A MICROMETHOD FOR THE SIMULTANEOUS DETERMINATION OF CORTISOL AND CORTICOSTERONE AND ITS APPLICATION TO EXPERIMENTAL AND CLINICAL CONDITIONS

By

GANJAM KUPPUSWAMY VENKATASESHU

Bachelor of Science
Sri Venkateswara University
Tirupati, India
1958

Bachelor of Veterinary Science
Sri Venkateswara University
Tirupati, India
1962

Master of Science
Washington State University
Pullman, Washington
1965

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
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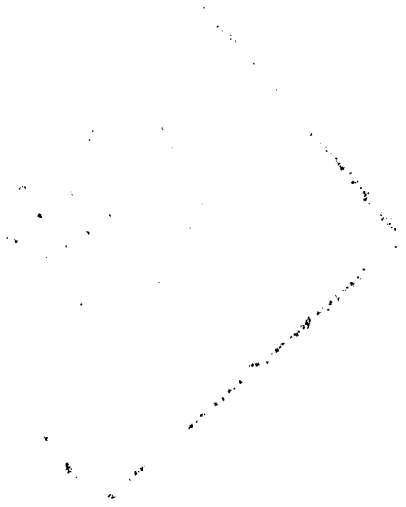

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Thesis Approved:

Larry Ewing
Thesis Adviser

Claude Desjardins

Kent E. Elmer

Calvin V. Beames, Jr.

J. Dunham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

The adrenal gland, though small in size, is essential for life. Studies in laboratory animals and man have done much to elucidate the two major functions of the many endocrines secreted by this gland. These biologically active steroids can be divided into those which aid in 1) the maintenance of electrolyte and water balance, and 2) the regulation of carbohydrate and protein metabolism. Mineralocorticoids include those steroids which exert an effect on electrolyte and water balance while those which influence the level of blood sugar are called glucocorticoids. The properties of these hormones overlap and a steroid may exert effects common to both functions. As the knowledge of adrenal function increased, much research was directed toward the assessment of adrenal activity in the living animal in health and disease. This was important in human medicine where early diagnosis of adrenal involvement meant therapy could be started sooner and recovery rate increased.

The multitude of adrenocorticoids secreted (approximately 30) warn against drawing unsupported conclusions as to the functional state of the adrenal gland when only one or two of its secretory products is determined. Conversely, estimation of 30 different steroids, classified as adrenocorticoids, is an enormous task and is not required to gain insight into the state of the adrenal gland. A compromise is necessary, and therefore, many investigators concentrated on the quantification of

the two major glucocorticoids (cortisol and corticosterone) and the mineralocorticoid aldosterone. These three compounds are for all practical purposes the naturally occurring, biologically active secretory products of the adrenal cortex and their measurement will often give decisive information as to the function of the adrenal cortex (Eik-Nes, 1960). Both cortisol and corticosterone are secreted by the adrenal cortex in different ratios in different species (Bush, 1953 and Hechter et al., 1954). The ratio of cortisol to corticosterone found in blood plasma is characteristic of a species, but within a species, the ratio seems to vary according to the magnitude and the type of adrenal stimulation (Kass et al., 1954 and Symington, 1960).

Alterations in the ratio of cortisol and corticosterone may thus represent a significant physiological adaptation of an animal to its internal or external environment. Hence, a simple quantitative method which allows for the individual determination of cortisol and corticosterone would be a valuable tool for studying adrenal function. Although, a variety of methods have been developed to estimate the concentration of 11-hydroxy-corticoids in small samples of crude plasma extracts, these methods have not been satisfactory because they lack the required specificity. The corticosteroid levels measured by these brief methods are considerably higher than those obtained by the more specific techniques employing chromatography, due to the presence of interfering fluorogens in the crude extracts. Although, the chromatographic techniques as developed by Sweat (1954), by Ely et al., (1958) and McLaughin et al., (1958) overcome many of the disadvantages, they are relatively complicated and not well adapted for routine use. Therefore, a simple, precise, sensitive and specific method for the simultaneous

determination of cortisol and corticosterone which is applicable to many species and will allow the routine investigation of adrenocortical function in small samples of biological material has to be developed.

The investigation reported herein describes in detail the development of such a micro-method, its extensive validation regarding precision, sensitivity and specificity and its applicability to both experimental and diseased conditions.

CHAPTER II

REVIEW OF LITERATURE

Cortisol (F), corticosterone (B) and aldosterone are the major secretory products of the adrenal gland in most animal species investigated (Yates and Urquhart, 1965). Corticosterone appears to be secreted by the adrenal gland of the echidna (Tachyglossus) while cortisol is the predominant adrenal hormone in the brush-tailed opossum (Trichosurus) (Chester-Jones et al., 1964). The monotremes are believed to be living examples of extremely archaic "premammalian forms" and appear to have remained unchanged over a period antidating the appearance and evolution of placental animals.

The adrenal enzymes involved in the production of hormones like cortisol and corticosterone could, therefore, be phylogenetically old and might thus have been involved in the maintenance and distribution of many species of animals throughout an indefinite period of time. Cell function is regulated by hormonal and nervous control and these regulatory mechanisms probably developed simultaneously in most animals. This development resulted in the occurrence of enzymes producing adrenocorticoids in a localized tissue of the multicellular organisms of high complexity. The functions of these enzymes are involved in many regulatory processes, and therefore, an understanding of adrenal secretion of steroids may often aid diagnosis and help therapy in different diseased states.

Requirements of an Assay Method for Adrenal Steroids

Although different assay methods may be used for the estimation of corticosteroids, regardless of their simplicity or complexity, all must satisfy the requirements of specificity, reproducibility, and sensitivity. Specificity, to a degree, will depend upon the methods used for extraction and the degree of purification of the adrenocorticoids achieved. The second requirement for an assay method is reproducibility. This means that a method should yield relatively constant values in replicate determinations of the same sample analyzed on the same or different days. In addition, when used by several groups of investigators, the results should be in agreement (Péron, 1962).

The choice of an assay method depends upon the concentration of adrenal steroids present in the tissue, blood or urine samples to be evaluated. If the concentration of adrenal steroids is in the milligram range, one could use a colorometric or bio-assay for quantitation. In the sub-microgramic range, one could use ultraviolet spectrophotometric methods. Radioisotopic, fluorometric or protein-binding techniques have to be used when they are in microgramic or sub-microgramic ranges. Regardless of whether the methods are to be used as research tools or in routine clinical assay, too much emphasis cannot be placed on the above criteria. Other points to be considered are rapidity, simplicity and cost. Obviously, assay methods must be chosen with regard to the information which is required (James et al., 1967).

Once the requirements for a good assay have been met, consideration must be given to the conditions under which the biological samples were collected. Diurnal variation in adrenal steroid secretion over a

24-hour period has been well established (Peron, 1962). Therefore, short-term urine collections cannot reflect daily secretion patterns since the values will vary with the time of collection. This also applies to blood and tissue studies (Reddy et al., 1956).

One of the apparent advantages of assaying blood corticosteroids is the possibility of differentiating between administered substances which can cause a protracted, a transitory, or a rapid rise in plasma corticosteroids as a result of an activation of the adrenal glands. Conditions which cause transitory rises in blood adrenocorticoids will not be detected by analyzing the urinary corticosteroids of a pooled 24-hour sample of urine. In general, however, good agreement exists between blood and urinary corticosteroid levels (Eik-Nes, 1960).

Methods for Determination of Adrenal Steroids in Blood

Adrenocortical hormones can be quantitated by bioassay or by direct chemical measurement. The biological response to a stressor initially served as the end point for bio-assay of corticosteroids. Evaluation of the potency of adrenal cortical extracts and of purified corticoids was based on the increased survival of adrenalectomized animals exposed to various stressors (Selye and Schenker, 1938; Zarrow, 1942; Dorfman et al., 1946). A decrease in circulating eosinophils following corticoid administration to normal animals has also been used (Denison and Zarrow, 1954; Zarrow and Denison, 1956). These early bio-assay methods for measuring corticosteroid concentrations in adrenal or peripheral venous blood suffered from the common defects of bio-assay techniques; lack of specificity and insufficient sensitivity. Thus, these methods were unsuitable for routine quantitative estimations of corticosteroids

(Bondy and Altrock, 1953). Moreover, such methods require a large number of test animals and may become an extremely expensive undertaking when a large number of samples need to be analyzed.

Chemical assay procedures have been in use since 1914, but until 1952 were only used successfully in the measurement of urinary corticoids (Callow, 1950). Cochran and Page (1948) attempted to use the periodic acid-formaldehydogenic technique (successfully used for urinary corticoids) for the measurement of adrenal steroids in the peripheral blood, but obtained very high values for blood corticoids due to the formation of formaldehyde by the phospholipids of blood corpuscles. Such problems were often encountered when urinary assay methods were applied to plasma. In the early 1950's, investigators at the University of Utah were able to measure corticosteroids in human peripheral plasma by chemical assay (Nelson et al., 1951; Nelson and Samuels, 1952; Eik-Nes et al., 1953) utilizing florisil chromatography for purification and phenylhydrazine reduction for quantitation.

Any method for the determination of steroids in biological material must, in some way, include the following steps:

1) Extraction of steroids from the biological material by the use of a suitable solvent.

2) Purification and separation of the steroids from the unwanted materials or interfering impurities.

3) Identification of the steroids.

4) Quantitative estimation by some suitable analytical method, of the amount originally present in the biologic material used.

Therefore in evaluating procedures now available, one must give consideration to these four areas.

Extraction of Free Adrenocorticoids

The first step in every procedure is to quantitatively extract the steroids from the biological material (urine, whole blood, plasma, serum, or tissue).

Most of the early methods for the estimation of adrenocorticoids in blood emphasized the use of blood plasma because the amounts in the red cells were small and enzymes of the red cells tended to alter the steroids. Blood serum was also used in such methods and it is the experience of some investigators that little difference exists between the level of corticosteroids in plasma compared to the serum of the same blood sample (Eik-Nes, 1960).

Admittedly, the use of whole blood rather than blood plasma complicates an assay for the estimation of circulating adrenocorticoids. Therefore, investigators prefer to use plasma for adrenocorticoid determination. The procedures reviewed herein are confined mostly to those that are applicable to blood plasma.

A variety of organic solvents have been used for the extraction of plasma adrenocortical steroids. Peterson et al., (1957) determined partition coefficients for cortisol between organic solvents and water and found that 98% of plasma cortisol could be removed in one extraction with five volumes of methylene chloride. In their original method, Nelson and co-workers (1951) used chloroform and this solvent has been used by many other investigators (Berliner, 1957; Bondy et al., 1957; Rongone, 1958; Matsumura et al., 1967). In addition to the above solvents, butanol (Reddy et al., 1956; Cohn and Bondy, 1959) has been used successfully. Various combinations of solvents such as methanol:

ether (Bondy and Altrock, 1953), ethyl acetate: ether (Reich, 1958; Baird and Bush, 1960; Zolovick et al., 1966), carbon tetrachloride: methylene chloride (Frankel et al., 1967), and ether: chloroform (Nelson and Samuels, 1952) have been employed by some investigators.

In their evaluation of different solvents, Peterson et al., (1957) found little to choose between ethyl acetate, choloroform, or methylene chloride. Many investigators prefer methylene chloride as a solvent for extraction because it is heavier than water, and thus, it is possible to aspirate and discard the top aqueous phase and alkali layers to avoid the use of separatory funnels and also removes less lipids than does ethylacetate or chloroform. Formerly, extraction procedures were carried out using separatory funnels. However, presently, most laboratories are using centrifuge tubes to accelerate the separation and solve emulsion problems (Silber and Porter, 1954; Bondy et al., 1957; Peterson, 1957).

The solvents described above extract only the free corticosteroids leaving the glucuronide and sulfate conjugated in the aqueous phase. In order to achieve the extraction of total corticosteroids, Reddy et al., (1956) prepared an acidified protein-free filtrate and extracted this filtrate with n-butanol. Bongiovanni (1954) measured total corticosteroids by incubating the plasma with glucuronidase followed by extraction with chloroform.

Purity of the solvents used to extract steroids is of extreme importance. It has recently been reported (Frankel and Nalbandov, 1966) that some unpurified solvents commonly used in the extraction of corticosteroids bring about steroid decomposition. The purified solvents which had little effect upon the stability of the steroid, were also

singularly free of fluorogenic contamination as these investigators noted by low extraction and reagent blanks in a fluorometric determination of corticosterone.

Purification and Separation

Purification of Extracts Containing Adrenocorticoids

The most variable and difficult stage in steroid analysis is the purification and concentration of the original extract. Though complex purification procedures usually increase the specificity of the method, each such step is accompanied by an unavoidable loss of steroid, thereby decreasing the sensitivity of the procedure. On the other hand, insufficient purification and a failure to remove interfering substances renders the quantitation of the desired steroid difficult and unreliable (Peron, 1962). Common methods of purification and separation are solvent partitioning, alkali washes, and chromatographic (column or paper or thin-layer) techniques. Counter-current distribution may also be applied (Carstenson, 1955).

Several investigators have pre-washed plasma with carbon tetrachloride (Silber and Busch, 1956), or isooctane (Silber et al., 1958; Guillemin et al., 1959) as a method of removing lipids. Such pre-extractions of plasma removes lipids and non-steroidal material less polar than C-21 steroids from plasma leaving behind the steroids of interest (Silber and Porter, 1957; Berliner, 1957).

Solvent partitioning between various organic solvent and aqueous solutions has been used as a method of removing unwanted materials from the extracts. One partition between equal volumes of a Shell petrol

fraction similar to "Skelly-solve B" and 70% aqueous ethanol removed 88% of plasma fats, leaving steroids of relatively low polarity in the aqueous phase. Emulsions formed when 70% methanol was used (Tamm et al., 1958) and more efficient purification resulted when the aqueous layer was acidified (Tamm and Starlinger, 1957). Another common and popular partitioning method which is especially applicable to the extraction of fats from blood plasma is based on the fact that aqueous solutions of adrenal steroids can be easily and safely partitioned between petroleum ether (ligroin) and 70% aqueous ethanol. Most lipids will dissolve in the petroleum ether resulting in an almost fat-free aqueous ethanolic phase containing the adrenal steroid (Peron, 1962). Partitions between aqueous ethanol or methanol at various concentrations and ethyl acetate (Morris and Williams, 1953), carbon tetrachloride (Morris and Williams, 1953), hexane (Kass et al., 1954; Carstensen, 1955; Robertson and Mixner, 1956), pentane (Kassenaar et al., 1954; Kassenaar et al., 1955), toluene-hexane (Kassenaar et al., 1954), and toluene-ligroin (Kassenaar et al., 1955) have also been used.

