is controlled mainly by humoral influences as is evident from studies in which the adrenal gland has been transplanted elsewhere in the body of animals, severing all nerve connections, and has been able to function normally over a period of years. For example, Blair-West et al (1962) transplanted the adrenal gland of Merino sheep to the capacious skin folds of the neck. The secretion rate of aldosterone, corticosterone and cortisol responsed normally to environmental stimuli.

Factors which are known to affect adrenocortical secretion are shown in Fig 9. The major influence on cortisol, corticosterone and probably DOC would appear to be ACTH whereas aldosterone secretion is less sensitive to ACTH and responds more rapidly, in man at least, to renal influence.

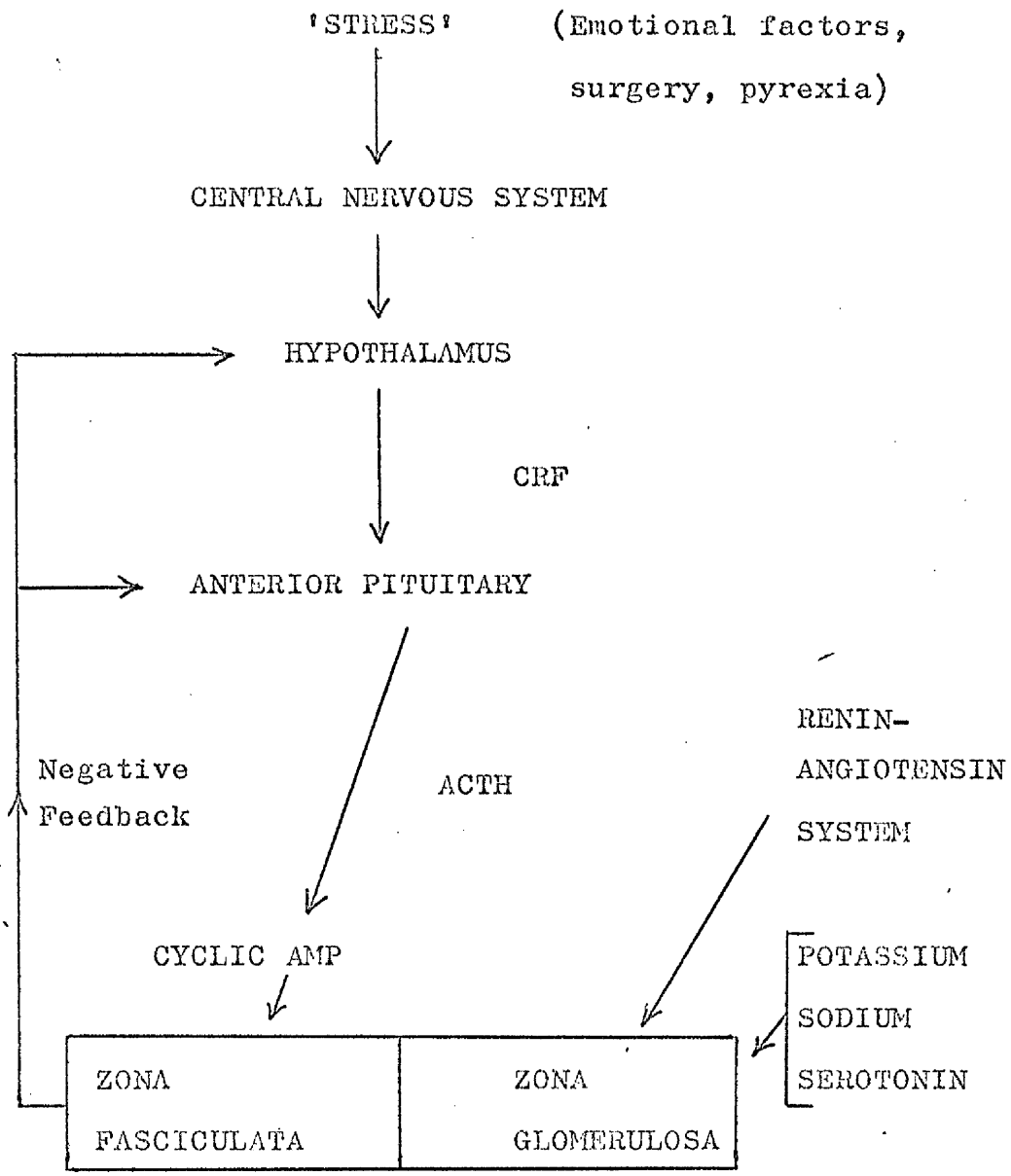

FIGURE 9.

Factors known to affect adrenocortical secretion in normal human subjects.

4.2. ANTERIOR PITUITARY AND ACTH.

ACTH is released from the pituitary as a result of a variety of stressful stimuli. Many events cause stress to man and animals; the effects of many of these have been tested and a few have been used for routine study of the sequence of events in the activation of the pituitary-adrenal axis. Emotional factors increase plasma cortisol concentration in man (Greenwood et al, 1966) and even mild stimuli such as movement of experimental animals may increase adrenocortical activity (Guillemin, 1967). Pain (Egdahl, 1967), surgery (Hume and Nelson, 1955; Liddle et al, 1962) and exposure to cold (D'Angelo, 1960; Boulouard, 1960) have similar effects. Pharmacological stress such as insulin induced hypoglycaemia and pyrexia (Bliss et al, 1954) have been extremely useful tools for testing the efficiency of the pituitary adrenal axis while vasopressin, which may be related to corticotrophin releasing factor, is useful for assessment of anterior pituitary function.

These stimuli cause parts of the central nervous system to activate the hypothalamus causing release of corticotrophic releasing hormone (CRF) which is carried to the anterior lobe of the pituitary by portal vessels. CRF rapidly provokes release of ACTH (Purves and Sirrett, 1967).

Mechanism of action of ACTH. Stone and Hechter (1954) postulated that ACTH stimulates the splitting of the cholesterol side chain by desmolase thereby increasing the rate of formation of pregnenolone. Haynes and Berthet (1957) later proposed that ACTH stimulates the formation of cyclic adenosine - 3, 5 - monophosphate (cyclic AMP) which in turn activates phosphorylase by a kinase reaction. Adrenal glycogen is then converted into glucose - 6 - phosphate which is metabolised by the hexose - monophosphate shunt pathway to yield $NADPH_2^+$, an essential cofactor in many of the hydroxylations involved in corticosteroid biosynthesis (see before). There is now a great deal of evidence to support this hypothesis. For example, using rat adrenal slices in vitro, it has been demonstrated that addition of gluocose - 6 - phosphate and NADP will enhance the production of

corticosteroids (Koritz and Peron, 1958). Dexter et al (1970) reported that ACTH also stimulates uptake of cholesterol from plasma. DOC secretion is promptly stimulated by administration of ACTH (Brown and Strott, 1971; Schambelan and Biglieri, 1972). Further studies of the effect of ACTH on DOC secretion are described in section 5.

Negative feedback control. The secretion of cortisol, corticosterone and DOC is mainly a consequence of ACTH stimulation (Cope and Black, 1958; Liddle et al, 1962). Conversely, administration of glucocorticoids in a dosage higher than physiological requirement causes suppression of ACTH release and subsequent adrenal atrophy (Ingle et al, 1938). Similarly, elevated plasma cortisol concentration inhibits production of ACTH by the pituitary (Yates et al 1961). James et al (1966) showed that infusion of glucocorticoids (Dexamethasone, .1 mg/hour) caused secretion of corticosteroids to cease within a few minutes of starting the infusion and concluded that, since such a rate of infusion of dexamethasone does not interfere with adrenal responsiveness to ACTH, the rapid decrease in plasma cortisol level on infusion of dexamethasone reflected the effect of dexamethasone on the secretion of ACTH. During stress, the level of glucocorticoids in plasma

required to inhibit ACTH release is set at a higher level. For example, even massive doses of glucocorticoids do not fully inhibit adrenal responsiveness to surgery, pyrexia and other stressful stimuli (Estap et al, 1963). Administration of dexamethasone has been shown to suppress DOC secretion (Bledsoe et al, 1966; Schambelan and Biglieri, 1972). Further studies are described in section 5.

Nycthemeral Rhythm. The secretion of cortisol and probably also DOC (James et al, 1971) does not occur at a constant rate during the day, but is high in the early hours of the morning, decreasing to a minimum at about midnight. This variation is reflected in the plasma concentrations of these steroids and is called a nycthemeral rhythm. The recent availability of accurate techniques for measuring plasma concentration of ACTH (Greenwood, 1968) confirm earlier reports (Ney et al, 1963; Clayton et al, 1963) that this corticosteroid rhythm is secondary to that of ACTH.

The rhythm is probably a manifestation of the cyclic functioning of the hypothalamus (Yates et al, 1961). Because of the variation of plasma concentrations of these steroids during the day, it is important that blood samples are taken at standard times.

Recently it has been shown that secretion of ACTH and consequently corticosteroids is phasic. The frequency of these phases is greatest in the early morning giving rise to maximal plasma concentrations of corticosteroids (Hellman et al, 1970).

4.3. THE KIDNEY

There is considerable evidence to suggest that the main product of the zona glomerulosa, aldosterone, which responds to changes of electrolyte status, is largely under the control of the kidney (Fraser et al, 1969). The zona glomerulosa, also synthesises DOC (see section 2) and the possibility of a renal involvement in the control of secretion of this steroid cannot be excluded.

Renin-angiotensin system. (Fig. 10). Renin is an enzyme produced by the kidney which acts upon a plasma globulin (renin substrate) to produce a decapeptide, angiotensin I which is subsequently converted to an octapeptide, angiotensin II, on passage through the lung. Angiotensin II is a powerful vasoconstrictor, and stimulates aldosterone secretion by the adrenal cortex (Fraser et al, 1969).

Angiotensin and DOC. Brown and Strott (1971) could detect no change in plasma DOC concentration on infusion of angiotensin II at a rate of 6-8 ng/Kg/min.

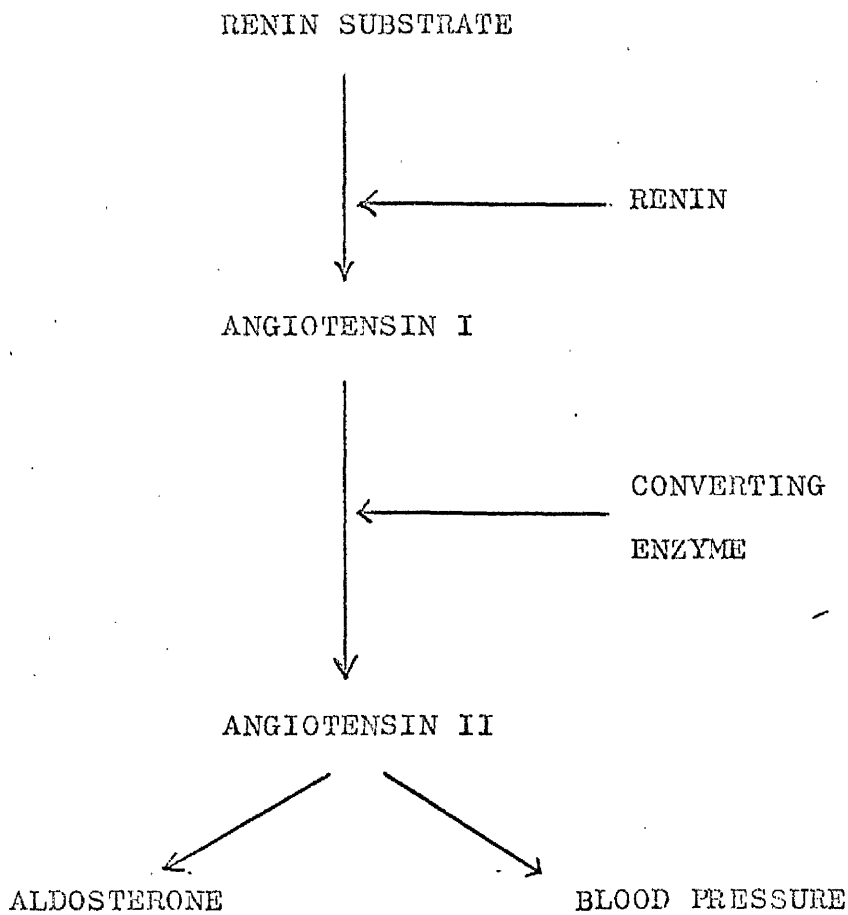

FIGURE 10

Renin Angiotensin system.

for 60 mins. This amount was sufficient to raise diastolic blood pressure by 20 mmHg. However, Davis et al (1968) demonstrated a four-fold rise in DOC secretion in the dog during infusion of angiotensin at a rate of 50 ng/Kg/min for 3 hours. They also reported that in chronic renal failure in the dog, when the plasma angiotensin should be raised, DOC secretion remained normal. In the various conditions associated with increased activity of the renin-angiotensin system, including malignant hypertension, juxtaglomerular-cell hyperplasia and some cases of essential hypertension, it is known that DOC secretion is within normal range (Biglieri et al, 1968).

The explanation for these conflicting results may be that physiological doses of angiotensin II, i.e. the dose required to cause a minimal rise in diastolic blood pressure may promote only aldosterone secretion whereas very large doses may also increase DOC secretion. This may be due to stress since cortisol and corticosterone are also raised in these circumstances (Blair-West et al 1968) or saturation of the capacity of the adrenal cortex to convert DOC to aldosterone.

Sodium Status. Changes in plasma sodium or total body sodium may alter the sensitivity of blood pressure to angiotensin II.

Brown and Strott (1971) and Oddie et al (1972) could detect no change in plasma DOC concentration during sodium depletion (10 m. equiv. sodium intake/day). However, under similar dietary sodium deprivation Bledsoe et al (1966) showed a rise in DOC secretion rate. In order to demonstrate this rise, the subjects were treated with dexamethasone and metapyrone throughout the study to inhibit 11β and 17α hydroxylation and also the production of cortisol as a result of ACTH stimulation. In this way the relatively small changes in DOC secretion caused by sodium depletion could be detected as they were not obscured by larger changes due to ACTH variation. Visser and Cost (1964) found low aldosterone secretion rate and high corticosterone and DOC secretion rates during sodium depletion of a subject with 18-hydroxylation deficiency. This would result in decreased aldosterone secretion rate and consequently increased secretion of DOC and corticosterone which are both precursors of aldosterone.

Studies of the effect of sodium depletion on plasma DOC concentration are described in section 5.5.

Other electrolytes. In vitro studies of corticosteroid biosynthesis have shown that several cations including sodium, potassium, rubidium,

ammonium, and caesuim may increase biosynthesis of aldosterone in adrenal slices (Muller, 1965a, b; Blair-West et al, 1968). Muller (1968) demonstrated that production of DOC by quartered rat adrenals was stimulated by a high potassium concentration.

Similar findings have been described in superfusion and pure cell preparation studies (Albano et al, 1972). The following section describes the investigation of factors affecting plasma DOC concentration.

5. STUDY OF FACTORS AFFECTING PLASMA DOC CONCENTRATION IN NORMAL HUMAN SUBJECTS

5.1. Introduction

As will be apparent from the preceding review, few studies have been made of the factors which influence plasma DOC secretion in man, presumably due to inadequate methodology. The aim of the following work is to study a number of factors which have been reported, or might be predicted to affect plasma DOC concentration in normal man.

5.2. General methods

Plasma 11-hydroxycorticosteroids were measured by the Steroid Biochemistry Department of the Royal Infirmary, Glasgow using a fluorimetric method (Mattingly , 1962, normal range 6 - 22, mean 12 ug/100 ml). Plasma renin concentration was measured by the method of Brown et al (1964; normal range 4-20, mean 8.4 units/ litre). Plasma angiotensin II was measured by radioimmunoassay (Dusterdieck and McElwee, 1971; normal range 5-35, mean 16 pg/ml). Serum and urinary electrolytes were measured by flame photometry. These estimations were carried out by other members of the staff of the MRC Blood Pressure Unit.

5.3.1. THE EFFECT OF ACTH ON PLASMA DOC CONCENTRATION.

Methods.

In order to study the effect of ACTH on plasma DOC concentration, the following study was undertaken in 5 normal subjects on unrestricted diet. The subjects remained supine throughout the study.

An indwelling cannula was inserted by a medical colleague into the subjects' peripheral arm vein under local anaesthetic and one hour later basal blood samples (40 ml) were taken. β^{1-24} ACTH (1 ug. Tetracosactrin; Ciba, Ltd.) was injected via the indwelling cannula and blood samples (25 ml) were taken at intervals. All samples were treated as previously described.

Results.

Plasma concentrations of DOC and 11-hydroxy-corticosteroids are illustrated in Fig. 11.

In these experiments, maximum DOC concentration (15 mins) always occurred before that of 'cortisol' (30 mins). The concentration of both substances had returned to basal levels after 90 mins.

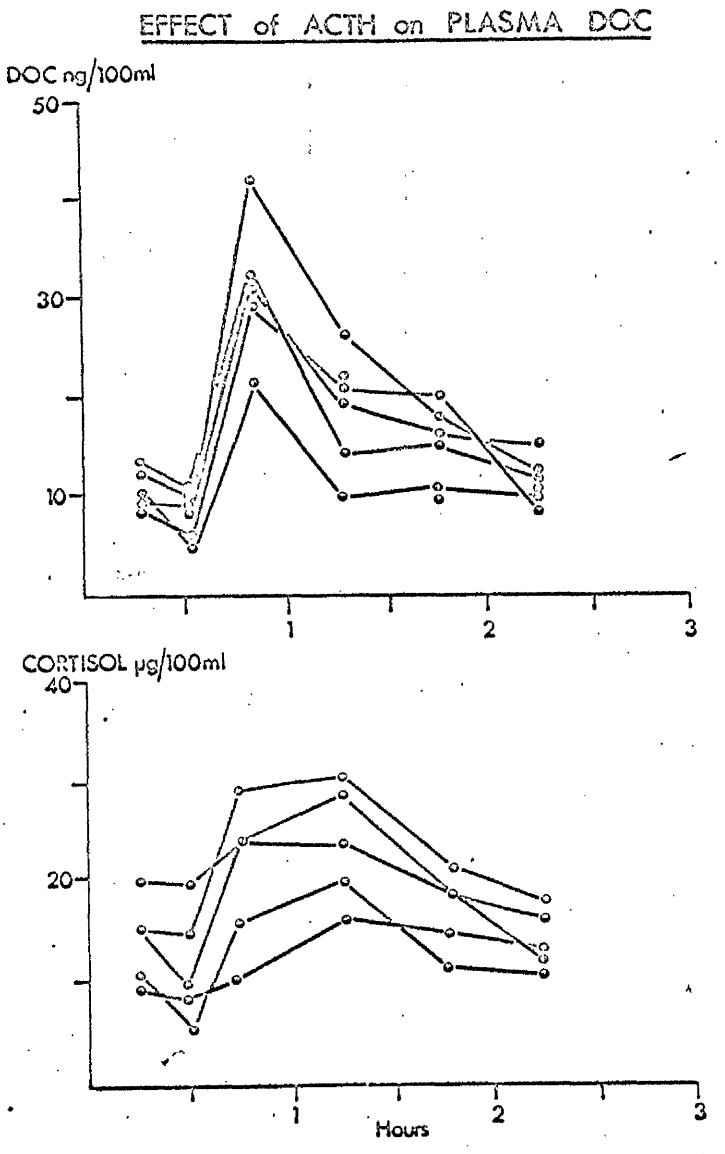

Figure 11.

Plasma concentration of DOC (ng/100 ml) and 'cortisol' (ug/100 ml) after injection of β^{1-24} ACTH (1 ug).

It is proposed to report these results with experiments 5.3.3. (Wilson, Love and Fraser, 1973).

5.3.2. THE EFFECT OF INSULIN INDUCED HYPOGLYCAEMIA ON PLASMA DOC CONCENTRATION.

Introduction

Reduction of blood glucose level by administration of insulin is an effective stress stimulus which causes release of corticotrophic releasing factor (CRF) from the hypothalamus. CRF stimulates release of ACTH from the pituitary (Bliss et al, 1954; Purves & Sirrett, 1967). This is a convenient means of studying the effect of physiological rises in endogenous ACTH concentration on plasma DOC concentration.

Method

Two normal, supine, fasting subjects were given insulin (0.15 units/insulin/Kg). Blood samples (50 ml) were taken for measurement of blood glucose levels and plasma concentration of DOC and cortisol.

Results.

A marked fall in blood glucose levels was observed in both subjects (Fig.12).
Plasma DOC concentration rose promptly to a maximum concentration after 30 minutes. Plasma cortisol also rose with maximum level occurring after 45 minutes. Both DOC and cortisol had returned to basal levels after 20 minutes.