A benzene-water partition (Gornall and MacDonald, 1953; Eik-Nes, 1957) achieved considerable purification of cortisol (Bierich, 1959), but losses of corticosterone into the organic phase were observed. The more polar estrogens, such as estriol and 16,17-ketols, when present, would remain in the cortisol fraction and must be removed in subsequent steps.

A feature common to most extraction procedures for adrenocorticoids, regardless of the solvent used, is the alkali wash (usually 0.1 N NaOH) of the extract. This procedure is usually carried out following solvent extraction of plasma. This removes some estrogens, as well as

other phenolic and acidic compounds. One-fifth to one-tenth volume of 0.1 N NaOH has been commonly used (Silber and Busch, 1956). Venning (1954) observed destruction of small amounts of adrenal steroids as a result of treatment with alkali. Milder conditions have been used, such as washing with sodium carbonate (Bush and Sandberg, 1953) or adjustment of the plasma to pH 9.0 $\pm$ 0.5 prior to extraction (Bondy et al., 1957).

Crude chromatographic purification on "florisil" and celite - commercial magnesium silicate preparations (Nelson and Samuels, 1952), and silica gel column (Reich, 1958; Eechaute, 1966a,b) have been used in some methods. It has been claimed (Reich, 1958) that silica gel yields fractions of greater purity and better recovery than partitioning between petrol and 70% ethanol.

Isolation of Adrenocorticoids in Purified Extracts

Different systems of adsorption or partition chromatography are widely used for the purification of extracts containing adrenocorticoids. In addition to purification, these techniques also result in separation of the individual steroids and in some instances even semi-quantification of the separated steroids (Bush, 1961). The techniques of steroid chromatography comprise an impressive literature. Many excellent reviews of different chromatographic methods for steroids have been published in recent years (Bush, 1961; Neher, 1964; Stahl, 1965; Randerath, 1966; Dominguez, 1967; Heftmann, 1967). Books by Bush (1961) and Neher (1964) are particularly valuable for investigators in the field of steroid chemistry.

Column Chromatography

This technique is particularly well suited for preparative purposes, but is often employed also for qualitative and quantitative analytical separations of adrenocorticoids. Either adsorption (solid and liquid phases) or partition (between liquids) chromatography may be used. The mixture, dissolved in a suitable solvent, is passed down a vertical column filled with an adsorbent or with another solvent on a carrier (the stationary phase). If the constituents of the mixture have different adsorption or partition coefficients, the individual substances will separate according to their varying mobilities (Neher, 1964).

Column chromatography is generally used for two purposes, either for removal of large amounts of impurities from an organic extract containing adrenocorticoids, or as a purification step after a steroid has been eluted from a paper strip. Such eluates can contain high amounts of non-steroidal material (Bush, 1964).

The following points are to be considered in deciding whether to employ adsorption or partition for a given purpose. Strongly polar steroids with carboxyl or several hydroxyl groups are suitable for adsorption chromatography only in the form of their esters, e.g. as acetates or as methyl esters. Adsorption chromatography has the advantage, however, of being easier to carry out, and more rapid than partition chromatography; moreover, the latter usually requires 10-30 times as large a column (by volume) as the former to separate a given weight of material. In making a choice between the two methods one must therefore consider: 1) polarity of the substance, 2) separating

power, and 3) the consumption of time and materials (Neher, 1964).

Chromatography in florisil columns (Nelson and Samuels, 1952; Ely et al., 1958; Saba, 1964) removes many contaminants and also separates the corticosteroids from the plasma 17-oxosteroids. After the chloroform extract is applied to a column of florisil, continued elution with chloroform removes pigments from the column, 2% ethanol in chloroform removes 17-oxosteroids, and 25% ethanol in chloroform elutes the corticosteroids (Gray et al., 1961). Column chromatography on silica gel has been reported by a number of investigators (Porter, 1958; Reich, 1958; Rongone, 1958). Weichselbaum and Margraf (1955) have reported consistently good separation of cortisol and corticosterone on silica gel using formamide, cyclohexane, and methylene chloride to develop the column. Eechaute (1966a,b) has also used silica gel columns for the determination of plasma cortisol and corticosterone. Generally, column chromatography has not given resolution of steroids equivalent to paper or thin-layer systems, nor can this procedure be used with success with the small amount of steroids often present in plasma extracts.

Paper Chromatography

Two types of paper chromatography systems are presently used. In the Zaffaroni system, the chromatography paper is impregnated with a nonvolatile solvent such as formamide or propylene glycol, which acts as the stationary phase (Zaffaroni et al., 1950; Burton et al., 1951a,b; Zaffaroni and Burton, 1951; Zaffaroni, 1953), while the Bush system uses a volatile solvent as the stationary phase. In the latter system, the stationary phase equilibrates with the paper prior to adding the mobile phase (Bush, 1952, 1954). In both systems, the mobile phase consists

of a volatile solvent selected according to the polarity of the steroids to be separated.

Extensive use has been made of several Zaffaroni type systems. Hexane-formamide has been used to remove the non-polar lipid contaminants (Zaffaroni, 1953; Hofmann, 1956), benzene-formamide for the separation of low and medium polar steroids from highly polar steroids (Zaffaroni et al., 1950; Burton et al., 1951; Zaffaroni, 1953), and chloroform-formamide (Caspi et al., 1953), toluene-propylene glycol (Burton et al., 1951; Unger et al., 1955), butyl acetate-water-formamide or butyl acetate-water-ethylene glycol (Mattox and Lewbart, 1958) have been used for the fractionation of mixtures of highly polar steroids into individual steroid components. Zaffaroni (1953) devised a system whereby the steroids of different polarities could be separated one after another by moving the paper containing the mixture through a series of chromatographic chambers. The steroid mixture was applied to a formamide impregnated paper and the nonpolar substances removed by development in the hexane system. After an appropriate time, the paper was removed, the volatile phase quickly evaporated, and the paper then placed in the chamber of the next higher polarity and so on through to the most polar system. The overflow from each system was collected and rechromatographed.

Bush (1952, 1954, 1961) developed a number of chromatography systems made up of a mixture of one or two solvents (ligroin, toluene, benzene, chloroform, xylene) together with methanol and water in varying proportions. After mixing, the water phase is placed in the bottom of the tank to saturate the atmosphere and equilibrate with the paper. Bush specified that the paper must equilibrate in the chamber several

hours or overnight at 30-38° C. He indicated that best speed was obtained if development was carried on at 35-38° C (Bush, 1954). The steroid mixture is applied to the dry paper which is then hung in the chromatography chamber saturated with both phases and allowed to equilibrate for some time. The paper absorbs the polar phase and the chromatogram is then developed with the weakly polar mobile phase. Alternately, the dry paper can be impregnated with aqueous solvents or stationary phases rendering equilibration unnecessary.

The Zaffaroni type systems are advantageous for separating large amounts of steroids and give good resolution between steroids which have similar chromatographic characteristics. However, in these systems, the low and medium polar steroids may run completely off the paper and must be rechromatographed. Also, the non-volatile stationary solvent takes considerable time to evaporate from the paper. The advantages of the Bush type paper chromatographic systems are: 1) short running time, 2) chromatograms are quickly and easily dried, 3) well adapted to micro quantities of steroids, and 4) all the steroids remain on the paper. Large quantities of steroids are not easily handled in the Bush systems and the low polarity steroids are not sharply resolved.

Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) techniques have the great advantage that qualitative and quantitative data can be obtained simultaneously. There have been some attempts made to separate and quantitate the corticosteroids by the use of gas chromatographic procedures. Corticoids containing 17-hydroxy groups tend to decompose due to the high temperatures at the flash heater zone (Vanden-Heuvel and Horning, 1960) and

various attempts have been made to convert the thermally unstable side chain of these corticosteroids to an alternative stable form using periodic acid oxidation (Merits, 1962; Kittinger, 1964, 1968), bismuthate oxidation (Bailey, 1963, 1964), and acetylation (Wotiz et al., 1956; Kliman and Foster, 1962; Carr and Wotiz, 1963; Brooks, 1965). Thermal decomposition (Luetscher and Gould, 1964; Gottfried, 1965) procedures and their quantitative aspects for the gas chromatographic analysis of corticosteroids have also been reported. It should be emphasized that, presently, no suitable method is available for gas chromatographic separation or quantitation of free adrenal corticosteroids.

Thin-Layer Chromatography

The development of thin-layer chromatography (TLC) has provided research workers with an important new method for the analysis of biological material. Thin-layer adsorption chromatography had been used to a limited extent in the field of lipid research prior to Stahl's classical description of TLC in 1956. However, it was only after Stahl used this technique for many compounds that extensive application was made of TLC, not only in the lipid field, but in various investigations requiring separations of inorganic materials.

The theoretical aspects of TLC closely parallel those of other types of chromatographic techniques. Stahl (1965) and Randerath (1966) have described TLC techniques, equipment, coating materials, and solvent systems and have given a great number of specific examples.

Research investigators in many fields soon recongized the simplicity, speed, low cost, and high resolving power of TLC. The

tremendous output of literature on the use of TLC is an index of its acceptance in a variety of applications (Konaka and Terabe, 1966; Attal et al., 1967; Race and Wu, 1967; Vandenheuval, 1967). As in any chromatographic procedure, steroid separations involve molecular weight, polarity, solubility, and structural isomerism. Eluting or developing power increases solvent polarity and the solubility of the steroid in the mobile phase (Wollish et al., 1961; Lisboa, 1966a,b). Of particular interest in steroid separations is the fact that minute amounts of mixtures may be efficiently resolved on thin-layer plates (Heftmann, 1967). As with other methods, TLC lends itself well to isotopic tracer methods.

Many solvent systems for the separation of corticosteroids have been reported (Lisboa, 1963; McCarthy et al., 1964; Quesenberry and Unger, 1964). These include various mixtures of organic solvents such as chloroform, ethyl acetate, methylene chloride, methanol, benzene, acetone, toluene, glycerol, dimethyl formamide, and water. Chloroform: methanol:water in a 90:10:1 ratio has been used for the separation of free corticosteroids (Bennett and Heftmann, 1962), while the same solvents in a ratio of 188:12:1 gave good resolution of corticosteroid acetates. Silica gel is the preferred adsorbent for these steroids.

Combining features like sample capacity, ability to separate closely related steroids, speed of execution and purity of eluted material, the technique of TLC is becoming more and more important for the purpose of separating the adrenocorticoids contained in a purified extract of a biological sample.

Identification of Adrenocorticoids

General

Identification comprises comparison of the physical and chemical properties of an unknown compound with that of a known compound. Physical properties like melting point, refractive index, solubility, infrared and other spectra, R_f values in different systems of chromatography, distribution k's, optical and crystallographic data, and chemical properties obtained through functional group tests and preparation of derivatives, are known for the adrenocorticoids (Bush, 1961). When isolating and identifying a compound for the first time in an extract from a biological source, rigorous proof of identity must be furnished. In routine investigation, however, the proof of identity is often only tentative and the existing concentrations of the adrenocorticoids in biological samples impose serious limitation even when only tentative proof of structure is required. The classic techniques of crystallization and melting point determinations are not possible with the small amounts of steroids isolated by paper or thin-layer chromatography. Therefore, other physical and chemical properties of steroids are used for positive identification (Estergreen, 1960).

In routine investigations, many of the adrenocorticoids are identified and qualitated by an organic reaction. One should, however, realize that in the process of sample isolation and characterization, the quantity of the sample will decrease. Since all organic reactions have lower operational limits, achievement of sample purity may infringe on this limit (Peron, 1962).

Identification by Chemical Reactions

Chemical reactions for the identification of corticosteroids have been described for various reactive groups (Mathews et al., 1962; Lisboa and Diczfulasy, 1963; Lisboa, 1963, 1966a,b; Diab and Gomoll, 1966), but the most sensitivie tests are often not the most specific (Lisboa and Diczfulasy, 1963). These reactive groups could be classified into four main groups, as follows:

Group 1 depends on the reducing properties of the α-ketolic side chain which will give rise to colored complexes after the addition of certain reagents. The reagent used most commonly in this type of reaction is blue tetrazolium (BT, 3,3'-dianisole bis[4,4'-(3,5-diphenyl) tetrazolum chloride] which forms a deep-blue-colored complex. Group 2 depicts the ability of periodate to oxidize and cleave the side chain of certain adrenal steroids. The products of the reaction, 17-ketosteroids, formaldehyde, or acetaldehyde are identified by standard Zimmermann and chromatropic acid reagents, respectively. Sodium bismuthate is used in Group 3 instead of periodate, the bismuthate oxidizing the side chain of certain 17α-hydroxy-C_{21} steroids to 17-ketosteroids.

The reaction given by Group 4 with a mixture of phenylhydrazine sulfuric acid forms the basis of a method of identifying 17α-hydroxy, 20-keto, 21-hydroxy steroids specifically and depends on the formation of a colored 3,20- or 3,21-bisphenylhydrazone (Silber and Porter, 1957) which has a maximum absorption in the 410 mμ region of the spectrum (Péron, 1962).

The above chemical reactions have been used with various modifications by a number of investigators to identify the adrenocorticoids on

paper and thin-layer plates (Bush, 1961).

Ultraviolet Fluorescence

Steroids possessing a Δ^{-4}-3-ketone group in the A ring exhibit fluorescence under ultraviolet light. The presence of a strong and characteristic chromophore grouping, namely, that of an α,β-unsaturated ketone (R-C(=O)-C=C) allows corticosteroids to strongly absorb ultraviolet light at 240 mμ (Unger et al., 1955). This ultraviolet absorption is taken to full advantage in identifying corticosteroids on both paper and thin-layer chromatograms (Berliner and Salhanick, 1956; Schedl et al., 1959). Samples containing as low as one μg of the steroid can be easily detected on the thin-layer and paper chromatograms by exposing them to ultraviolet light (Diab and Gomoll, 1966). Fluorescence under ultraviolet light after treatment with NaOH (Bush, 1952) or isonicotinic acid hydrazide (Weichselbaum and Margraf, 1957) has also been used for identification of corticosteroids on the chromatograms. The absorption spectra, in sulfuric acid, of many steroids have been determined and can be used as another aid in identification of unknown samples (Zaffaroni, 1953; Goldzeiher and Besch, 1958).

Chromatographic Techniques

Chromatographic mobility of a given steroid in a given solvent system can be used for identification purposes. Chromatographic R_f (distance from origin to the center of the sample spot/distance from origin to the solvent front) values can be calculated for different solvent systems, but since slight variations in mobility occur due to variations in temperature and handling procedures, it is usual to run

reference standards simultaneously with the unknowns for a more positive identification (Bush, 1952; Zaffaroni, 1953; Kunze and Markham, 1961).