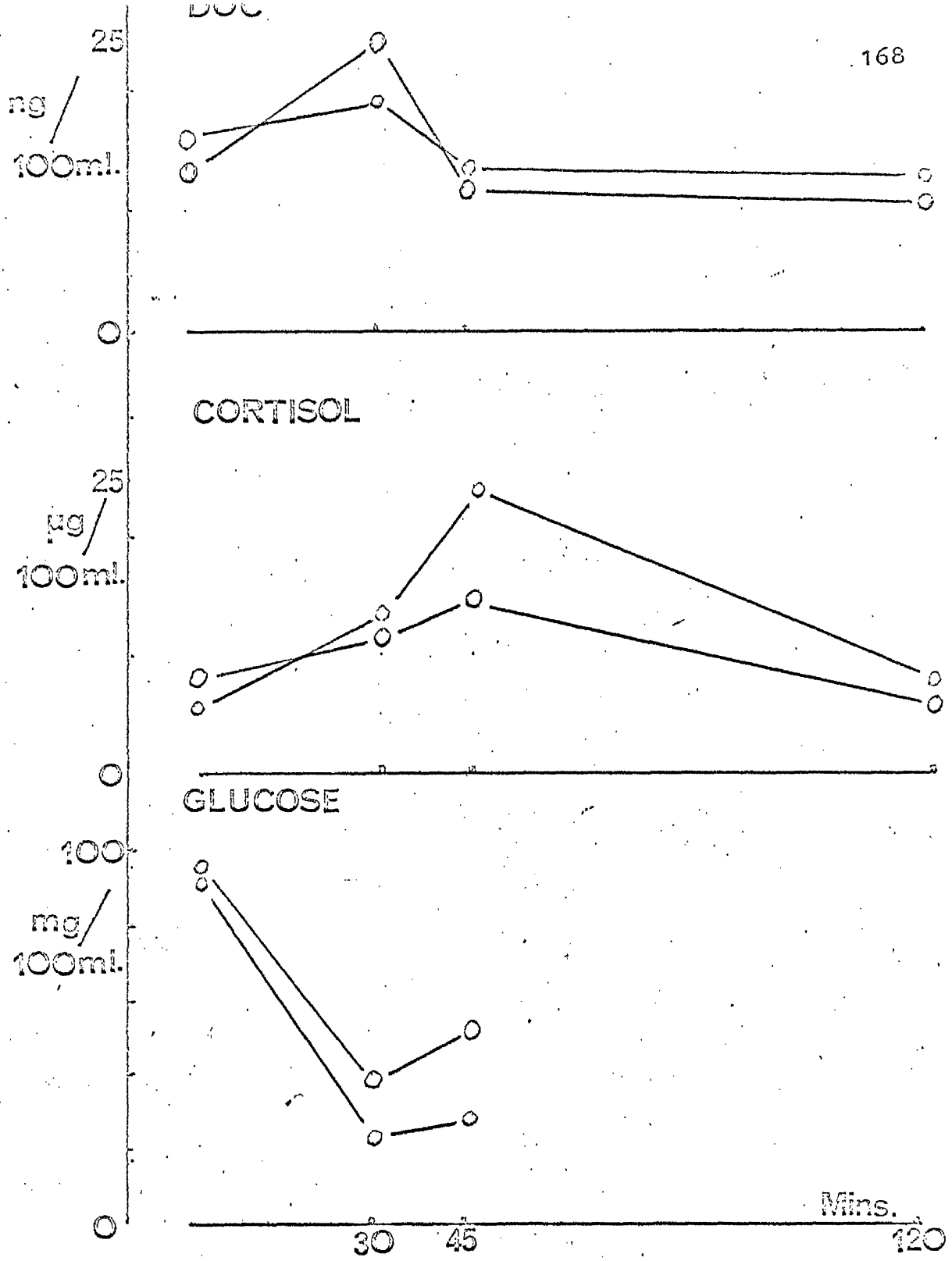

FIGURE 12.

Plasma concentration of DOC (ng/100 ml) 'cortisol' (ug/100 ml) and blood glucose (mg/100 ml) after injection of insulin.

5.3.3. DISCUSSION

The ability of ACTH to increase DOC secretion is well documented (see before) and this small series of experiments confirms such a relationship. The experiments involving administration of synthetic ACTH may have been pharmacological since in two of the subjects very high levels of 11-hydroxycorticosteroids were achieved. However since plasma ACTH levels were not measured the extent of adrenocortical stimulation is difficult to assess.

However it is clear, firstly, that DOC secretion is promptly increased and, secondly, that the duration of the response is shorter than that of cortisol.

Endogenous release of ACTH, induced by hypoglycaemia, results in an increase in circulating ACTH levels approximately to high physiological levels, although such gross hypoglycaemia would rarely be encountered in normal subjects. In the two patients subjected to this experiment plasma cortisol and DOC levels were again increased. Again it appeared that plasma DOC concentration rose more quickly than cortisol and may have been maximal before the first available sample (30 mins). Further studies

are necessary to establish this point.

From these preliminary experiments therefore, it would appear that the response of plasma DOC concentration to a rise in plasma ACTH concentration is

more rapid and
of shorter duration than
that of cortisol.

ACTH is known to exert its effect early in the biosynthesis of corticosteroids. A possible explanation for these results is that the capacity of the adrenal cortex to hydroxylate at the 11β position is temporarily exceeded and that there is a consequent increase in the tissue concentration and hence secretion of 11 deoxy-compounds. This is supported by the fact that 11-deoxy-cortisol behaves in a similar fashion to DOC during insulin induced hypoglycaemia (Mason, personal communication, 1971) whereas the response of corticosterone resembles that of cortisol. Also the corticosterone response to stress and to administered ACTH is indistinguishable from that of cortisol (Fraser 1967), but no reliable comparison of DOC and 11-deoxycortisol is yet available.

ACTH therapy is commonly used in the treatment of inflammatory conditions such as arthritis and the electrolyte and body fluid disturbances occurring during such therapy may in part be due to increase in the circulating levels of DOC

5.4. THE EFFECT OF ANGIOTENSIN II ON PLASMA DOC CONCENTRATION.

Methods

The effect of angiotensin II on plasma DOC concentration was studied in 3 normal male subjects on unrestricted diet. The subjects remained supine throughout the study. An indwelling cannula was inserted into a peripheral vein in each arm under local anaesthetic. Dextrose (5% aqueous) was infused for one hour. Two basal blood samples (50 ml.) were then taken with an interval of 15 minutes between samples. Angiotensin II (Hypertensin; Ciba Ltd.) was then infused continuously for 1 hour at 2 ng/Kg/min. and then for a further hour at 8 ng/Kg/min. Blood samples (50 ml) were taken after 15,30, 60 and 120 minutes of angiotensin II infusion.

Results

Blood pressure did not increase in any subject on the lower rate of infusion of angiotensin II, but increased significantly during the higher rate of infusion. Plasma concentrations of angiotensin II and DOC are shown in Figure 13. Despite the prompt and sustained rise in angiotensin II concentration to levels above the

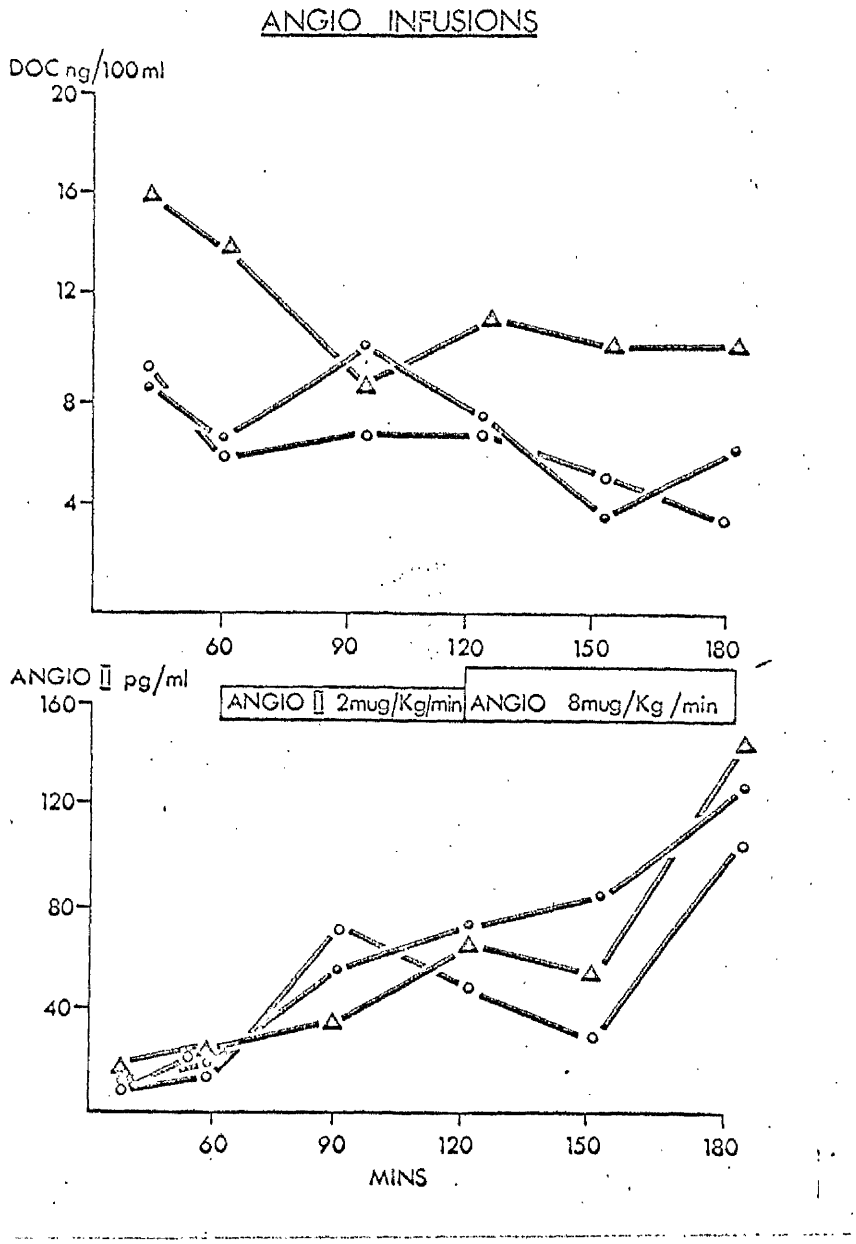

FIGURE 13.

Plasma concentrations of DOC (ng/100 ml) and angiotensin II during angiotensin II infusion.

normal range, plasma DOC concentrations did not rise.