When isotope dilution methods are used, autoradiography and scanning of the chromatograms have been used in locating the radioactivity and for the identification of the unknown compound (Stahl, 1965). Chromatography to a constant specific activity using mixtures of isotopically labelled and unlabelled steroids has been used as a criterion of identification (Peterson, 1957).

Preparation of Derivatives

The preparation of derivatives and the comparison of the chromatographic behavior of these derivatives with that of standards has also served as a further means of identification of steroids (Caspi and Hechter, 1956; Berliner, 1957; Touchstone, 1958; Schedl et al., 1959). Acetylation is a common procedure for corticosteroids. For this purpose, Dominguez et al., (1963) recommended a 1:5 mixture of acetic anhydride and pyridine. This is added to the dry sample of the steroid and incubated at room temperature in the dark for 24-hours. Following acetylation, the chromatographic mobility of the acetylated steroid in question markedly changes from that of the non-acetylated steroids. Identification is achieved by comparing the R_f of the acetylated unknown to that of the standard-acetate in any chromatographic solvent system.

Infrared Spectrometry

The infrared spectrum of an unknown steroid serves as a good criteria in steroid identification. In order to obtain a conclusive spectrum, from 1-2 mg of steroid is needed by the conventional potassium

bromide pellet technique. The use of additional optical beam condensing systems, allows the amount of steroid to be reduced by eliminating the nonradiated parts of the sample. A potassium bromide pellet with dimension equal to that of the slit of infrared spectrophotometer will give good spectra from about 20 μg of steroid (Caspi and Hecter, 1956). Using beam condensing units even less sample size will give a spectrum which can be interpreted and if scale expansion can be applied, about 0.1 μg of a steroid should produce a reasonable spectrum. The main problems encountered in micro infrared spectrophotometry of small amounts of steroids isolated from biological sources are purity of the final sample and complete transfer of the sample onto a potassium bromide pellet without introducing impurities. Moreover, when recording infrared spectra of small amounts of steroids isolated from a biological sample, it is needed to compare the spectrum of the isolated compound with that of the same amount of authentic compound subjected to the same isolation procedure (Silverstein and Bassler, 1967).

Mass Spectrometry

A method which allows one to gain positive information on steroid structure from submicrogram quantities is the mass spectrum of an isolated compound. The relatively low concentrations of some of the adrenocorticoids in blood necessitates increased sensitivity in the identification procedures. Gas chromatographs using the detector device of the mass spectrometer appear to be one possible solution to this problem. The retention characteristics of the adrenocorticoids (or their derivatives) in gas chromatography serve much the same purpose for their isolation and identification as their R_f values in paper or

thin-layer chromatography (Horning and Vanden Heuvel, 1965). Mass spectrometry, besides determining the precise molecular weight, also provides information regarding the location of various functional groups (Djerassi et al., 1965).

Quantitative Determination

There are a number of methods reported in the literature for the quantitative determination of corticosteroids.

Colorimetric Methods

The chemical reactions usually used in the colorimetric assays react with adrenocorticosteroids by virtue of reactive grouping(s) on the steroid nucleus. The resulting reactions can give rise to the formation of colored complexes, the liberation of volatile products, the formation by transformation of new steroid products, and the formation of fluorescent substances. These can all be measured with relative simplicity and accuracy with the methods which are presently available.

The first successful colorimetric procedure for blood corticosteroid analysis was introduced by Nelson and Samuels (1952) and quantitation was based on the Porter-Silber reaction (Poter and Silber, 1950). This reaction involves the formation of a yellow phenylhydrazone when the 17,21-dihydroxy-20-ketone grouping of certain steroids reacts with phenylhydrazine in an acid solution. The Porter-Silber reaction has been used extensively for the determination of 17-hydroxycorticosteroids in plasma and urine and also for the specific determination of cortisol and cortisone where these can be separated from other steroids (Nelson and Samuels, 1952; Reddy et al., 1956; Robertson and Mixner, 1956;

Shaw et al., 1960; Kruger et al., 1965; Franks, 1967) but suffers from a lack of specificity.

Of the methods briefly mentioned in the preceding paragraphs, the relatively simple technique of Porter and Silber (1950) and the more complex procedure of Nelson and Samuels (1952) and Eik-Nes et al. (1953) are commonly used. Muller et al. (1955) compared these two methods for determination of 17-hydroxycorticosteroids in normal human subjects, subjects following physical effort, in patients with hepatic coma, anuria, babiturate coma, and Addison's disease, and in patients before and after administration of ACTH, and after the administration of cortisol. A good correlation was obtained between the two methods, although somewhat more variability was found with the simpler Porter-Silber technique. Gemzell (1955) reviewed the methods of assay of adrenal corticosteroids and concluded that the Nelson-Samuels procedure was a reliable method, particularly for multiple determinations.

Another colorimetric procedure which has been widely used for quantitation of C-21 steroids utilizes the reduction of α-Ketolic side chain by blue tetrazolium (Mader and Buck, 1952; Chen et al., 1953; Beigelman et al., 1956; Izzo et al., 1957; Mattox and Lewbart, 1958; Lidner, 1959). There have been reports of other colorimetric procedures for steroid estimations (Burstein, 1953; Gornall and MacDonald, 1953; Steyermark and Nowaczynski, 1955; Umberger, 1955; Mazarella, 1957), but they have not been used as extensively.

Colorimetric methods are capable of measuring accurately 1 μg of cortisol or corticosterone and detecting as little as 0.4 μg of these substances. However, the interfering substances in extracts of biological material due to their reaction with some or all of the reagents

used in the colorimetric assay results in high blank values, which reduces the sensitivity of these methods. In addition, the blank color fades on standing and a wide variation in optical densities obtained with different steroids limits the quantitation of complex mixtures of adrenocorticoids found in biological extracts by colorimetric methods.

Radioisotopic Methods

The application of isotopic techniques in conjunction with reliable colorimetric or spectrophotometric assay methods to quantitate steroids has been extremely helpful in resolving problems concerned with rates of metabolism, apparent distribution volumes and turnover rates of some steroid substances (Eik-Nes, 1960). The technique of isotope dilution for the measurement of secretion rates of steroids depends upon estimation of the extent to which a labelled steroid is diluted by endogenous production of this steroid.

Most of the above mentioned methods have been modified by the use of isotope dilution techniques for the quantitative determination of steroids in biological fluids. A common procedure consists of the addition of a known quantity of radioactive steroid of a known specific activity to plasma before extraction (Bondy et al., 1957; Peterson, 1957; Cohn and Bondy, 1959), determination of the radioactivity as well as quantitative estimation of the analogous steroids, and determination of the original concentration of steroid from the recovery of radioactive compound (Calvin et al., 1949; Kamen, 1957). These methods offer a special advantage in that it is possible to carry out an extensive purification of the plasma extract without undue concern about losses of steroid.

Following the work of Avivi et al. (1954), Peterson and his collaborators (Peterson et al., 1957; Kliman and Peterson, 1960; Peterson, 1962) introduced methods for the determination of several steroids by the "double isotope derivative" assay. This approach presents great advantages of precision, sensitivity, and specificity over other methods. Isotope derivative methods make use of the property of primary and secondary alcohols to react readily with acetic anhydride. The procedure essentially consists of the addition of a known amount of radioactive steroid to the sample, solvent extraction of plasma, separation of steroids by chromatography, preparation of derivatives of the unknowns and the added tracers labelled with a second isotope, purification of the derivatives, and counting of the doubly labelled derivatives by a channel ratio technique. Compounds labelled with either carbon-14 or tritium can be used for derivative formation (Kliman and Peterson, 1960).

After derivative formation, purification of the mixture is very important and it is usually necessary to chromatograph at least four times to reach a constant $^{3}H/^{14}C$ ratio in the final product. This is the most time consuming step of the entire procedure and is of utmost importance for accurate results. The double isotope dilution derivative technique has been used for the specific determination of cortisol, corticosterone, and aldosterone (Hollander and Vinecour, 1958; Bojesen and Degn, 1961; Porter, 1963; Porter and Klaiber, 1964; Stachenko and Giroud, 1964; Benrad and Kloppenborg, 1965; Cade and Perenich, 1965; Hillman and Giroud, 1965; Seely, 1965; Whipp et al., 1967).

In general, the isotope dilution derivative methods are quite specific, sensitive, and accurate. On the other hand, these procedures

are time consuming, expensive, and require highly skilled technical help.

Gas-Liquid Chromatographic Methods

Thermostable derivatives of the adrenocorticoids can be quantitated by the technique of gas-liquid chromatography. Any biological extract applied to the gas chromatograph should be in a high state of purity. Furthermore, the relatively low concentration of some of the adrenocorticoids in blood will probably necessitate increased detection sensitivity of the gas chromatograph. Gas chromatographs using the detector device of the mass spectrometer appear to be one possible solution to this problem. Admittedly, one could extract a relatively large sample of blood, but this is a dubious way of solving assay problems when dealing with end point analysis of extreme sensitivity since it tends to create a gross disturbance of the ratio between "background noise" and "sample". Also, the application of extracts from large amounts of blood to the gas chromatograph would probably poison the detector system. Detection of derivatives of the adrenocorticoids by the electron capture cell following gas chromatography should be investigated. This technique has been successfully applied to the assay of plasma testosterone (Brownie et al., 1964), and plasma progesterone (van der Molen and Groen, 1965), by preparing chloroacetate derivatives of these compounds. The sensitivity in these methods was reported to be as low as 1 mμg. Unpublished reports from the laboratories of Dr. Eik-Nes (Utah) indicate that the acetates of some of the adrenocorticoids can capture electrons. If so, small amounts of these derivatives can be determined following gas-liquid chromatography on a SE-30 (1%) column.

Protein-Binding Methods

The α-globulin fraction of human plasma contains a protein with a high binding affinity for cortisol (Slaunwhite and Sandberg, 1959). This protein has been named "transcortin" or CBG. A method for the measurement of corticosteroids, utilizing the steroid-binding properties of transcortin, was described by Murphy et al. (1963). The technique involved dialysis and required 2 days for completion. Subsequently, gel filtration using sephadex was substituted for the dialysis step and the time was shortened from 2 days to 2 hours (Murphy and Pattee, 1964). It has been reported that through the use of tritiated steroids in place of ^{14}C-steroids and by using CBG of species other than man, with adsorption on florisil in place of dialysis or gel filtration, adrenocorticoids in subnanogramic levels could be quantitated (Murphy, 1967). Dextran-coated charcoal has also been employed in place of florisil to measure the concentrations of plasma corticosteroids in other protein-binding procedures. The results obtained by these procedures agreed closely with those obtained using a chromatographic fluorometric technique which specifically measured cortisol (Nugent and Mayes, 1966).

Fluorometric Methods

Molecules which are excited by incident radiation sometimes emit light. The light-emitting process is due to a change of the energy states of the molecules going from an unstable to a stable form. In this transition, the excitation energy from the incident light which was absorbed by the molecules is dissipated as light immediately after irradiation ceases and is called fluorescent light. Thus, even though fluorescence is intimately related with the absorption spectra of a

substance or solution, the fluorescence spectra usually differ in many respects. Under ideal conditions, absorption of incident radiation by solutions or substances of known concentrations follow Beer's and Lambert's laws. On the other hand, under the same conditions, fluorescence is directly proportional to the concentration of the fluorescing material unless the quenching phenomenon interferes. This phenomenon is believed by many investigators to be due to collisions between excited and stable molecules, in which excitation energy is dissipated other than by fluorescence. Such collisions may involve molecules of a foreign substance (giving quenching by impurities) or those of the fluorescent substance itself (self-quenching).

Difficulties arising from quenching as well as from other causes may be experienced in the fluorometric measurement of adrenal steroids in biological extracts. One of these is due to the fact that structurally related steroids have different fluorescent spectra on a qualitative and quantitative scale. For this reason methods and procedures have been devised which will measure one or a small group of adrenal steroids specifically. If a more quantitative picture is desired, it may be obtained by separating all the steroids in the extract by chromatography. Each steroid is then treated individually with fluorescence-inducing reagents. Measurement, however, is limited by the availability of a sufficient number of fluorometric methods which can measure all the steroids.

Mineral acids are usually added to biological extracts or to pure adrenal steroids before fluorescence is induced by incident radiation. When working with extracts, this creates another difficulty which is inherent in the use of fluorometric methods. Because acids react

non-specifically with many nonsteroidal substances, the extract must be meticulously purified before the addition of the acid reagents (Peŕon, 1962).

Sweat (1951) utilized fluorescence to measure the enzymatic synthesis of cortisol from 11-desoxycortisol and, later, Sweat and Farrell (1952) used the fluorescence technique for the determination of cortisol and corticosterone in adrenal venous blood of dogs and rats. Following chloroform extraction of plasma, a partial chromatographic purification of the extract was done on a silica gel microcolumn. This procedure separated estradiol, estrone, cholesterol (these compounds develop some fluorescence in sulfuric acid), and other fats from cortisol. The results obtained by this procedure corresponded fairly accurately with those found with the Porter-Silber reaction (Nelson and Samuels, 1952). Since then, sulfuric acid induced fluorescence has been used by numerous investigators (Silber et al., 1958; Zenker and Bernstein, 1958; Guillemin et al., 1959; Moncola et al., 1959; Moor et al., 1960; Vermeulen and Straeten, 1964; Solem and Brinck-Hohnsen, 1965; Spencer-Peet et al., 1965; Eechaute, 1966a,b; Solem, 1966; Frankel et al., 1967; Jansen et al., 1967; Matsumura et al., 1967; Schou et al., 1967) for the determination of cortisol and corticosterone in plasma. These fluorometric methods are extremely sensitive and a small amount of plasma can be used. In 1958, it was demonstrated by Silber et al. that in a fluorometric determination of corticosterone in rat plasma, the preliminary silica gel chromatography may be replaced by washing with petroleum ether and NaOH. With certain modifications, Moor et al. (1960) and Moor and Steeno (1963) adapted this analytical method to the determination of cortisol (plus corticosterone) in human plasma. The

steroids were extracted from undiluted plasma by methylene chloride. The extract was washed with 0.1 N NaOH to remove estrogens and other nonspecific fluorogens. Thereafter, the methylene chloride phase was extracted with sulfuric acid-ethanol (75:25)v/v, and fluorescence was measured.