Discussion

Infusion of angiotensin II increases plasma aldosterone concentration (Fraser 1967), and secretion rate (Laragh et al, 1960) although its effects may vary from species to species (Mulrow and Ganong, 1961). Currently it is thought that this effect occurs early in the biosynthetic pathway of aldosterone possibly during the conversion of cholesterol to pregnenolone (Muller, 1970) and it may therefore seem surprising that plasma DOC concentration does not rise during angiotensin II infusion. However, if as seems likely, the conversion of cholesterol to aldosterone via DOC is enhanced no increase in plasma DOC concentration would be expected if the rate of conversion of DOC to corticosterone increased in proportion to the rate of pregnenolone formation. There is evidence that conversion of corticosterone to aldosterone is more efficient in situations of high aldosterone production (Blair West et al, 1968; Tait et al, 1961).

Few studies of the effect of angiotensin II *in vivo* have been carried out in man. Brown et al

(1972) infused angiotensin (6-10 ng/Kg/min; 30 mins) into 5 normal subjects receiving metapyrone in whom ACTH secretion had been abolished by dexamethasone administration. Plasma DOC concentration rose in the absence of any effect on the 17 hydroxy analogue of DOC, 11-deoxycortisol, which is secreted only by the zona fasciculata. This would suggest that angiotensin II exerts its effect only on the zona glomerulosa and that the increased quantity of DOC in the circulation originated solely from this source. Possibly in conditions where the function of the zona fasciculata is not suppressed, as for example in the experiments described here, DOC may diffuse from the zona glomerulosa to the inner zones and be consumed within the gland. In a previous study (Brown and Strott, 1971) dexamethasone was not used and plasma DOC concentration fell to levels undetectable by the competitive protein binding method used. This may also be due, to increased efficiency of conversion of DOC to corticosterone and aldosterone.

Some studies of the effects of angiotensin II in vitro have been carried out in the rat and beef (Saruta et al, 1972) adrenal tissue, but as far as the author is aware no

measurements of DOC have been made. In any case, the effects of aldosterone production have remained in dispute and, particularly where incubation techniques have been used, conclusions are difficult to draw if the rapid destruction of angiotensin II *in vivo* and *in vitro* is borne in mind.

5.5. THE EFFECT OF SODIUM DEPLETION ON PLASMA DOC CONCENTRATION

Method

The effect of sodium depletion on plasma DOC concentration was studied in 4 normal subjects (3 male) who were placed on a low sodium diet (20 m.equiv./day) for 5 days. Blood samples (50 ml) were taken and 24 hour urine collections were taken during the period of unrestricted diet prior to the sodium depletion, during the period of sodium depletion and then during a further 3 days of unrestricted diet for the measurement of plasma concentrations of DOC, angiotensin II, aldosterone and electrolytes and urinary electrolyte excretion.

Results

Electrolyte balance was achieved after two days of dietary sodium restriction (Table 21 a) The mean cumulative sodium loss for the four subjects during the restriction was 98 m.equiv.

Plasma angiotensin II and aldosterone both rose in all subjects above the normal range (Table 20, Fig.14 while plasma sodium concentration fell (Table 21b) No consistent change in plasma DOC concentration was observed (Fig 14, Table 19).

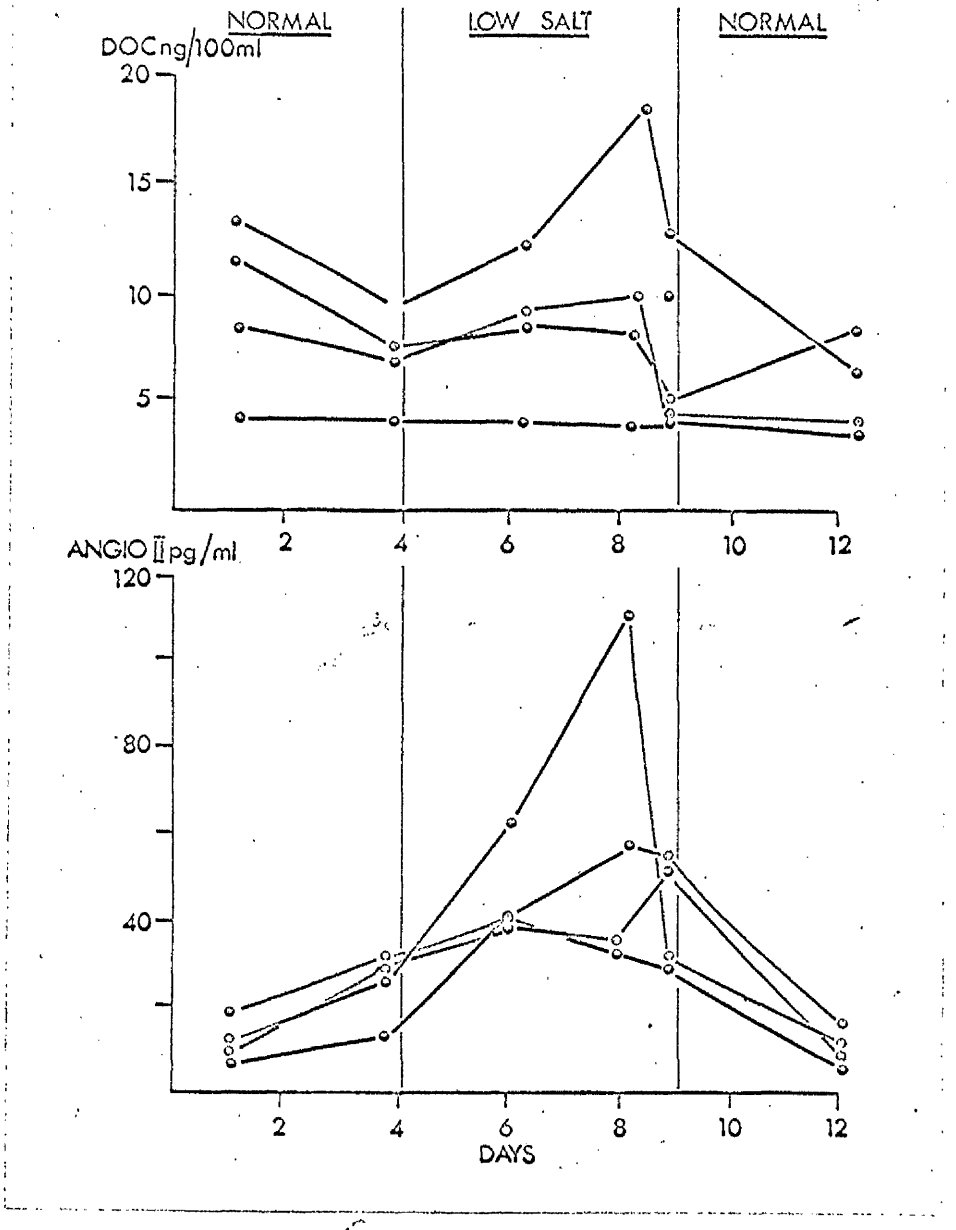

Figure 14

Plasma concentrations of angiotensin and DOC during dietary sodium depletion.

Sample No	DRL		PM		JY		IM	
	Angio II	DOC	Angio II	DOC	Angio II	DOC	Angio II	DOC
1	12	13.3	10	4.0	9	8.0	18	11.4
2	24	9.0	26	4.0	14	6.8	29	7.3
3	61	12.0	36	4.0	38	9.7	38	9.6
4	111	18.8	34	4.0	33	10.2	59	8.3
5	35	13.1	51	4.2	31	4.9	56	5.2
6	14	7.3	12	4.0	5	4.3	18	9.4

TABLE 19

Levels of angiotensin II (pg/ml) and DOC (ng/100 ml) in plasma during dietary sodium depletion.

Subject	Plasma aldosterone concentration ng/100 ml					
DAYS	1	4	6	8	9	12
IM	14	14	14	17	17	6
PM	6	6	26	66	55	7
JY	11	10	26	38	36	28
DRL	8	7	36	57	51	7

TABLE 20

Plasma aldosterone concentrations (ng/100 ml) during dietary sodium depletion. Samples were taken at times shown on Figure

Normal range of plasma aldosterone is 4 - 17 ng/100 ml.

Subject	Day 1	Day 2	Day 3	Day 4	Day 5
IM	-36	-24	-7	+3	+4
PM	-95	-23	-8	+8	+9
JY	-74	-10	+2	+8	+4
DRL	-90	-55	-10	0	+2

TABLE 21.(a)

Sodium balance in 4 subjects during 5 days dietary sodium depletion.

Sodium loss denoted -

Sodium retention denoted +

Results are expressed in m.equiv. sodium.

Subject	1 Na	K	2 Na	K	3 Na	K	4 Na	K	5 Na	K	6 Na	K
IM	143	3.8	139	3.6	139	3.6	139	3.6	-	-	142	3.4
PM	141	3.3	141	3.3	139	3.3	139	3.1	-	-	140	3.3
JY	142	3.8	140	3.8	139	3.6	139	3.6	-	-	142	3.6
DRL	142	3.5	138	3.4	139	3.4	138	3.4	-	-	140	3.5

TABLE 21.(b)

Plasma electrolyte concentration (m.equiv./l). during dietary sodium depletion. Samples were taken as shown in Figure 14.

Discussion

Although the degree of sodium depletion in the subjects studied was sufficiently severe to reduce plasma sodium levels by a mean of 3.5 m.equiv./litre and to raise aldosterone and angiotensin levels to 2-6 times normal, no significant changes occurred in plasma DOC concentration. This is compatible with the results of the previous experiment in which angiotensin II failed to increase plasma DOC concentration. It could perhaps have been predicted that stimulation of the renin-angiotensin system within physiological limits would not increase plasma DOC concentrations. Also as in the previous experiment, any increase in DOC biosynthesis may have been absorbed by increased efficiency of aldosterone biosynthesis which increased.

Relatively mild changes in sodium status also do not appear to alter DOC secretion which is in common with cortisol and corticosterone (Fraser, 1967). Brown and Strott (1971) report similar findings in a small group of normal human subjects. Of the major corticosteroids therefore, only aldosterone responds to dietary sodium deprivation in normal man although small increases in plasma corticosterone have been

reported in sodium depletion associated with sodium-losing renal disease (Fraser 1967). Plasma DOC concentration was not measured in this study but plasma cortisol was not affected suggesting that the effect on the pituitary adrenocortical axis was not significantly altered. Further studies of the effect of more severe sodium depletion on plasma DOC concentration would therefore be of interest. Such a study including also a period of total fasting in obese subjects is described in section 9 –

Recent work both in vitro and in vivo indicates that potassium concentration may be an important factor in the control of steroid biosynthesis by the zona glomerulosa (Muller, 1970). While it seems likely that, like angiotensin II, the effect of potassium may be early in the metabolic pathway of pregnenolone to aldosterone (Muller 1970) a later effect cannot be excluded. The effect of potassium and the inter-relationship of sodium, potassium and mineralocorticoid secretion remains to be evaluated.