These "so-called" brief fluorometric methods have often been criticized for giving high readings which are claimed to be due to background fluorescence from either nonsteroidal or other steroidal materials. On the basis of theoretical considerations, some investigators (Moncola et al., 1959; Rudd et al., 1961; Stewart et al., 1961; Spencer-Peet et al., 1965) propose an approximate correction for this non-steroidal fluorescence without elimination of the interfering substances. Others have tried to separate the background material from the steroid fraction (Eechaute, 1966a,b). Braunsberg and James (1962) have eliminated a great deal of background material by means of a benzene:water partition. However, their recovery data after addition of cortisol to 4 ml of plasma was quite variable (53.3-114% with a mean of 80.9%). The method developed by van der Vies (1961) for the separate determination of cortisol and corticosterone in plasma is based on the different partition coefficient of these hormones between water and carbon tetrachloride. Vermeulen and van der Straeten (1964) investigated the method of van der Vies and found it to be a reliable method for the determination of plasma cortisol concentrations for routine clinical application. Nielsen and Asfeldt (1967) studied, in detail, the method of Moor and Steeno (1963) in an attempt to elucidate the problem of nonspecific fluorogens, and found that, in spite of its lack of specificity, the method was well suited for following spontaneous diurnal variations in

plasma cortisol, and variations during ACTH stimulation and medication. In short, these brief fluorometric methods are less specific if applied in cases where cortisol and corticosterone occur together in the same mixture (James et al., 1967).

A more complete purification of plasma extract and simultaneously a quantitative separation of cortisol and corticosterone can be obtained by the use of chromatographic methods (Eik-Nes, 1957; Eechaute, 1966a,b). Eechaute (1966a,b) found a more complete separation of cortisol from corticosterone when the dried methylene chloride extract was dissolved in 10% ethyl alcohol in chloroform and applied onto the silica gel microcolumn, using the same combination of solvents for elution. Matsumura et al. (1967) have described a fluorometric method for the simultaneous determination of cortisol and corticosterone in plasma. It is comprised essentially of a solvent partitioning extraction, Girard separation, fluorescence, and calculation of concentration by experimentally derived simultaneous equations. The fluorescence was developed with 75 and 60% ethanolic sulfuric acid reagent on two halves of the same extract separately at 45° C for twenty minutes after which the fluorescence was read using the Aminco-Bowman spectrophotofluorometer. Frankel and his associates (1967) compared four brief fluorometric methods with an extended fluorometric method which included chromatographic purification of steroids and was thoroughly validated by them. These investigators found variable influence of interfering chromogens on the development of fluorescence using extracts of avian adrenal plasma. These results obtained by the extended fluorometric procedure closely compared with those obtained by the double isotope dilution derivative technique.

In conclusion, it can be said that brief fluorometric methods appear to reflect changes in plasma steroid concentrations quite faithfully and may, therefore, be adequate for many clinical purposes. It is important, nevertheless, to realize that it is not cortisol alone which is being measured. The extended methods which include chromatographic separation are, however, quite accurate and cortisol and corticosterone can be separately and specifically measured.

CHAPTER III

MATERIALS AND METHODS

Reagents

1) Benzene, methanol and toluene used were nanograde in quality (Mallinckrodt Chemical Works).

2) Iso-octane, chloroform, and carbon tetrachloride were spectro-grade in quality (Mallinckrodt Chemical Works).

3) Dichloromethane (analytical reagent grade, Mallinckrodt) was glass distilled two times.

4) Water used in the procedure was glass distilled two times followed by ether extraction and finally redistilled over glass.

5) Sodium hydroxide (0.1 M) was freshly prepared by mixing 0.4 g of NaOH pellets (analytical grade, Mallinckrodt) in 100 ml of water.

6) Anesthetics used: Sodium Pentabarbitol (Haver-Lockhart Laboratories, Kansas City, Missouri); Surital (Thiamylal Sodium, Parke Davis and Co., Detroit, Michigan); and Ether (anesthesia grade, Fisher Scientific Co., Fair Lawn, New Jersey).

7) Hormonal preparation used: Azium (dexamethasone - two mg/ml, Schering Corp., Bloomfield, New Jersey); Adrenomone (40 IU ACTH/ml, Armour Baldwin Laboratories, Nebraska); ACTH-peptide (chromatographically isolated and free from LH contamination - Sigma Chemicals), suspended in physiological saline (10 IU/ml) for intravenous administration; suspended in sterile peanut oil with 5% beeswax (401 U/ml)

for intramuscular administration.

8) Thin-layer plates: Silica gel (Silicar TLC-7GF, Mallinckrodt) was washed three times with dilute acetic acid over a sintered glass filter followed by three rinses with boiling triple distilled water and finally washed two times with boiling nanograde methanol. It was dried 18 to 24 hours at 110° C. Thin-layer chromatography plates were 8" x 8" in size and were 0.25 mm thick (Brinkmann & Co. Desaga thin-layer spreader was used) and had a ratio of 33 g silica gel to 78 ml water.

9) Scintillation mixture: Prepared by mixing 15.14 g 2,5 diphenyloxazole (PPO) and 0.1514 g 1,4-bis-[2-(5phenyloxazolyl)]-benzene (POPOP) per 3.79 liters of tolune (analytical grade, Mallinckrodt) and equilibrated for 24-hours before using.

10) Acid-alcohol mixture: Freshly prepared by mixing very slowly, 65 ml of concentrated sulfuric acid (from Dupont) and 35 ml of absolute alcohol (200 proof, U. S. Industrial Chemical Co.,) in an ice-bath.

Steroids

Ten milligrams of corticosterone (11β,21-dihydroxy-pregn-4-ene-3, 20-dione) or cortisol (11β,17,21-trihydroxy-pregn-4-ene-3,20-dione) obtained from Sigma Chemicals were recrystallized to constant mp (182° and 220°, respectively) and dissolved in 100 ml of ethanol.

Corticosterone-1,2-^{3}H (having specific activity of 0.25 mc/0.00348 mg) and hydrocortisone-1,2-^{3}H (with a specific activity of 0.25 mc/ 0.00165 mg) were obtained from New England Nuclear Corp., Boston, Mass., and were chromatographically purified. Purity was checked by a radio-chromatogram scanner and identity by the use of authentic standards in adjacent lanes on the thin-layer plate. They were stored in ethanol at

a dilution of about 1500 dpm/100 μl at 5° C. The purity of isotopically labelled steroid standards were checked at 4 month intervals.

Equipment

1) Fluorometer: Turner, Model 110 with accessories including: (a) a high sensitivity conversion kit and a micro-adapter, (b) a blue-lamp which has a continuous emission from below 400 mμ to over 520 mμ, and (c) a primary filter combination of 48 + 48 + 3 for 470 mμ activation and a secondary filter combination of 2A - 15 + 4 = 94 for 570 mμ emission.

2) Scintillation Counter: Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003 with automatic standardization model 574.

3) Radiochromatogram Scanner (Packard, Model 7201).

4) Kofler Micro-Melting Point Apparatus (Metro Industries, New York).

5) Cahn Electronic Balance (Cahn Instrument Co., Paramount, Calif.).

6) Mass Spectrometer-gas Chromatograph: LKB-9000, prototype (Karolinska Institute, Stockholm, Sweden).

Cleaning of Glassware

Glassware was rinsed immediately after use, soaked in detergent, brushed and placed in chromic acid overnight. Following seven to eight tap water rinses, the glassware was soaked in detergent, rinsed again with tap water and placed in dilute hydrochloric acid. After being rinsed ten times with tap water, ten times with distilled water and twice with nanograde methanol, the glassware was air-dried. The

micro-cuvettes used were soaked in concentrated nitric acid instead of chromic acid and following rinses with tap and distilled water were air-dried following an ethanolic wash.

Animals and Collection of Blood

Animals in this investigation were comprised of rabbits, three cattle, and 23 swine. Unless otherwise stated, all rabbits used as experimental animals were mature New Zealand white male rabbits. Upon receiving the rabbits from the supplier, they were placed singly in different cages and allowed to remain in these cages approximately 2 weeks prior to beginning the experiments. In the 2 week rest period and throughout the experiment, the rabbits were offered water and a commercial grade of rabbit pellets of free choice. The details about the experimental cattle and swine are described in detail in the particular experiments in which they were used. Blood was collected from these animals under sterile conditions. Blood collection procedures that were least stressful to the animals were chosen in all cases. A post mortam examination was made on all the animals that were bilaterally adrenalectomized to ascertain that there was no adrenal tissue left intact.

Experimental Procedures

Surgical Procedures Involved in the Physiological Validation of the Extended Fluorometric Method

Nine rabbits were used in this study. The rabbits were randomly placed into three groups of three animals each. Bilateral gonadectomy was performed on two groups. A rest period of 1 week was given before

one of the gonadectomized groups and one group with intact testes were subjected to bilateral adrenalectomy. The surgical technique for bilateral adrenalectomy involved a mid-line incision following anesthesia and cutting through fascia, muscles, peritoneum, and finally locating the adrenals. The left adrenal was removed first, followed by the right adrenal by retracting a portion of the postcava. Continuous sutures were applied to both the peritoneum and to the layer of muscles separately, and interrupted sutures were applied to the skin. One ml of Azium was given intravenously to all of the animals 1 hour prior to surgery to reduce the mortality rate, and 0.5 ml of Azium was administered intravenously for 2 days post-surgery. On the fourth day following post-surgery, animals were decapitated and blood collected. To the remaining group an intramuscular injection of 2 ml of Azium daily was given for 7 days and on the eighth day animals were decapitated and blood collected. Blood was collected into glass tubes, rinsed with 10% potassium oxalate, immediately chilled and centrifuged. The plasma was deep frozen and stored at -20° C until analyzed for corticosteroids by the extended fluorometric method.

Comparison of the Extended Fluorometric With Two Other Widely Used Brief Fluorometric Methods

Rabbit blood was obtained from each of nine animals by cardiac puncture after anesthetizing the animals with surital. The syringes used for cardiac puncture, as well as the tubes into which blood was later transferred, were rinsed with 10% potassium oxalate. Blood samples were centrifuged immediately and supernatant plasma aspirated and stored at -20° C until extracted for corticosteroids. The plasma corticosteroid concentrations were determined in triplicate by three

different fluorometric methods for each of the nine rabbits. The experiment was arranged as a Latin Square so that variation due to methods, samples and animals could be evaluated.

Effect of Different Blood Collection Procedures on the Corticosteroid Levels in Rabbit Peripheral Blood Plasma

Twenty-four rabbits were used in this study. The rabbits were randomly placed into individual cages. On each day of the week, starting with Tuesday, four rabbits were randomly selected and bled by any of the four different blood collection procedures. The type of blood collection was either by decapitation or by mild vacuum suction from a cut in the ear vein. The latter was used either with or without anesthesia. The anesthetics used were either sodium pentabarbitol or ether. In the decapitated animals, blood was collected within 30 seconds from the time the animal was removed from the cage. Whereas with the vacuum alone or vacuum following either sodium pentabarbitol or ether anesthesia, the time interval was approximately 3 to 5 minutes. Blood was collected into glass tubes rinsed with 10% potassium oxalate, immediately chilled and centrifuged. The plasma was deep frozen and stored at -20° C until extracted and quantitated by the extended fluorometric method for corticosteroids.

Effect of Acute Stress on the Corticosteroid Levels in Rabbit Peripheral Blood Plasma

Two mature male rabbits were used in this experiment. Blood was collected by mild vacuum suction of the ear vein from each rabbit, immediately before and at ½, 1, 1½, 2, 2½, 3, 4, and 5-hour intervals following intravenous administration of ACTH-peptide (10 IU suspended

in 1 ml of physiological saline). Blood was collected into glass tubes rinsed with 10% potassium oxalate, immediately chilled and centrifuged. The plasma samples were extracted the same day by the extended fluorometric method to quantitate corticosteroids.

Effect of Chronic Stress on the Corticosteroid Levels in Rabbit Peripheral Blood Plasma

Eighteen rabbits were used in this experiment. The rabbits were randomly placed into three groups of six animals. Each animal was individually caged. One of the groups served as controls and received 0.5 ml of sterile peanut oil with 5% beeswax intramuscularly daily for 28 days. The adrenals were chronically stimulated in another group by injecting 0.5 ml ACTH-peptide (40 IU/ml suspended in sterile peanut oil with 5% beeswax) intramuscularly daily for 28 days. The remaining group received 0.5 ml Azium intramuscularly daily for the same length of time. At the end of the treatment period, the animals were decapitated and blood collected. Blood was collected in glass tubes rinsed with 10% potassium oxalate, immediately chilled in an ice bath and centrifuged within 5 minutes of collection. The plasma was deep frozen and stored at -20° C until extracted for corticosteroid quantitation by the extended fluorometric method.

Corticosteroid Levels in Porphyric Cattle

Animals in this study consisted of one porphyric cow, one porphyric bull, and one normal cow. All 3 animals were Holsteins, approximately 2 years of age and weighed around 1200 lbs. The animals belonged to the Department of Pathology, School of Veterinary Medicine, Oklahoma State University. One to three liters of blood were collected from the

jugular vein of each of these animals immediately before and 1 hour after an intramuscular injection of 200 IU ACTH (Adrenomone). The blood was collected into bottles containing 10% potassium oxalate (20 ml/l), immediately chilled in an ice bath and centrifuged within 15 minutes of collection. The plasma was deep frozen and stored at -20° C until extracted. The corticosteroid levels in these samples were assayed via a spectrophotometric method.

Corticosteroid Levels in Pigs Suspected of a Porcine Stress Syndrome

Animals in this investigation belonged to the swine herd of the Institute of Animal Science and Industry, Oklahoma State University, and were maintained under uniform husbandry practices. Animals weighed 40-50 kilograms, were about 3 months old and comprised of 3 groups. Group I consisted of 8 Yorkshire swine from the herd in which the stress syndrome was of frequent occurrence, Group II consisted of 10 Hampshires representing a strain in which the disease had not been recognized, and Group III consisted of 5 swine of mixed breeding that had not reported or observed incidence of the disease. Groups I and II were designated as the high-incidence and low-incidence groups, respectively, and Group III served as the controls. All animals in each of the 3 groups were randomly selected. Blood serum samples were obtained by the Department of Pathology, School of Veterinary Medicine, Oklahoma State University. The corticosteroid levels in these serum samples were determined by the extended fluorometric method.