5.6. THE EFFECT OF POSTURE ON PLASMA DOC CONCENTRATION

Method

The effect of posture on plasma DOC concentration was studied in two normal male subjects on unrestricted diet. An indwelling cannula was inserted in a peripheral arm vein under local anaesthetic. The subjects remained supine on a tilt table for 2 hours and then were tilted to an upright position for a further 2 hours. Blood samples (50 ml), were taken at times shown in Fig. 15 for the measurement of plasma DOC and angiotensin II concentrations.

Results

As shown in Fig. 15, plasma angiotensin II concentration rose above normal range on tilting to upright position, but plasma DOC concentration showed no significant change.

Discussion

Under these experimental conditions, the rise in plasma angiotensin II is well documented and again shown in these experiments. As discussed in previous sections, an increase in plasma angiotensin II within physiological

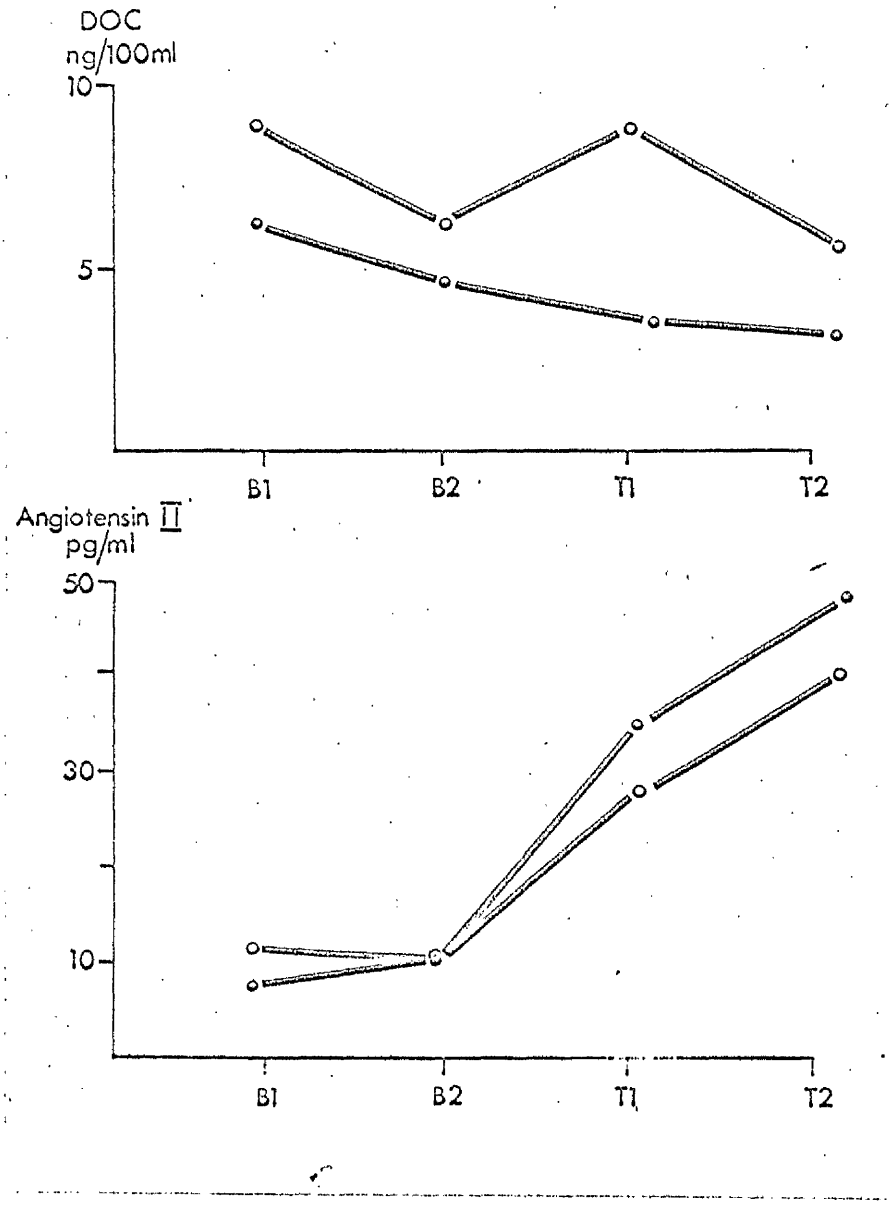

Figure 15.

The effect of posture on plasma concentrations of DOC and angiotensin II.

limits is unlikely to produce an increase in plasma DOC concentration.

In addition to the rise in plasma angiotensin II, the assumption of an upright position causes a redistribution of blood in the vascular system which is counteracted to some extent by increasing peripheral blood vessel constriction. A fall in splanchic blood flow should also result in a reduction of metabolic clearance rate and consequently a rise in plasma DOC concentrations such as have been shown for plasma aldosterone (Bougas et al, 1964). This did not occur. A possible explanation may be that although DOC does not operate the negative feedback mechanism whereby ACTH secretion is halted, it is nevertheless ACTH-dependent. Thus a rise in cortisol levels due to a general fall in corticosteroid metabolic clearance rate by suppressing ACTH secretion will also prevent plasma DOC concentration from rising. Aldosterone concentration which rises in these circumstances (Balikian et al, 1968) is largely independent of ACTH.

5.7. GENERAL COMMENTS AND SUMMARY

In section 5 studies of some of the factors known to affect aspects of adrenocortical secretion have been described. It was found that of the potential stimuli tested, only ACTH significantly affected plasma DOC concentration and that the rise in the level of DOC may be more rapid and of shorter duration than that of cortisol. Therefore in any investigation of DOC in man the same precautions must be taken when sampling blood as are important in the study of cortisol. Samples must be taken at the same time of day, preferably early in the morning to avoid the effects of diurnal variation and under conditions of minimal stress.

On the other hand changes in posture and dietary sodium intake did not alter plasma DOC and unless other substances such as aldosterone or angiotensin are under investigation the standardisation of these factors may be less important. The degree of haemorrhage involved in the experiments described here did not affect plasma DOC concentration.

Several known products of the adrenal cortex (see section 1) possess mineralocorticoid activity and may contribute to sodium homeostasis

To be effective such hormones must, in addition to promoting sodium retention by the kidney, respond to small changes in some parameter of the body's sodium status. While DOC is a mineralocorticoid of some potency and must contribute to the total effect of the adrenal cortex on the renal tubules its secretion and therefore plasma concentration do not respond to changes in sodium status at least within the normal physiological range. It is therefore unlikely that DOC is important in the fine control of sodium status in normal man.

However it is well known that administration of large doses of DOC derivatives to animals particularly when salt intake is high, may lead to severe electrolyte disturbances and hypertension. It is therefore conceivable that abnormally high DOC secretion in man, as in congenital adrenal hyperplasia due to partial or total absence of adrenal 11β-hydroxylase activity or following therapeutic administration of ACTH, may lead to similar electrolyte and blood pressure disturbances.

The following sections describe studies of plasma DOC concentration in hypertensive rats and man.

6. SOME STUDIES OF THE EFFECTS OF DOC ADMINISTRATION IN MAN AND THE RAT

Administration of derivatives of DOC

Much of the pharmacology of DOC in man and animals is based on results of experiments using derivatives such as DOC acetate (DOCA) or DOC pivalate. Experiments in man and rats were conducted to test the efficiency of absorption and the rate of hydrolysis of DOC esters *in vivo* and also to determine the plasma concentration of DOC attained by injection of DOC esters in quantities sufficient to produce hypertension in rats.

6.2. THE EFFECT OF DOCA ADMINISTRATION ON PLASMA DOC CONCENTRATION IN MAN.

Methods.

DOCA (5 mg/oil, i.m.) was given to two normal subjects. Blood samples (25 ml) were taken immediately before the injection and at intervals thereafter for the measurement of plasma concentrations of DOC and DOCA.

After extraction of the plasma the neutral extract was applied to a paper chromatogram (Bush, B5, 5 hours). The area corresponding to DOC and DOCA were eluted with nanograde methanol and the DOC extract was treated as detailed in gas chromatographic method 2. The DOCA extract was hydrolysed using saturated methanolic sodium carbonate (see gas chromatographic method 1) and then re-chromatographed in the same system (Bush B5, 5 hours). DOC area was eluted and after esterification with HFBA was applied to gas chromatography with electron capture detection.

Results.

Plasma DOC concentration rose rapidly to a maximum concentration after 1 hour and returned to basal levels within 24 hours. No DOCA was detected in any of the samples.

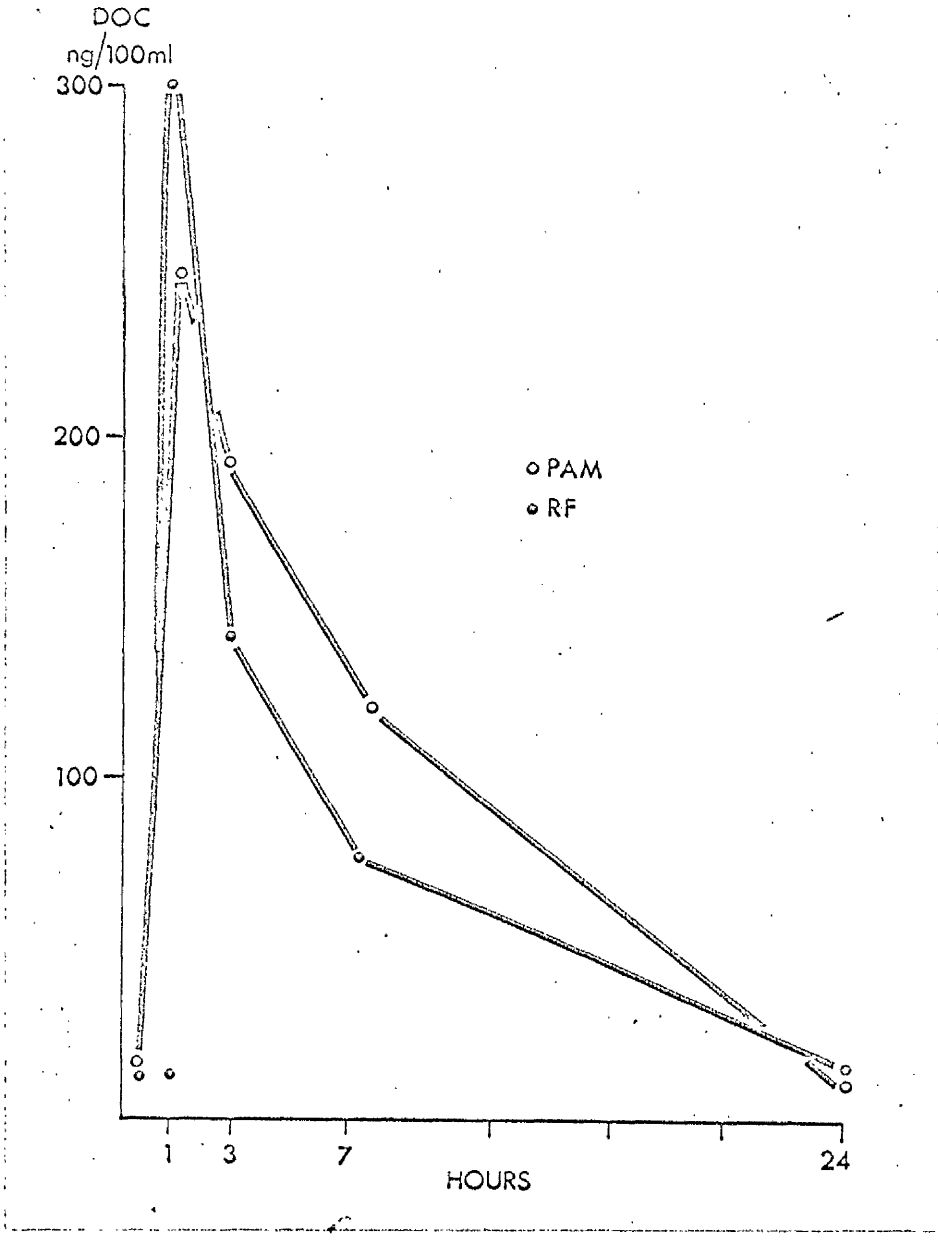

Figure 16.

Plasma DOC concentrations (ng/100 ml) in two normal subjects after injection of DOCA (5 mg/ coconut oil, i.m.).

It is hoped to publish this work as part of a study of DOC induced hypertension in rats conducted in our department in cooperation with several colleagues.

6.3. THE EFFECT OF DOC PIVALATE ADMINISTRATION ON DOC CONCENTRATION IN THE BLOOD OF THE RAT

Method

Under general anaesthesia a small indwelling polythene cannula was inserted in the jugular vein in 6 Sprague-Dawley rats. Twenty four hours later a basal blood sample (0.25 ml) was taken in a heparinised syringe from each rat while conscious. DOC pivalate (12.5 mg, i.m. 25-30mg/Kg) was injected in 3 rats and saline (0.5 ml, i.m.) in 3 control rats. Blood samples were then taken at intervals. Whole blood DOC concentration was measured by haemolysing the red cells with an equal volume of water prior to extraction. Purification and assay were as in gas chromatographic method 2.

Results.

The concentration of DOC in whole blood in both sets of rats are shown in Fig. 17. Maximum DOC concentration was attained 12 hours after the injection. DOC concentration in the control rats showed no rise but varied slightly throughout the period of study.

This experiment was a preliminary study set up in order to investigate the feasibility of

Whole blood DOC
concentration ng/ml.

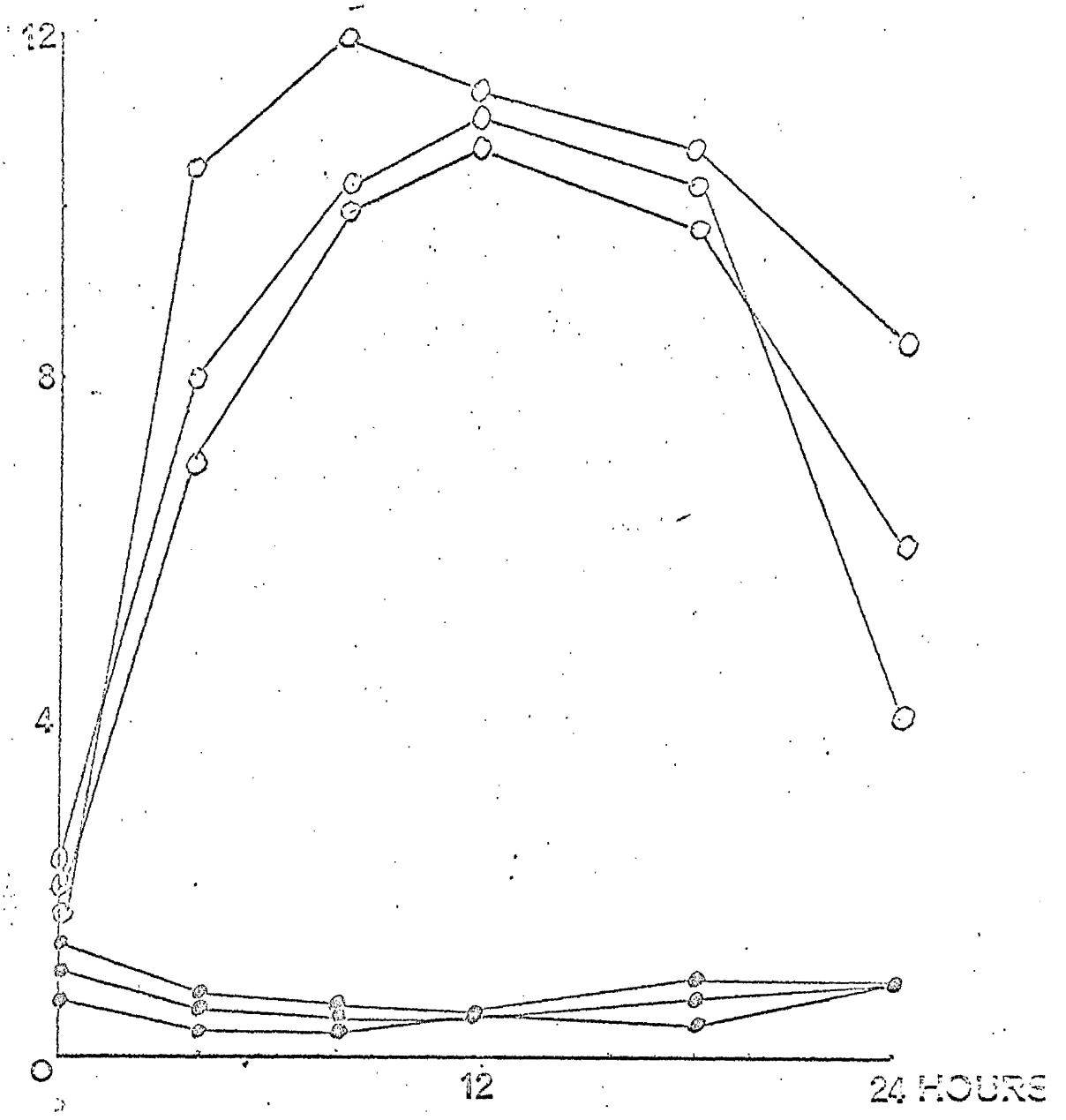

Figure 17

Whole blood DOC concentration in (o) DOCA and saline (o) injected rats.

measuring DOC concentration in small blood samples in the rat.

As previously stated the results of this study will be published later (Schalekamp et al, 1973).

6.4. DISCUSSION

The experiments with DOCA in man demonstrate that DOC is rapidly absorbed and hydrolysed *in vivo* giving rise to very high levels of DOC in plasma within 1 hour of administration of DOCA. Blood esterases must be capable of very rapid hydrolysis of the absorbed DOCA since DOCA was not detectable in any sample.

The injected DOCA is likely to have the same effects as DOC.

In experiments with rats, whole blood was used in preference to plasma because repeated sampling of blood from each rat was required. Smaller blood samples were more likely to avoid stimulating corticosteroid secretion. As separation of plasma from such small samples is difficult whole blood was used in this study.

The concentration of DOC in whole blood in the control rats remained normal throughout the study indicating that insertion of the plastic cannula and repeated blood sampling did not appear to influence plasma DOC concentration. Very high levels of DOC in whole blood were obtained in the DOC-pivalate treated rats indicating again that absorption and hydrolysis of the administered DOC ester occurs.

This experiment also demonstrated the suitability and validity of the gas chromatographic method for the study of hypertension and DOC in the rat. The high sensitivity of the method permits repeated measurement of DOC concentration in individual rats and consequently provides an efficient monitor of the levels of DOC, maintained over a prolonged period, which are sufficient to cause hypertension in the rat. In studies of DOCA-induced hypertension, the rats are normally unilaterally nephrectomised and then given saline as drinking water. Injections of DOCA given 2-3 times week induce hypertension within 6-8 weeks (Hall and Hall, 1968).

This present study indicates that under these circumstances the level of DOC in whole blood in the rat is on average several times higher than in control rats. However, in the rat DOC is the major mineralocorticoid and thus direct extrapolation of these results to the study of hypertension in man may not be valid. It is not entirely clear whether plasma DOC levels in man similarly elevated over a period of years would cause hypertension (see section 7).

7. PLASMA DOC CONCENTRATION IN HUMAN HYPERTENSION

7.1. Introduction

In hypertensive disease in man, low plasma concentrations of renin and angiotensin are often but not always related to high plasma levels of aldosterone (Brown et al, 1971). It is possible that under certain circumstances other corticosteroids such as 18-hydroxy-DOC (Melby et al, 1971; Genest and Nowaczynsky, 1970) or DOC may be involved in the aetiology of the disease. The following section describes the investigation of the levels of plasma DOC in such cases of hypertensive disease with low plasma renin and angiotensin and low or normal aldosterone.

7.2. Methods

Plasma DOC levels were measured in 31 hypertensive patients (21 female). The average blood pressure in the individual cases as outpatients before treatment ranged from 149/106 to 230/145 mmHg (mean for the whole group was 191/120 mmHg) and their ages ranged from 20 to 65 years. There were no symptoms or signs in any of the patients to suggest Cushing's syndrome, phaeochromocytoma or aortic coarctation. No abnormal sex characteristics were noted. None of the patients had peripheral oedema, papilloedema, retinal

haemorrhage or exudates. No evidence of renal disease was noted.

21 of these patients were selected for plasma DOC measurement because earlier investigations had shown evidence of mineralocorticoid excess (i.e. low plasma level of renin and/or potassium with normal plasma aldosterone concentration. The remaining 10 hypertensive patients were included for comparison because their plasma renin and aldosterone concentrations were known to be normal.

7.3. Results

Plasma concentrations of DOC, renin, aldosterone and potassium and also blood pressure are shown in Table 22.