Spectrophotometric Method

Before extraction, about 10,000 CPM each of tritiated corticosterone and cortisol were mixed with 350 ml thawed plasma and allowed to equilibrate for about 30 minutes. The plasma was extracted 3 times with 1½ volumes of chilled dichloromethane (CH_2Cl_2). The pooled CH_2Cl_2 extract was evaporated to dryness under nitrogen following alkali (0.1N NaOH) and water washes until neutral. The residue was dissolved in hexane and partitioned 3 times with an equal volume of 70% aqueous methanol. The methanol was reduced to an aqueous phase which was re-extracted 3 times with an equal volume of CH_2Cl_2. The CH_2Cl_2 was filtered through anhydrous sodium sulfate and concentrated under a stream of nitrogen for chromatography. The extract was chromatographed on paper in a system consisting of petroleum ether:benzene:methanol:water (100:100:60:400). Quantitative analysis was accomplished by spectrophotometry in methanol and isotope dilution as follows:

$$= 2A_{240} - (A_{230} + A_{250})$$

$$\text{sample } \mu g = \frac{\text{sample}\Delta \text{ x sample ml}}{\text{standard}\Delta / \mu g/ml}$$

$$\mu g/ml \text{ plasma} = \frac{\dfrac{\text{sample } \mu g}{\% \text{ recovery of isotope}}}{\text{ml plasma}} \quad .$$

Fluorometric Methods

The fluorometric methods used to quantitate corticosteroids in this study, consisted of two brief methods and the present extended fluorometric method. Depending upon the presence or absence of a chromatographic purification step, these fluorometric methods were

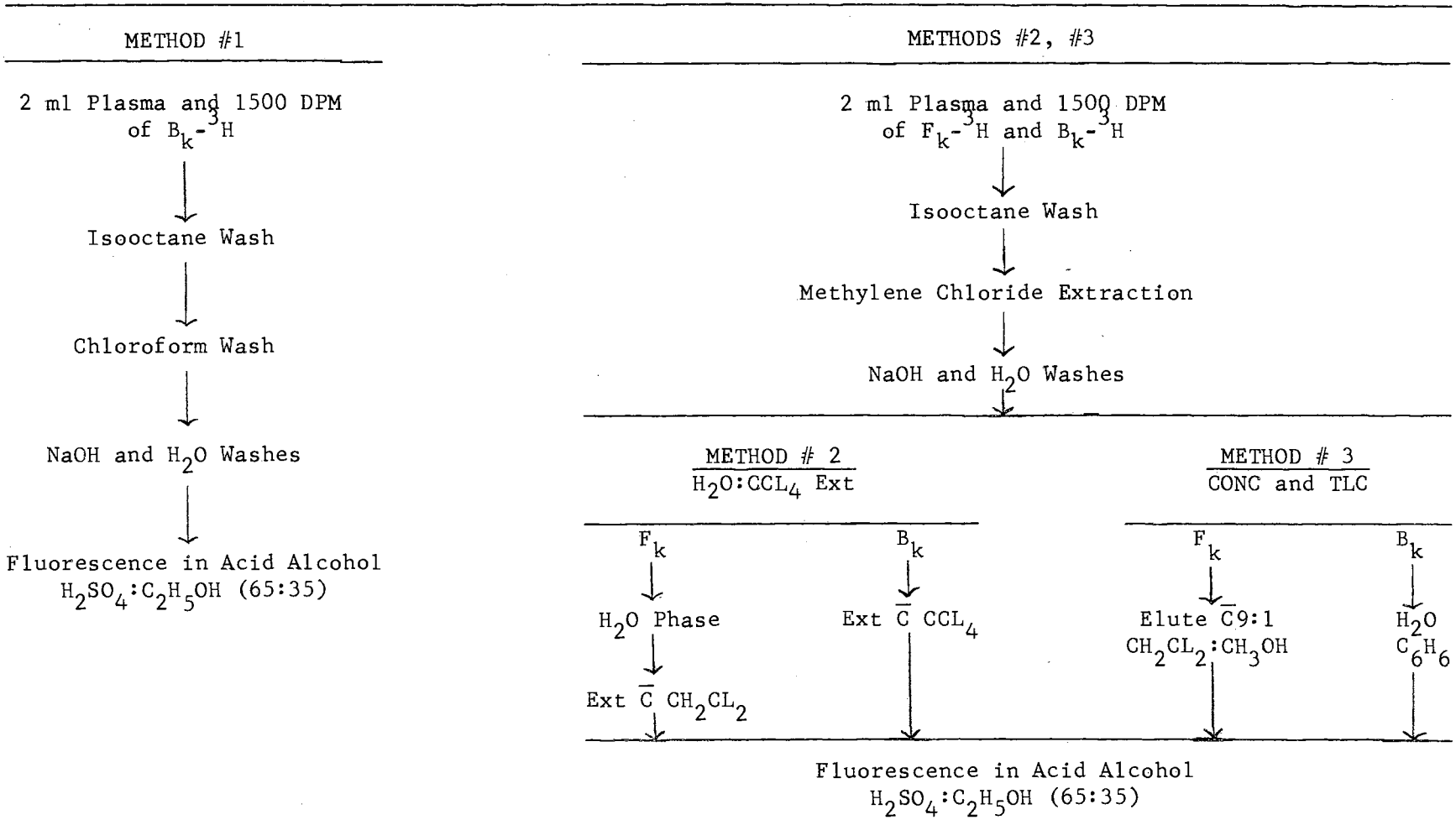
designated either "brief" or "extended". The brief fluorometric methods used were essentially that of Guillemin et al. (1959) and that of van der Vies (1961) with minor modifications such as addition of isotopically labelled standards to monitor for losses through the method. The protocol followed for all three fluorometric methods in this investigation regarding extraction, saponification and quantitation by combined fluorometry and liquid scintillation spectrometry were similar and are as shown in Figure 1.

The Extended Fluorometric Method in Detail

Preparation of Plasma for Solvent Extraction

About 100 µl of labelled steroid standards (1500 dpm of both cortisol-1, 2-^{3}H and corticosterone-1, 2-^{3}H) were pipetted into 80 ml extraction tubes and evaporated to dryness under a thin stream of nitrogen. An aliquot of the same amount of the radioactive standards was placed in scintillation vials (in duplicate) to permit calculation of recovery and to correct for losses through the method.

The plasma samples were thawed and recentrifuged to eliminate any fibrin clots. Two ml of plasma were accurately measured and added to the extraction tubes containing the labelled standards. The tubes were gently vortexed and the contents allowed to equilibrate for at least 15 minutes. Following which the plasma samples in each tube were washed with 5 ml of iso-octane. The iso-octane layer was apirated by a pasteur pipette using mild vacuum suction and discarded. The prewashing of plasma with iso-octane removes lipids less polar than C-21 and C-19 steroids and reduced emulsion formation during the extraction step.



Method #1 = Guillemin et al.
Method #2 = van der Vies
Method #3 = Extended Fluorometric Method

Figure 1. Protocol for the Micro Determination of Cortisol (F_k) and Corticosterone (B_k) in Blood Plasma

Extraction and Saponification

Plasma prewashed with iso-octane was extracted 1 time with 40 ml (20 volumes) of ice cold dichloromethane. Extractions were carried out by hand-shaking the tubes gently for about 2 minutes. Emulsion formation between plasma and solvent interphases were kept to a minimum by using large volumes of ice cold solvent, hand shaking and by centrifugation at 1200 g for 5 minutes. The extracted plasma (top layer) was aspirated and discarded.

The dichloromethane extract was saponified by the addition of 4 ml (1/10 volume) of freshly prepared 0.1N sodium hydroxide. The alkali phase was aspirated and the extract washed twice with water (20 ml aliquot each time). The saponification step was designed to eliminate estrogens, as well as other pheolic and acidic steroids. The organic extract was exposed to sodium hydroxide less than 2 minutes, and then evaporated to dryness under a thin stream of nitrogen. After drying, the tubes were washed with 3, 2, 1 and 0.5 ml of benzene to concentrate the residue into the tip of the tube. If the tubes containing unknown samples had to be stored, they were stored at this stage in 0.5 ml benzene. The extracts in the tubes were completely dried before subjecting them to chromatography.

Chromatographic Isolation of Corticosteroids

Thin-layer plates (TLP) were set up with 9 lanes (2 cm wide), 6 for samples, 1 for blank and 2 end lanes, one on either side, for cold standards. The samples were spotted with a capillary tube within a 1 cm diameter on the TLP utilizing 3, 2, and 1 drops chloroform:methanol (1:1)v/v. Development was in chloroform:methanol:toluene:water

(60:20:120:1)v/v. Areas corresponding chromatographically to standard cortisol and corticosterone were eluted. The exposure of the TLP to ultraviolet light was less than 10 seconds. Cortisol had an R_f of 0.25 and corticosterone 0.50 in this solvent system.

Elution of Corticosteroid From TLP

Cortisol was eluted from the TLP using a fritted disc eluter (Dependable Scientific) with about 10 ml of a mixture of dichloromethane:methanol (9:1)v/v. Corticosterone was eluted by placing the sample in a 35 ml tube and extracting the silica gel with a benzene-water partition system three times using 1 ml benzene with 0.5 ml water. The benzene was pooled and the eluates evaporated to dryness under nitrogen.

Fluorometric Quantitation

One ml of methanol was added to the eluate residue and two-tenths ml were removed for a scintillation counting. The remainder was evaporated and 2 ml of acid-alcohol mixture was added. Fluorescence was measured against appropriate blanks at the end of 30 minutes for cortisol and at the end of 45 minutes for corticosterone. Fluorescence of both cortisol and corticosterone was measured using a wavelength of 470 mμ for activation and 590 mμ for emission.

Monitoring for Losses Through the Method

Corticosteroid loss prior to fluorometry was determined by measuring the loss of tritiated standards added to the plasma sample before extraction. All samples (0.2 ml aliquots) were evaporated to dryness

in the counting vials and 10 ml of scintillation mixture added. Radioactivity was measured in all samples for at least 5 minutes.

The following formula was used to calculate the % recovery for the corticosteroids through the method (aliquot factor being 5).

$$\% \text{ recovery} = \frac{\text{net sample CPM}}{\text{net standard CPM}} \times 100 \times 5$$

Calculation of Corticosteroid Concentration

The following formula was used to calculate the amount of endogenous cortisol or corticosterone in 100 ml of plasma.

$$\text{Steroid } (\mu g/100 \text{ ml plasma}) = \frac{S}{St} \times C \times \frac{A}{V} \times \frac{100}{R}$$

where

S = Reading (fluorescence units) of the sample

St = Reading (fluorescence units) of the standard

C = Concentration of the standard

A = Aliquot factor (volume of acid-alcohol reagent)

V = Volume of sample extracted

R = % Recovery.

CHAPTER IV

RESULTS

Failure to detect corticosteroids in the peripheral blood plasma of individual rabbits by ultraviolet spectrophotometry following solvent extraction and paper chromatography (Venkataseshu and Estergreen, 1965) indicated low levels of circulating corticosteroids in these animals. An attempt to increase the sensitivity of the above method by using microcuvettes was partially successful in that only corticosterone could be quantitated in the maximum amount of plasma obtained from one rabbit.

The suitability of fluorometric methods to our study was indicated since these procedures are sensitive and require only small volumes of blood. A literature search revealed that there was no adequate fluorometric method for the simultaneous determination of cortisol and corticosterone in peripheral plasma. Therefore, we have developed a micromethod for the simultaneous determination of cortisol and corticosterone, henceforth referred to as the extended fluorometric method (EFM). The extraction procedures, chromatography systems, elution techniques and various aspects of quantitation, physiological and physico-chemical validation, specificity, precision and application to experimental and pathological conditions are presented below.

Details of the Extended Fluorometric Method

Extraction

Plasma was pre-extracted with iso-octane prior to solvent extraction. Dichloromethane was selected as the extraction solvent because it removed a minimal amount of pigment from rabbit blood plasma. Because of the difference in partition coefficients between cortisol and corticosterone, it was necessary to test the effect of solvent volume and number of extractions on recovery rate of both the corticosteroids.

The solvent volume and the number of extractions needed to give the maximum recovery rates of cortisol and corticosterone were determined in a series of experiments with physiological saline. This was followed by repeating the same type of experiments employing rabbit blood plasma in place of physiological saline. Since the procedure involved saponification and water washes of the organic extract containing corticosteroids, the recovery rates of the same were checked with similar experiments. Finally, recovery rates of both cortisol and corticosterone carried through the entire method were determined.

Extraction of Corticosteroids From Physiological Saline

Both tritiated cortisol and corticosterone previously purified by thin-layer chromatography were added to 2 ml of physiological saline extracted, and the recovery rate of the corticosteroids measured by liquid scintillation counting. The results shown in Table I indicate that when 30 volumes of solvent were distributed among 3 extractions (series #1), 98% of cortisol and 99% of corticosterone were recovered. A single extraction comprised of 20 volumes of the solvent (series #4)

resulted in equally good recoveries.

TABLE I

EFFECT OF SOLVENT VOLUME (DICHLOROMETHANE) AND NUMBER OF EXTRACTIONS ON RECOVERY RATE OF CORTICOSTERONE-H^3 AND CORTISOL-H^3 ADDED TO TWO ML OF PHYSIOLOGICAL SALINE

Amt. of Solvent (ml) and No. of Volumes Per Extraction		% Recovery			
		Cortisol-H^3		Corticosterone-H^3	
Series #1	20:20:20* 10:10:10**	98	(8)***	99	(8)
Series #2	20:20 10:10	94	(8)	96	(8)
Series #3	20 10	88	(8)	90	(8)
Series #4	40 20	95	(8)	97	(8)

*The first figure in each series indicates ml of solvent/ extraction.

**The second figure in each series indicates volume of solvent.

***Number of experiments carried out in each series.

Extraction of Corticosteroids From Rabbit Blood Plasma

Emulsion formation between the solvent and plasma was partially solved by using ice cold methylene dichloride, by keeping a large volume ratio between organic and aqueous phases, and by hand extractions (inverting the tubes carefully instead of vortexing). A single extraction comprising 20 volumes of the solvent (series #4) yielded

recoveries from plasma comparable to that of 30 volumes of solvent distributed in 3 extractions (series #1) as shown in Table II. The recovery rates from blood plasma were similar to that obtained with physiological saline except that they were 2-3% lower in corresponding series. This could be due to the binding of the labelled steroids to the plasma proteins.

TABLE II

EFFECT OF SOLVENT VOLUME (DICHLOROMETHANE) AND NUMBER OF EXTRACTIONS ON RECOVERY RATE OF CORTICOSTERONE-H^3 AND CORTISOL-H^3 ADDED TO TWO ML OF RABBIT BLOOD PLASMA

Amt. of Solvent (ml) and No. of Volumes Per Extraction		% Recovery			
		Cortisol-H^3		Corticosterone-H^3	
Series #1	20:20:20* 10:10:10**	96	(10)***	98	(10)
Series #2	20:20 10:10	92	(10)	94	(10)
Series #3	20 10	85	(10)	89	(10)
Series #4	40 20	93	(10)	95	(10)

*The first figure in each series indicates ml of solvent/ extraction.

**The second figure in each series indicates volume of solvent.

***Number of experiments carried out in each series.

Effect of Saponification and Water Washes on the Recovery Rate of Corticosteroids

Saponification of the organic extract is necessary to eliminate non-specific fluorogens extracted along with the corticosteroids from plasma. The alkali wash should be followed by water washes to neutralize the extract.

The recovery rates of both cortisol and corticosterone when added to plasma and carried through solvent extraction and saponification of the extract including two water washes are presented in Table III. The lower recovery rate of cortisol compared to corticosterone could be due to the difference in polarity of these two compounds. Note that there was approximately a 10% reduction in corticosteroid recovery due to the saponification and water washes.

Recovery Rates of Corticosteroids Known Amounts of Which Were Added to Plasma and Carried Through the Entire Method

The overall recovery rates of both cortisol and corticosterone through all the steps of the EFM was determined. Two ml of rabbit blood plasma obtained from adrenalectomized, orchidectomized, dexamethasone treated rabbits containing 100, 150, 175 and 200 mμg of added cortisol and corticosterone were carried through the EFM. There was a considerable loss of both cortisol and corticosterone following extraction, saponification, neutralization by water washes, chromatography, and elution as shown in Table IV. The overall recovery rates ranged from 63 to 68% for cortisol and from 75 to 84% for corticosterone, respectively. The lower recovery rate of cortisol could most probably be attributed to the polarity of the compound and its binding to the thin-layer plate.

TABLE III

EFFECT OF SOLVENT VOLUME (DICHLOROMETHANE) AND NUMBER OF EXTRACTIONS ON CORTICOSTERONE-H^3 AND CORTISOL-H^3 ADDED TO TWO ML OF RABBIT BLOOD PLASMA (PROCEDURE INCLUDED SAPONIFICATION AND TWO WATER WASHINGS)

Amt. of Solvent (ml) and No. of Volumes Per Extraction		% Recovery	
		Cortisol-H^3	Corticosterone-H^3
Series #1	20:20:20* 10:10:10**	85 (6)***	90 (6)
Series #2	20:20 10:10	83 (6)	87 (6)
Series #3	40 20	81 (6)	86 (6)

*The first figure in each series indicate ml of solvent/ extraction.

**The second figure in each series indicates volume of solvent.

***Number of experiments carried out in each series.

TABLE IV

RECOVERY OF CORTICOSTEROIDS FROM RABBIT BLOOD PLASMA*

Steroid	Amount (mμg)		Percent Recovered
	Added	Recovered	
Cortisol	100	66	66
	150	111	68
	175	102	63
	200	126	63
Corticosterone	100	75	75
	150	121	80
	175	148	84
	200	155	78

*Each value is the average of two determinations made with 2 ml of plasma obtained from adrenalectomized, orchidectomized, and dexamethasone treated rabbits.

Chromatographic Isolation and Purification

Following pre-extraction with iso-octane, methylenedichloride extraction and saponification, the plasma extract was rendered relatively free from most of the non-steroidal material. The chromatographic step in our procedures in addition to further purification also resulted in separation of corticosterone and cortisol.

Choice of a Thin-Layer Chromatography (TLC) Solvent System for Fractionation of Cortisol and Corticosterone

The R_f values for both cortisol and corticosterone in different TLC solvent systems are shown in Table V. The solvent system consisting of chloroform:methanol:toluene:water (60:20:120:1) was selected. There was good separation between cortisol and corticosterone in this system, corticosterone having twice the R_f value as that of cortisol. In addition both cortisol and corticosterone moved sufficiently away from the non-steroidal fluorogenic material which is either polar and at the origin or non-polar and at the solvent front.

TABLE V

R_f VALUES FOR CORTICOSTEROIDS IN DIFFERENT SOLVENT SYSTEMS

System		Cortisol	Corticosterone	R_f
No. 1; C:M:W	(188:12:1)	0.26	0.48	0.22
No. 2; C:M:W	(90:10:1)	0.46	0.64	0.18
No. 3; C:M:W	(80:18:2)	0.60	0.70	0.10
No. 4; C:M:T:W	(60:120:20:1)	0.25	0.50	0.25
No. 5; A:B	(30:70)	0.24	0.37	0.13

A = Acetone C = Chloroform T = Toluene
B = Benzene M = Methanol W = Water

Difficulties Encountered in Eluting Corticosteroids From the Thin-Layer Plate (TLP) - Choice of Specific Elution Techniques for Cortisol and Corticosterone

Eluting corticosteroids from the coating material of the TLP presented problems. Hence, quantitative recoveries for cortisol and corticosterone by different techniques of elution from TLP were determined in Table VI.

TABLE VI

COMPARISON OF DIFFERENT TECHNIQUES OF ELUTION FOR CORTISOL AND CORTICOSTERONE

Method of Elution	% Recovery	
	Cortisol	Corticosterone
Column Chromatography	25*	71.4
Partition of Silica Gel Between Benzene and Water	1	96
Cold Ethanol	15	55
Hot Ethanol	15	60
Cold Methaonl	16	62
Hot Methanol	25	65
Chloroform:Methanol (1:1)v/v	33	50
Dichloromethane:Methanol (9:1)v/v	90	92

*Values represent mean % recoveries obtained from eight experiments in each.

The elution procedures for progesterone and testosterone established in our laboratory, namely scraping the zone of silica gel containing the steroid and partitioning between benzene and water resulted in 96% recovery of corticosterone and about 1% recovery of cortisol. A method of elution for corticosteroids based on the principle of column chromatography (Attal et al., 1967) failed to give the high recoveries as reported by these authors. Both cold and hot, ethanol and methanol or a cold mixture of chloroform:methanol (1:1)v/v, which are usually employed in quantitative elution of corticosteriods from paper chromatograms also resulted in low recoveries, especially so in the case of cortisol. When a relatively non-polar solvent mixture dichloromethane: methanol (9:1)v/v, (Idler et al., 1966) was used, 90% of cortisol and 92% of corticosterone were recovered.

In this study, cortisol was eluted by the non-polar solvent mixture of dichloromethane:methanol (9:1)v/v, and corticosterone by a partition of the silica gel between benzene and water. The latter method was very specific for corticosterone in that very little cortisol, if any, is soluble in benzene.

Fluorometric Quantitation

Both cortisol and corticosterone were quantitated fluorometrically using the principle of sulfuric-acid-induced fluorescence of these in a Turner (Model 110) Fluorometer, with a 470 mμ emission wavelength. The amount of fluorescence developed by the corticosteroids varies according to the difference in ratio of acid:alcohol in the reagent mixture. Also the time interval to attain maximum fluorescence for cortisol and corticosterone are different from each other. Therefore, optimum

conditions regarding the acid to alcohol ratio and the time interval required for both cortisol and corticosterone to reach maximum fluorescence had to be determined.

Determining the Suitable Ratio of Acid:Alcohol That Gives Maximum Fluorescence of the Corticosteroids

The effect of different ratios of acid to alcohol in the reagent mixture on the fluorescence development of both cortisol and corticosterone were examined and the results are presented in Table VII. The maximum fluorescence intensities for both cortisol and corticosterone were obtained by 70% sulfuric acid-ethanol solution. But the 65% ethanolic acid mixture was chosen in this study because of a comparatively low blank reading. The fluorescence intensity of corticosterone was observed to be almost twice that of cortisol in all cases.

TABLE VII

RELATIVE FLUORESCENCE INTENSITIES FOR CORTICOSTERONE AND CORTISOL IN VARIOUS REACTION MIXTURES

Sulfuric Acid:Ethanol (v/v)	Relative Fluorescence Intensity*		
	Cortisol	Corticosterone	Blank
80:20	21	44	1.0
75:25	29	47	1.0
70:30	38	65	3.0
65:35	33	62	1.5
60:40	30	56	1.5
55:45	20	50	1.0
50:50	22	48	1.0

*Each test tube contained 0.1 μg of cortisol or corticosterone. Each value represents a mean of five observations. (Wavelength 470 mμ for activation, 590 mμ for emission.)

Determining the Time Interval Required for the Development of Maximum Fluorescence Intensity for Corticoids Following the Addition of Acid-Alcohol Mixture

The effect of time interval following the addition of acid-alcohol mixture on the fluorescence intensity of the coricosteroids was determined and the results are presented in Table VIII. For corticosterone fluorescence developed at the end of 10 minutes, reached the maximum at 50 minutes and remained so at the end of 1 hour. The disparity between cortisol and corticosterone in the time interval for attaining the maximum fluorescence was taken to advantage in the EFM. Thus, the addition of acid-alcohol mixture to the corticosterone batch of samples preceded that of cortisol, but fluorometric measurements were made in the reverse order to save time.

TABLE VIII

RELATIVE FLUORESCENCE INTENSITIES FOR CORTISOL AND CORTICOSTERONE AT VARIOUS TIME INTERVALS

Time From Addition of Reaction Mixture (Minutes)	Relative Fluorescence Intensity*	
	Cortisol	Corticosterone
10	31	16
20	33	34
30	34	55
40	32	60
50	30	62
60	28	61

Values represent mean of three observations. Each test tube contained 0.1 µg of corticosterone or cortisol. The reaction mixture used was H_2SO_4:Ethanol (65:35) v/v; (Wavelength: 470 mµ for activation and 520 mµ for emission.)

Sensitivity of the EFM

Sensitivity of the EFM was determined by measuring fluorescence intensity of standard cortisol and corticosterone solution (1 μg/ml) diluted appropriately to concentrations ranging from 5 to 100 mμg/ml. As low as 10 mμg of corticosterone and 15 mμg of cortisol could be measured by the EFM. Fluorescence as a linear function of corticosteroid concentration can be seen from the standard curves obtained and are as shown in Figure 2.

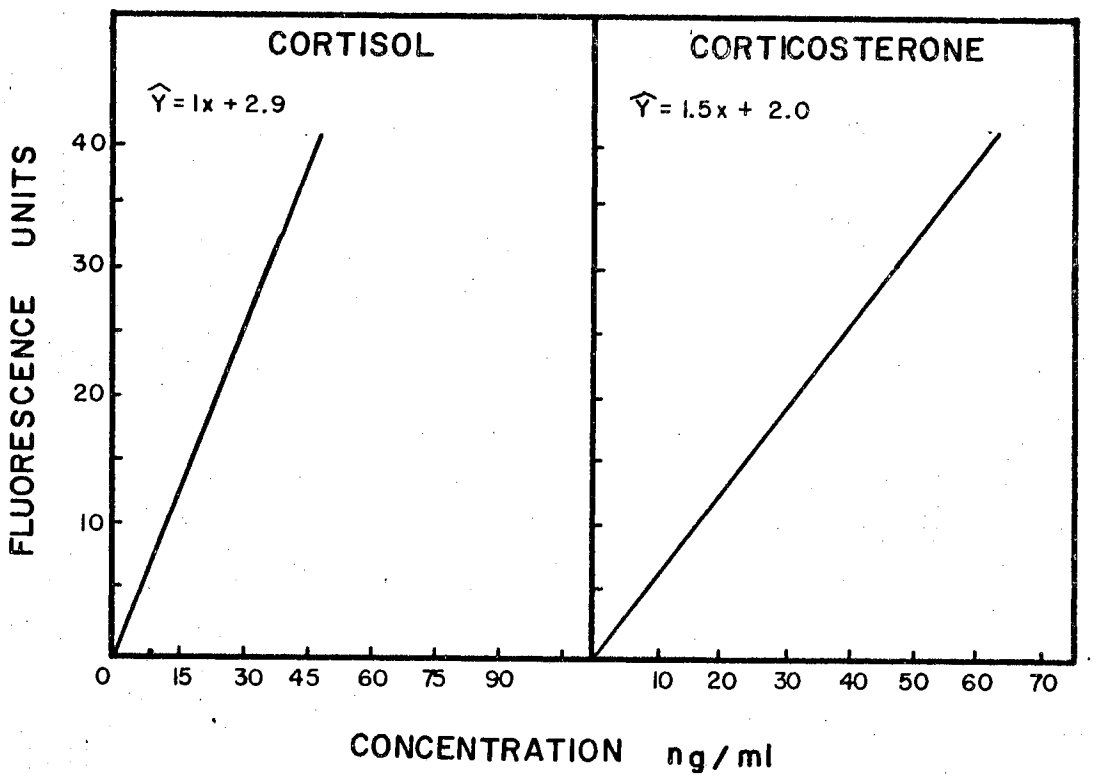


Figure 2. Sensitivity of the Extended Fluorometric Method

Validity of the EFM

Blood plasma obtained from animals that were adrenalectomized is presumed to be corticosteroid free. In addition to adrenalectomy, gonadectomy is also performed by some investigators to eliminate any possible gonadal contribution. The fluorescence intensity of plasma from such animals should be at minimum with respect to corticosteroids and represents the background fluorescence or the so-called residual activity.

If high fluorescence values are obtained by a fluorometric method designed to quantitate the corticosteroids in plasma from such animals, the physiological validity of such a method is dubious. Since physiological validation alone is not enough to rigorously prove the specificity of the method, physico-chemical criteria were employed in this study to establish that the compounds quantitated were cortisol and corticosterone.

The EFM has been thoroughly validated by using both physiological as well as physico-chemical criteria.

Physiological Validation

The specificity of the EFM was demonstrated by examining plasma obtained from animals that had combined adrenalectomy and gonadectomy or adrenalectomy alone or animals that were simply dexamethasone suppressed as shown in Table IX. Duplicate determinations were made on 2 ml of plasma from animals in each group. Physiological saline of the same volume was extracted and carried through the EFM similarly to serve as a blank. The residual activity was least in animals that had combined adrenalectomy and gonadectomy.

TABLE IX

PHYSIOLOGICAL VALIDATION OF THE METHOD

Treatment	1×10^{-2} µg/ml of plasma	
	Cortisol	Corticosterone
Physiological Saline Blanks	0.000	0.000
Adrenalectomized and Gonadectomized	0.002	0.003
Adrenalectomized	0.008	0.006
Dexamethasone Suppressed	0.008	0.005

Possible gonadal contribution in the residual activity could be seen in animals that were only adrenalectomized. Dexamethasone suppressed animals had comparatively higher values. Although the rabbit adrenal cortex secretes predominatly corticosterone, the cortisol values in these animals were higher and could be attributed to the dexamethasone administered. When dexamethasone was extracted and run in our thin-layer system, it had the same R_f value as that of cortisol.

Physico-Chemical Validation

Whenever cortisol and corticosterone from rabbit blood plasma were quantitated by the EFM, an aliquot of these samples was saved and eventually pooled into either "cortisol-pool" or "corticosterone-pool". The objective was to subject these pools to various physico-chemical criteria to demonstrate that the isolated compounds were cortisol and corticosterone.