Plasma DOC concentration was found to be consistently high in 6 of the 21 patients with low plasma renin concentration and in none of the 10 in whom normal renin levels were found. Intermittently raised plasma DOC levels were found in 3 patients with low plasma renin concentration and in two patients with normal plasma renin levels. In one of the 6 patients with consistently high DOC level, the estimation was repeated on the 14th day of treatment with dexamethasone (0.5 mg, 6 hourly)

	Low Renin Group		Normal Renin Group		t	p<
	RANGE	MEAN	RANGE	MEAN		
No. and Age of patients (female)	21(16) 22 - 65	46	10 (5) 20 - 63	43	0.57	0.6
Blood Pressure mmHg.	177/199 - 230/145	193/120	163/106- 200/140	186/119	0.78 0.16	0.5 0.9
Plasma Renin Units/litre	0.4 - 15.3	43	4.6- 20	10.0	6.39	0.00
Plasma deoxycorticosterone ng/100 ml	N.D. - 92.6	16.1	N.D. - 17.0	8.1	1.35	0.2
Plasma aldosterone ng/100 ml	N.D. - 17	10.4	N.D. - 17.5	8.5	1.16	0.3
Serum Potassium mEq/litre	1.9 - 4.7	3.8	3.4 - 5.7	4.1	2.32	0.05

TABLE 22. Clinical details in patients with raised plasma DOC levels.

and a similarly high result was obtained (34 ng/100 ml before and 31 ng/100 ml during treatment). In one patient DOC levels of 5.2 and 213 ng/100 ml were obtained in right and left adrenal venous plasma respectively.

7.4. Discussion

High plasma DOC concentrations have been described in several syndromes with hypertension, hypokalaemia, suppression of renin and low plasma aldosterone levels. These include tumours of the adrenal cortex (Crane and Harris, 1966; Biglieri et al, 1968; Wolff et al, 1968), defects in either 17α-hydroxylation (Biglieri et al, 1966; Goldsmith et al, 1967; Mallin 1969) or 11β-hydroxylation (Eberlein and Bongiovanni, 1956; Imai et al, 1968) and also ectopic corticotrophin syndrome (Crane and Harris, 1966; Schambelan et al, 1971).

The present results appear to indicate a new syndrome in which hypertension is associated with a low plasma level of renin, normal plasma aldosterone concentration, a raised plasma DOC concentration and hypokalaemia. High plasma DOC concentrations were found only in a minority of the low renin hypertension group and so these results

do not necessarily conflict with findings of consistently normal secretion or plasma concentration of DOC in smaller series of similar patients (Woods et al, 1969; Brown and Strott, 1971; Crane et al, 1972).

Aetiology of excess DOC in hypertension.

Adrenal venography and analysis of adrenal venous plasma was performed in two of the six patients with excess plasma DOC concentration. In one of these the appearances suggested an adrenocortical tumour; in the other there was a two-fold difference in DOC level in venous plasma from the two adrenal glands. More detailed studies are necessary in these patients.

11-hydroxy-corticosteroids and reponse to injected tetracosactrin were within normal range in all of these patients. Dexamethasone therapy did not alter serum electrolytes or blood pressure and did not reduce plasma DOC concentration in one patient studied. These findings indicate that the mechanism involved in this syndrome is not corticotrophin dependent.

Role of DOC in pathogenesis

It is not apparent whether or not the levels of DOC noted in these subjects would be sufficiently high maintained over a prolonged period to cause

the biochemical abnormalities and hypertension. Successful treatment of 5 of these patients with spironolactone, which acts as a competitive inhibitor of mineralocorticoids, suggests that DOC may be involved in the pathogenesis. High plasma DOC concentration have also been implicated in the pathogenesis of adrenal regeneration hypertension (Rapp, 1969) and can cause hypertension in animals (Ferebee et al, 1941; Gross et al, 1955; Hall and Hall, 1967) and man (Perera et al, 1944; Perera, 1948; Relman and Schwartz, 1952; Thorn et al, 1953).

DOC and suppression of renin.

Renin suppression is often associated with sodium retention (Brown et al, 1972) . The mechanism causing this suppression is not known. Despite the fact that the sodium retaining potency of DOC is thought to be less than that of aldosterone (Hall and Hall, 1967) it is reasonable to expect low renin in cases of DOC excess.

These present results appear to indicate a new syndrome in patients with high blood pressure. However illucidation of the mechanism causing high plasma DOC levels and determination of the extent to which this

mineralocorticoid is responsible for the hypertension merits further study.

This work has been published (Brown et al, 1972) and further investigation of this syndrome is being conducted in our department.

8. PLASMA DOC CONCENTRATION DURING NORMAL PREGNANCY

8.1. Introduction

During pregnancy there is apparent need for sodium retention and this is partly achieved by increased mineralocorticoid activity. There is also a gradual increase in protein bound corticosteroids in plasma throughout pregnancy.

However, in normal and hypertensive pregnancy plasma aldosterone concentration may not be elevated. It would appear that some other mineralocorticoid or mechanism is responsible for the increased sodium retention. The following study was designed in order to investigate plasma DOC concentration during pregnancy.

8.2. Methods and Subjects.

Plasma DOC concentration was measured in 29 women at various stages during pregnancy and in 10 women in the first week post-partum.

All were single pregnancies. Ages ranged from 17 to 33 years (mean 24.6 years). Blood pressure remained normal in every case and no other abnormalities were observed. Blood samples were taken after the women had lain supine for 30 mins. All the women were on unrestricted diet.

8.3. Results

Individual plasma DOC concentrations are shown in Table 23. The mean plasma DOC concentration was increased at 20.4 ng/100 ml (range 7.0 - 35.0).

Comparison of the mean concentration of plasma DOC during each trimester of pregnancy are shown in Table 23. There was no significant difference between stages of gestation. Plasma DOC concentration fell quickly to normal in the puerperium (Table 23).

8.4. Discussion

During pregnancy, plasma DOC concentration rises in a similar manner to plasma 11-hydroxycorticosteroids. The increase in 11-hydroxycorticosteroid concentrations during pregnancy is composed largely of a rise in the bound fraction probably due to a general increase in plasma protein concentration. However, there is also a rise in free cortisol (Brown et al, 1972) indicating possibly stimulation by ACTH. Since DOC is also bound to proteins (Burke, 1969) and sensitive to ACTH stimulation (see section 5.3) it is possible that these changes also contributed to the total rise

PLASMA DOC CONCENTRATION (ng/100 ml).

GESTATION (WEEKS)			POST-PARTUM	
0 - 12	13 - 26	27 - Term	0 - 2 WEEKS	
19.0	26.3	19.8	17.3	
14.0	24.4	34.7	10.1	
19.0	14.6	20.0	20.1	
15.0	20.6	20.0	11.5	
13.0	17.1	35.0	9.8	
15.0	16.3	10.0	14.8	
	33.3	12.0	14.7	
	9.1	35.0	11.6	
	16.0	7.0	9.2	
	18.0	16.0	6.1	
	15.0	11.0		
		25.0		
15.8	19.2	20.5	12.4	MEAN
2.6	6.7	10.1	4.3	± 1 S.D.
6	11	12	10	n

TABLE 23.

Plasma DOC concentration during pregnancy. There was no significant difference in plasma DOC concentration between each trimester. However there was a significant difference between plasma DOC concentration during the third trimester and post-partum levels ($t = 2.3445$, $p < 0.05$).

in plasma DOC. If bound DOC remains hormonally active it could conceivably contribute to sodium retention in pregnancy and thus an abnormally high concentration could possibly lead to hypertension particularly in a sodium loaded condition.

In a recent study of DOC in pregnancy (Brown et al, 1972) it was suggested that the raised maternal plasma DOC levels originate from the fetoplacental unit rather than the maternal adrenal cortex as dexamethasone administration did not suppress the maternal plasma DOC levels. The rapid disappearance of DOC from maternal plasma following delivery also supports this theory. These workers also found that in toxaemia of pregnancy plasma DOC levels were as high as those found in hypertension due to 11β hydroxylation deficiency (Brown and Strott, 1971). This suggests that DOC may play some role in toxaemia of pregnancy and it is therefore intended to extend this work to the study of hypertension in pregnancy.

9. PLASMA DOC CONCENTRATION IN SODIUM DEPLETION, TOTAL FASTING AND REFEEDING IN OBESE SUBJECTS

9.1. Introduction

Previous studies (section 5) showed that mild sodium depletion does not alter plasma DOC concentration in normal man. During the course of these studies the opportunity arose of studying a group of 4 obese subjects undergoing the more severe regime of total starvation as therapy for obesity.

9.2. Subjects and Methods

The subjects were placed on a sodium-restricted diet (10-20 m.equiv./day) for 5 days, followed by 12 days total starvation when only water was allowed to drink. A normal diet was then resumed.

At intervals throughout the study blood samples (50 ml.) were taken for measurement of plasma concentration of renin, DOC and electrolytes. Twenty four hour urine collections were also taken for measurement of urinary electrolyte excretion.

9.3. Results.

Weight.

The mean weight loss in the 4 obese subjects during the period of dietary sodium restriction was 3.4 Kg. (range 2.1 - 4.5 Kg) and during

total fast was 7.0 Kg (range 4.5 - 9.1 Kg).

Urinary electrolytes.

Sodium (Table 24).

The mean sodium loss during 5 days of dietary sodium restriction was 182 m.equiv. (range 113-210 m.equiv) and during 12 days of total fasting was 319 m.equiv. (range 72 - 875 m.equiv.). The mean cumulative sodium loss over the period of sodium restriction and total fasting was 501. m.equiv. In the refeeding period there was marked sodium retention. At the end of 3 days of refeeding the mean cumulative sodium gain was 262 m.equiv.

Potassium (Table 24)

During the 5 days of sodium restriction, the mean cumulative potassium loss was 18 m.equiv. (range + 14 to - 36 m.equiv) and during 12 days of total fast was 428 m.equiv (range 371 - 490 m.equiv.).

In the first 3 days of refeeding, the mean cumulative potassium gain was 57 m.equiv. (11 - 82 m.equiv).

Plasma renin concentration.

At the end of 5 days of sodium deprivation plasma renin concentration rose in all 4 subjects (Table 25). In the first few days of

	Sodium depletion Cumulative loss		Total Fast Cumulative loss		Refeeding Cumulative gain	
	Mean	Range	Mean	Range	Mean	Range
Na	182	113 - 210	319	72 - 875	475	406 - 510
K	18	14 - 36	428	371 - 490	100	46 - 135

TABLE 24.

Cumulative changes in urinary electrolyte excretion (m.equiv.) during sodium depletion, total fasting and refeeding in obese subjects.

Day	A	B	C	D
1	10.3	10.2	16.0	6.8
6	20.6	14.8	22.8	17.6
7	18.3	29.2	11.2	11.5
8	16.3	25.5	24.7	9.7
9	-	27.0	18.6	9.7
14	17.6	16.2	25.7	16.3
15	-	42.5	19.9	18.2
18	92	60.7	17.9	19.8
20	40.3	52.5	17.9	21.3
22	22.8	27.7	14.4	8.5

TABLE 25.

Plasma renin concentration (units/litre) during sodium depletion, total fasting and refeeding in obese subjects.

total fast, plasma renin fell, rising again towards the end of the period of total fast. During the refeeding period plasma renin concentration again fell rapidly.

Plasma DOC concentration

At the end of the period of dietary sodium restriction plasma DOC concentration rose in all four subjects. During the period of total fast it fell rapidly to very low levels below the normal range (Figure 18, Table 35). In the refeeding period plasma DOC rose towards or above the normal range.

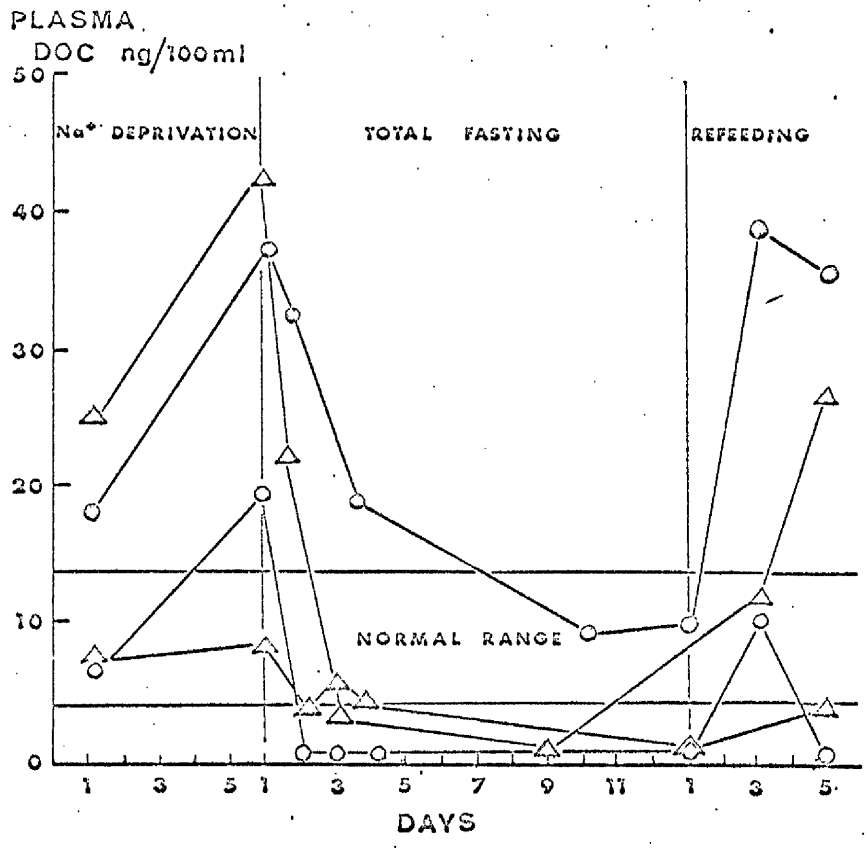

FIGURE 18

Plasma DOC concentration (ng/100 ml) in obese subjects during sodium deprivation, total fasting and refeeding.

9.4. Discussion

1. Effects of sodium depletion.

In this study the mean cumulative sodium loss (182 m.equiv.) was considerably greater than that achieved by sodium depletion of normal subjects (section 5.5). This may be due to the effect of the lower sodium content of this diet used in this present study (10-20 m.equiv.) compared with that used in the previous study of normal subjects and to the effect of the weight loss of the obese subjects.

During the period of sodium depletion plasma renin rose in all 4 subjects. This indicates that the renin angiotensin system would be activated and as a result aldosterone secretion would probably be raised although it was not measured in this case. However, the relationships of renin and aldosterone is known to be valid in obese subjects (Chinn et al, 1970).

Plasma DOC concentration rose during sodium depletion in contrast to the results of sodium depletion in normal subjects (section 5.5). It is not clear whether this difference is due to the effects of greater sodium loss or to some particular endocrinological aberration in obesity. For example distinguishing between obesity and

Cushing's syndrome by adrenal function tests occasionally proves difficult as obese subjects may have high resting corticosteroid levels. The ACTH secreting mechanism is more sensitive in obese subjects although this does not cause a rise in cortisol (Chinn et al, 1970). However, DOC may be marginally more sensitive than cortisol to ACTH.

Renin-DOC relationship

Angiotensin II infusion in normal subjects does not increase plasma DOC concentration. This means that either there is no relationship between the increase in plasma renin concentration and the increase in plasma DOC concentration or some mechanism peculiar to obese subjects is responsible for these changes in renin and DOC. Further study is obviously required to investigate this point.

II. Effects of total starvation.

It has been shown that plasma aldosterone concentration rises during total starvation as a result of sodium depletion (Chinn et al, 1970) However, there is a temporary fall in renin associated with a diuresis when calorie intake is reduced to zero. This dissociation of renin and aldosterone is discussed by Chinn et al (1970)

The 4 subjects continued to lose sodium and at the end of the period of starvation were much more severely sodium depleted than after uncomplicated sodium depletion.

Total starvation causes a fall in blood glucose which, as in the insulin stress test, is an effective stress stimulus. However, Chinn et al (1970) found no change in plasma cortisol during fasting.

Plasma DOC concentration falls to low levels during total starvation while plasma aldosterone concentration rises (Chinn et al, 1970). This may be due to an increased efficiency of 11β of 18 hydroxylation of DOC. This could not be caused by stimulation by angiotensin II as the concentrations of renin and therefore of angiotensin are low. In any case angiotensin II acts early in the biosynthesis of aldosterone during the conversion of cholesterol to pregnenolone (Muller, 1970). The decrease in DOC concentration and simultaneous increase in plasma aldosterone concentration may be due to some unknown factor acting on the biosynthetic pathway between DOC and aldosterone. Study of corticosterone and 18-hydroxycorticosterone

would be necessary to ascertain the extent of the impairment of secretion.

III. Refeeding.

Sodium retention occurred rapidly during the period of refeeding (475 m.equiv.) and plasma DOC concentration quickly attained pre-treatment levels.

10. GENERAL CONCLUSIONS.

10. GENERAL CONCLUSION

Techniques. The development of new techniques and methods of assay of steroid hormones has provided a quantitative basis for study of the role of the adrenal cortex in health and disease. The discovery of new disease situations such as Conn's syndrome has followed the development of methods of measuring plasma concentrations or secretion rates of aldosterone and other corticosteroids. In the present work methods of measuring the plasma concentration of DOC have been developed and compared and these methods have been instrumental in the discovery of an apparently new syndrome in which hypertension is associated with low plasma concentrations of renin and angiotensin II, normal plasma aldosterone concentration, raised plasma DOC concentration and intermittent or persistent hypokalaemia.

Methods developed for the analysis of large numbers of samples need to be convenient, precise and highly specific. The gas liquid chromatographic method and radioimmunoassay are convenient and precise. However, the gas-liquid chromatographic method is more specific and would be the method of choice for analysis of unknown samples or samples from patients who have been

prescribed steroid drugs such as spironolactone. The specificity of the radioimmunoassay, which gave results comparable to the gas liquid chromatographic method, can not be assumed in such a situation where unknown drug metabolites may be present. Both methods possess high sensitivity adequate for analysis of human plasma and also small volumes of whole blood in animal studies. This has proved to be of particular benefit in the study of hypertension in the rat where the level of blood pressure attained can now be compared with the concentration of DOC prevailing in whole blood at that time.

Application of electron capture detection of steroids to analysis of biological samples has until recently been hindered by the problem of contamination of the detector. The introduction of a by-pass valve into the gas chromatograph has greatly increased the applicability and sensitivity of the technique for analysis of biological extracts by overcoming the problem of detector contamination.

Obviously, there is yet great scope for improvement of these techniques. For example, use of capillary columns coated with stationary phase would improve the quality of the chromatography and minimise column bleed.

This may improve the sensitivity of the GLC method by minimising losses due to column absorption.

In the techniques of radioimmunoassay, more specific antisera are yet required in order that prior extraction and chromatography of sample may be omitted. The more successful means of achieving the end may not be the improvement of immunisation procedure and techniques but purification and isolation of particular antibodies in antisera presently in use.

10. Control of DOC secretion.

DOC is secreted by the adrenal cortex as a result of stimulation by ACTH and is in a true sense a secretory product of the gland. Present findings indicate that DOC secretion is controlled mainly by ACTH in contrast to aldosterone which is governed mainly by the renin angiotensin system. Moderate electrolyte changes due to dietary sodium depletion does not alter plasma DOC concentration in normal subjects, although in obese subjects this produces an increase in plasma DOC concentration. DOC is to some extent bound to plasma proteins such as albumen or corticosteroid binding globulin and as a result raised plasma DOC concentration is

found in conditions such as pregnancy where there is an increase in plasma protein concentration.

Role of DOC in sodium retention.

DOC is thought to be a much less potent mineralocorticoid than aldosterone in man (Hall and Hall, 1967) and as it is found in plasma in similar concentrations to aldosterone it is assumed that DOC does not play a major role in normal electrolyte and water balance but probably contributes to the overall control. However, the evidence that DOC is a less potent mineralocorticoid than aldosterone is not conclusive as no controlled pharmacological studies has been done in normal man.

Role of DOC in pathogenesis of hypertension

In this present study raised plasma DOC levels have been found in patients with hypertension. Whether the levels of DOC measured in these subjects would be sufficient to raise blood pressure and cause the electrolyte abnormalities has yet to be established. However it is known that DOC will correct the electrolyte abnormalities associated with Addison's disease. Also in nephrectomised rats given saline as drinking water DOCA injections given frequently will induce severe hypertension.

Further Studies.

Investigation of the control of electrolyte and water homeostasis in man requires a knowledge not only of the behaviour of each individual mineralocorticoid but also an understanding of their relationship and relative contribution to this total control. The development of gas liquid chromatographic techniques for measurement of all the known mineralocorticoids (Mason, 1973) using a small volume of plasma is in progress and may prove useful in the investigation of patients with hypertension presumably due to excess of one or more mineralocorticoids in plasma.

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