Chromatography to a Constant Specific Activity. The preliminary evidence as to the chemical purity of the material isolated from rabbit blood plasma was shown by establishing a constant specific activity of cortisol (Table X) and corticosterone (Table XI) following TLC in a number of different solvent systems. Maintenance of a constant specific activity through repeated chromatography showed the homogeneity of the endogenous steroids extracted from plasma by the method with that of the added tritiated corticosteroid standards. R_f values of the unknown compounds and the standard cortisol and corticosterone were identical in all TLC solvent systems.

TABLE X

SPECIFIC RADIOACTIVITY OF CORTISOL ISOLATED FROM RABBIT BLOOD PLASMA FOLLOWING REPEATED CHROMATOGRAPHY IN DIFFERENT SOLVENT SYSTEMS

Solvent System	R_f	cpm/μg
No. 1; C:M:T:W (60:20:120:1)	0.25	21,193
No. 2; C:M:W (80:18:2)	0.60	20,916
No. 3; C:M:W (90:10:1)	0.46	21,075

C = Chloroform
M = Methanol
T = Toluene
W = Water

TABLE XI

SPECIFIC RADIOACTIVITY OF CORTICOSTERONE ISOLATED FROM RABBIT BLOOD PLASMA FOLLOWING REPEATED CHROMATOGRAPHY IN DIFFERENT SOLVENT SYSTEMS

No. 1;	C:M:T:W (60:20:120:1)	0.50	13,740
No. 2;	C:M:W (188:12:1)	0.48	13,682
No. 3;	A:B (30:70)	0.37	13,586

A = Acetone C = Chloroform T = Toluene
B = Benzene M = Methanol W = Water

Mass Spectral Analysis. A more rigorous proof of structure and purity of the isolated cortisol and corticosterone from rabbit blood plasma by the EFM was established by comparing their mass spectra with those of the authentic standards. The samples (0.5-1 μg in size) were injected by a direct probe into a mass spectrometer-gas chromatograph. Details of its operation have been reported by Waller (1967) and Ryhage (1967).

The molecular ion peak was seen at 362 for both the unknown and standard cortisol.

Figure 3 presents the mass spectrum of the standard cortisol and Figure 4 presents the mass spectrum of the unknown compound.

For both the unknown and the standard corticosterone, the molecular ion peak was 346. The mass spectrum of the standard corticosterone is presented in Figure 5 and that of the unknown in Figure 6. The similarity in fragmentation patterns and the finding of a molecular weight of 362 and 346 are conclusive proofs that the isolated compounds were

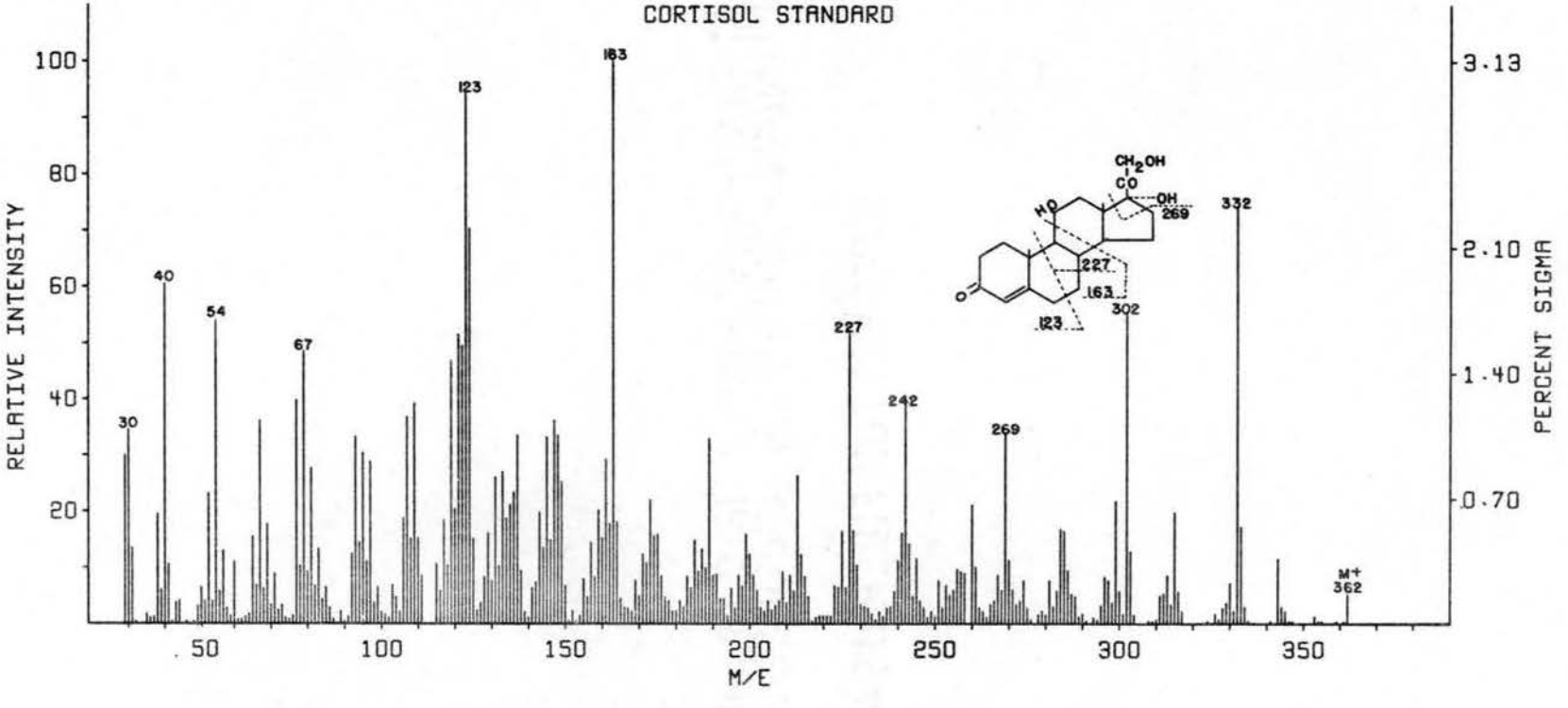


Figure 3. Mass Spectrum of Standard Cortisol

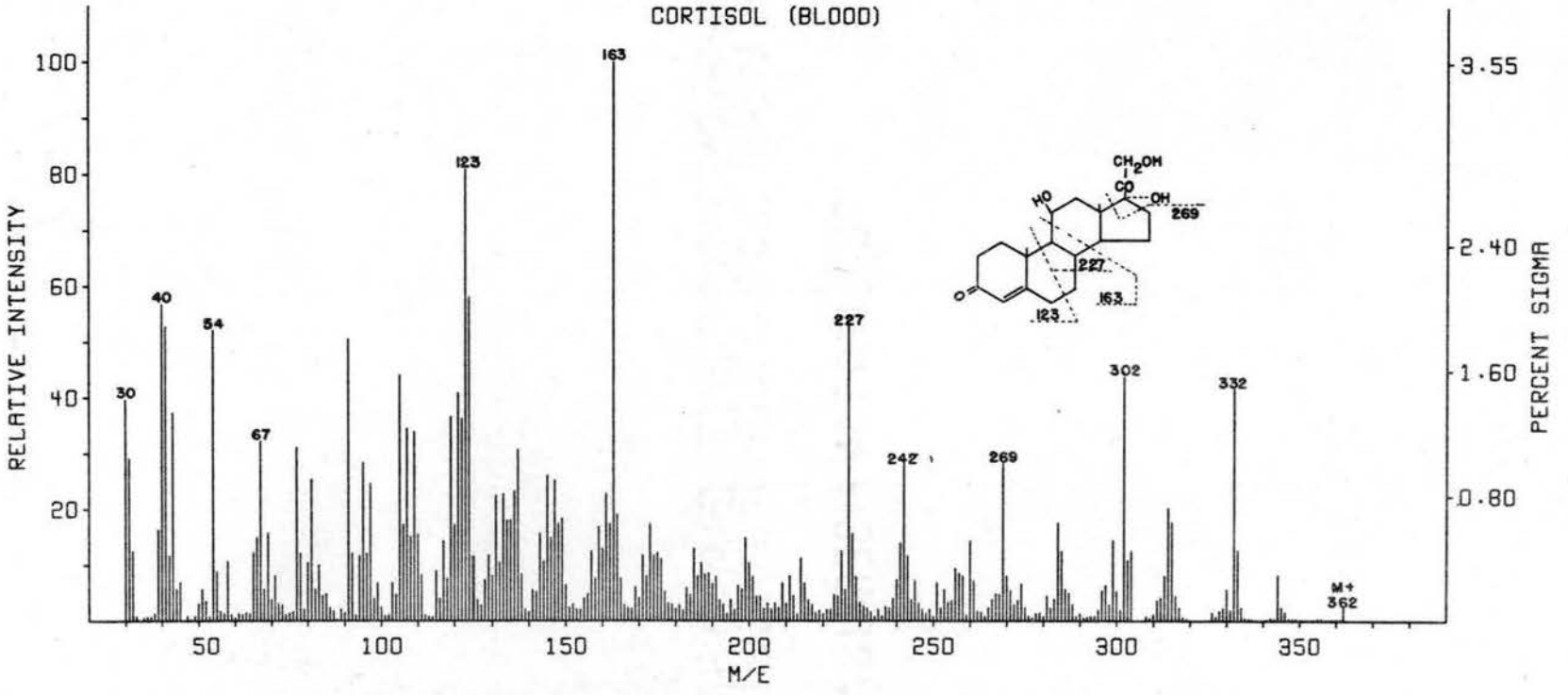


Figure 4. Mass Spectrum of Cortisol Isolated From Rabbit Blood Plasma

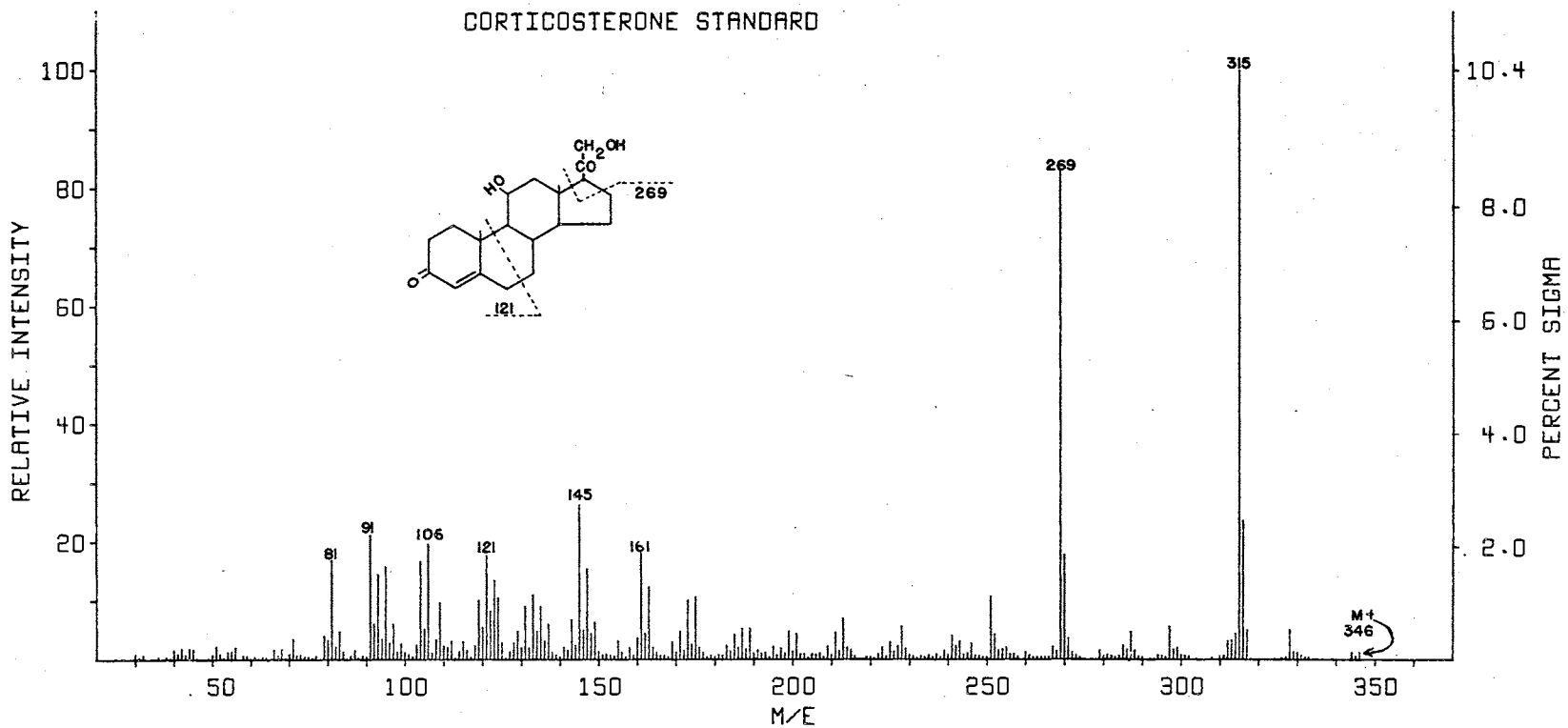


Figure 5. Mass Spectrum of Standard Corticosterone

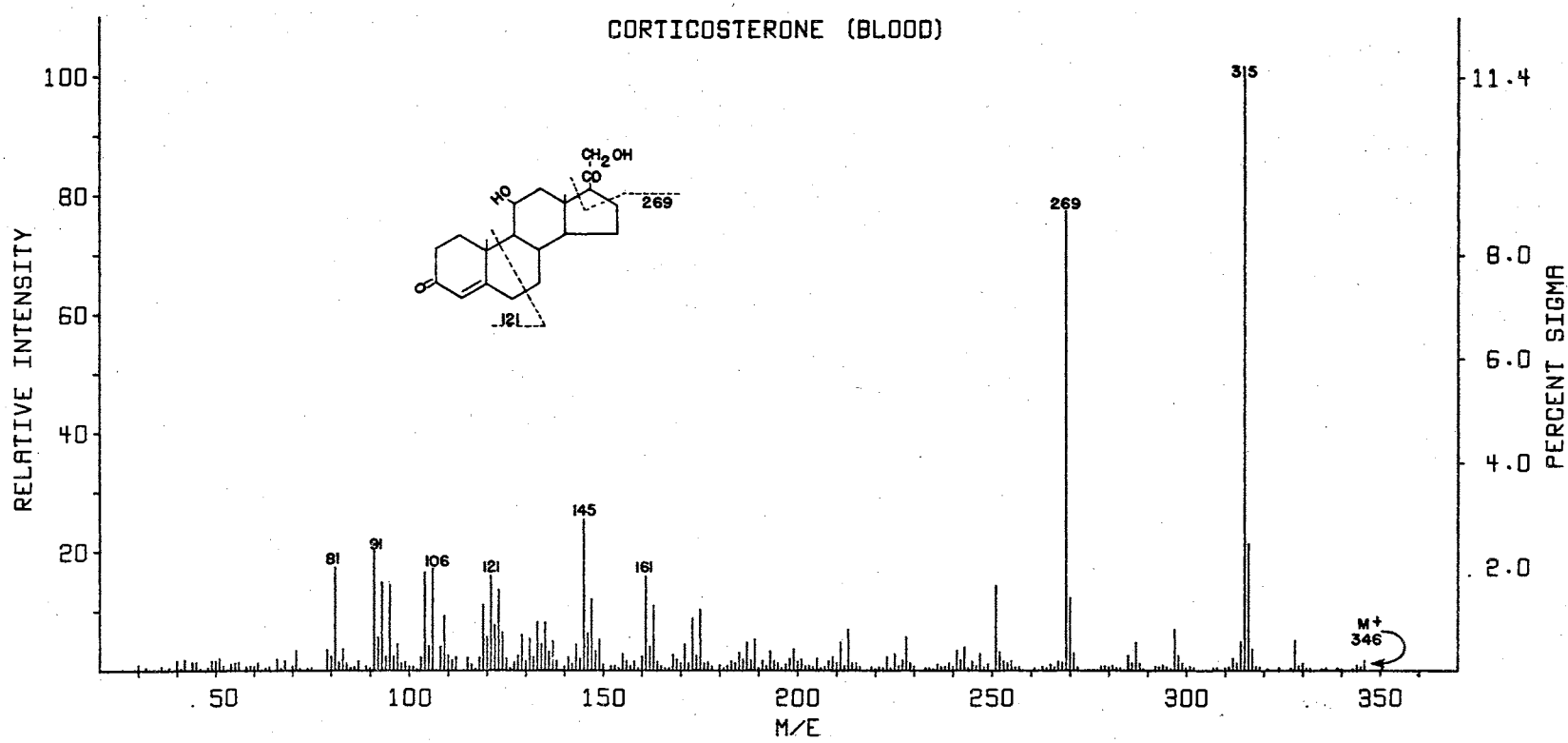


Figure 6. Mass Spectrum of Corticosterone Isolated From Rabbit Blood Plasma

cortisol and corticosterone, respectively. Also, the suggested fragmentation pattern and the mass spectra of our unknown as well as the standard cortisol and corticosterone agree with those obtained by Genard and co-workers (1968).

Comparison of the EFM With Two Other Widely Used Brief Fluorometric Methods

Following validation of the EFM regarding sensitivity and specificity, it was important to determine its precision. It would also be meaningful to see whether the precision of the EFM was greater than the brief fluorometric methods. Therefore, plasma corticosteroid concentrations were determined in triplicate by three different methods for each of nine rabbits and their precision compared. The three fluorometric methods employed were EFM and two brief methods, namely that of Guillemin et al. (1959), and of van der Vies (1961) with slight modifications. The modifications were definite improvements which included addition of labelled compounds as internal standards to correct for losses through these methods and measuring the final concentrations from our standard curves where there were no Y-intercept values as background fluorescence.

Corticosteroid Levels in Rabbit Peripheral Blood Plasma as Measured by Three Methods

Table XII shows the plasma corticosteroid levels in rabbits as determined by the three different fluorometric methods. The mean cortisol and corticosterone levels were 1.80 and 7.70 µg%, respectively, as determined by the EFM.

TABLE XII

CORTICOSTEROID LEVELS IN RABBIT PERIPHERAL BLOOD PLASMA AS DETERMINED BY THREE DIFFERENT FLUOROMETRIC METHODS

	Method of Guillemin	Method of van der Vies		Extended Fluorometric Method	
No.	Corticosterone	Cortisol	Corticosterone	Cortisol	Corticosterone
1	12.4*	1.5	9.5	1.8	6.7
2	15.1	1.9	12.0	1.9	8.2
3	13.0	2.0	11.9	2.5	6.6
4	13.1	1.7	9.3	1.4	7.2
5	16.9	2.2	11.3	1.3	8.1
6	15.0	1.8	10.6	1.5	6.2
7	16.1	2.4	9.7	1.8	9.1
8	15.2	2.0	10.0	2.0	9.3
9	17.0	1.9	10.6	2.4	8.2
Mean**	14.8±0.6	1.9±0.2	10.5±0.4	1.8±0.1	7.7±0.3

*Values expressed on a μg% basis and represents the mean of three determinations.

**The mean ± standard error of nine adult male rabbits.

Most of the non-specific fluorogens are eliminated by chromatography in the EFM. The corticosteroid levels measured by the EFM were comparatively lower, presumably, because of its improved specificity over the brief methods.

The brief fluorometric method of Guillemin and co-workers which does not differentiate between cortisol and corticosterone resulted in

56% more corticosteroids on an overall basis, and 92% more corticosterone in particular compared to the EFM.

The brief method of van der Vies where fractionation between cortisol and corticosterone is achieved through solvent-partitioning, resulted in 36% more corticosterone when compared to the extended method. There does not seem to be much difference in cortisol levels between these two methods. However, in the method of van der Vies, a complete quantitative separation between cortisol and corticosterone cannot be achieved by a carbon tetrachloride/water partition as both the compounds are slightly soluble in both phases.

Comparison of Precision of the Extended and Brief Fluorometric Methods

The comparative precision of the three fluorometric methods used to quantitate corticosteroids in rabbit peripheral blood plasma in this study is presented in Table XIII. The coefficient of variation for both cortisol and corticosterone was least in the extended method. The 95% confidence limits for corticosterone were significantly less in the EFM. The upward bias for corticosterone values in the brief fluorometric methods could be due to the interferring fluorogens resulting in non-specific fluorescence.

Effect of Different Blood Collection Procedures on the Corticosteroid Levels in the Rabbit Peripheral Blood Plasma

To procure meaningul information about adrenocortical function, it is important to know the conditions under which the blood samples are collected. Time of collection, type of anesthetic used, the route of collection and the amount of time involved during blood collection, all seem to influence the hypothalamo-pituitary-adrenal axis.

Therefore, an experiment was designed testing the effects of blood collection procedures on the level of cortisol and corticosterone in rabbit peripheral blood plasma as shown in Table XIV.

TABLE XIII

COEFFICIENTS OF VARIABILITY AND 95% CONFIDENCE LIMITS ASSOCIATED WITH CORTICOSTEROID DETERMINATIONS BY THE THREE FLUOROMETRIC METHODS

Corticosteroid	Statistic	Method		
		1***	2****	3*****
Cortisol	CV*	---	4	3
Corticosterone		35	10	9
Cortisol	95%** limits	---	1.5-2.3	1.6-1.9
Corticosterone		13.2-16.4	9.6-11.4	7.3-8.2

*Coefficient of variation = $100(s)/\bar{x}$.

**95% confidence interval = $t_{0.95}s_{\bar{d}}$

***Guillemin et al. (1959).

****van der Vies (1961).

*****Extended fluorometric method.

An analysis of variance showed that the corticosterone values were significantly higher ($P < 0.01$) after ether anesthesia than after the other three treatments. The other three groups were not significantly different from each other ($P > 0.25$) in corticosterone values. Neither cortisol values nor the F/B ratio showed any significant difference

between treatments ($P > 0.25$). The effect of ether on the corticosterone level is readily apparent in Figure 7 which shows the effect of different blood collection procedures on the corticosteroid levels. These findings with rabbits agree with observations of Greer and Rockie (1968) in rats that ether is a powerful stimulus for ACTH release acting directly on the hypothalamic eminence presumably to release corticotrophin releasing factor which in turn activates ACTH secretion.

TABLE XIV

EFFECT OF BLOOD COLLECTION PROCEDURES ON PLASMA CORTICOSTEROID LEVELS*

Type of Collection	μg per 100 ml plasma		F_k/B_k
	Cortisol (F_k)	Corticosterone (B_k)	
Decapitation	1.9±0.2	6.0±0.7	0.34±0.04
Vacuum	2.2±0.1	7.6±1.0	0.31±0.03
Sodium Pentabarbitol	1.7±0.2	6.4±0.6	0.30±0.05
Ether	2.5±0.3	11.9±0.8	0.21±0.03

*Each value represents the mean ± standard error of six adult male rabbits.

Corticosteroid levels in the decapitated rabbit could be taken as the resting peripheral levels since there was neither an anesthetic used nor was the time interval for collection greater than 30 seconds in these animals. Blood collected from animals following sodium

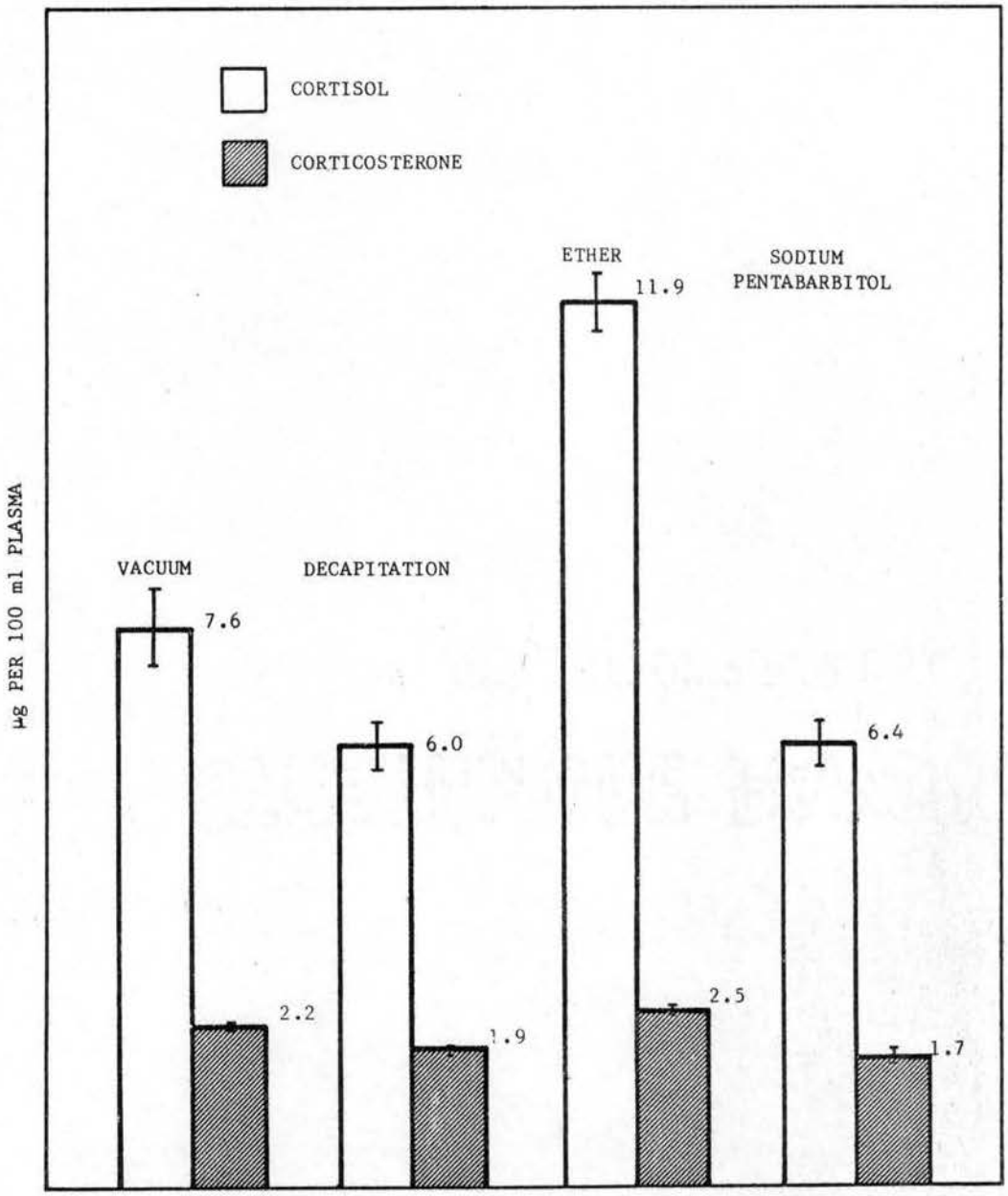


Figure 7. Effect of Different Blood Collection Procedures on the Corticosteroid Levels in the Rabbit Peripheral Blood Plasma

pentabarbitol anesthesia are very close to those of the decapitated animals pointing to the possibility of its routine use, without expecting any undue changes in the hypothalamo-pituitary-adrenal axis.

Effect of Exogenous ACTH on Corticosteroid Levels in Rabbit Peripheral Blood Plasma

Acute Effects

The study to be described here was designed to assess the magnitude of the adrenocortical response to acute stress in rabbits and to provide information regarding specific effects of ACTH on cortisol and corticosterone levels.

An experiment to simulate "acute stress" was designed where 10 IU ACTH was injected intravenously into two rabbits and blood collected from an ear vein by mild vacuum suction. Cortisol and corticosterone levels in peripheral blood plasma of these two rabbits were quantitated using the EFM and are as shown in Table XV. The administration of this level of ACTH more than doubled (6.00 to 13.00 μg%), the concentration of corticosterone within one half-hour post-injection. The level of corticosterone dropped slightly (13.00 to 12.00 μg%) at the end of one hour, drastically declined at the next half-hour interval and continued to fall three hours post-injection. Pre-injection levels of corticosterone were observed by the end of the fourth and fifth hours post-injection. On the other hand, cortisol levels were held nearly constant and at no time did they markedly differ from pre-injection levels. Figure 8 shows the changes in cortisol and corticosterone levels following intravenous administration of ACTH.

TABLE XV

EFFECT OF ACUTE STRESS ON THE CORTICOSTEROID LEVELS IN RABBIT PERIPHERAL BLOOD PLASMA

Hours following ACTH Injection	μg/100 ml plasma	
	Cortisol	Corticosterone
0	2.0*	6.0
0.5	2.2	13.0
1	1.6	12.1
1.5	1.8	7.9
2	1.8	7.9
2.5	1.9	6.5
3	1.9	5.6
4	2.3	6.8
5	1.8	6.7

*Values represent mean of duplicate determination in each of two rabbits.

Chronic Effects

In 1954, Kass and co-workers reported that 21-28 day stimulation of rabbits with 12.5-25 units of porcine ACTH per day caused an increase in cortisol and a decrease in corticosterone secretion. These results were of interest since cortisol is normally produced in only trace amounts by rabbit adrenals, but is the predominant corticosteroid secreted by the adrenal gland of the pig, the species from which ACTH is isolated. Yudeav and Anfinogenova (1960), and Krum and Glenn (1965)

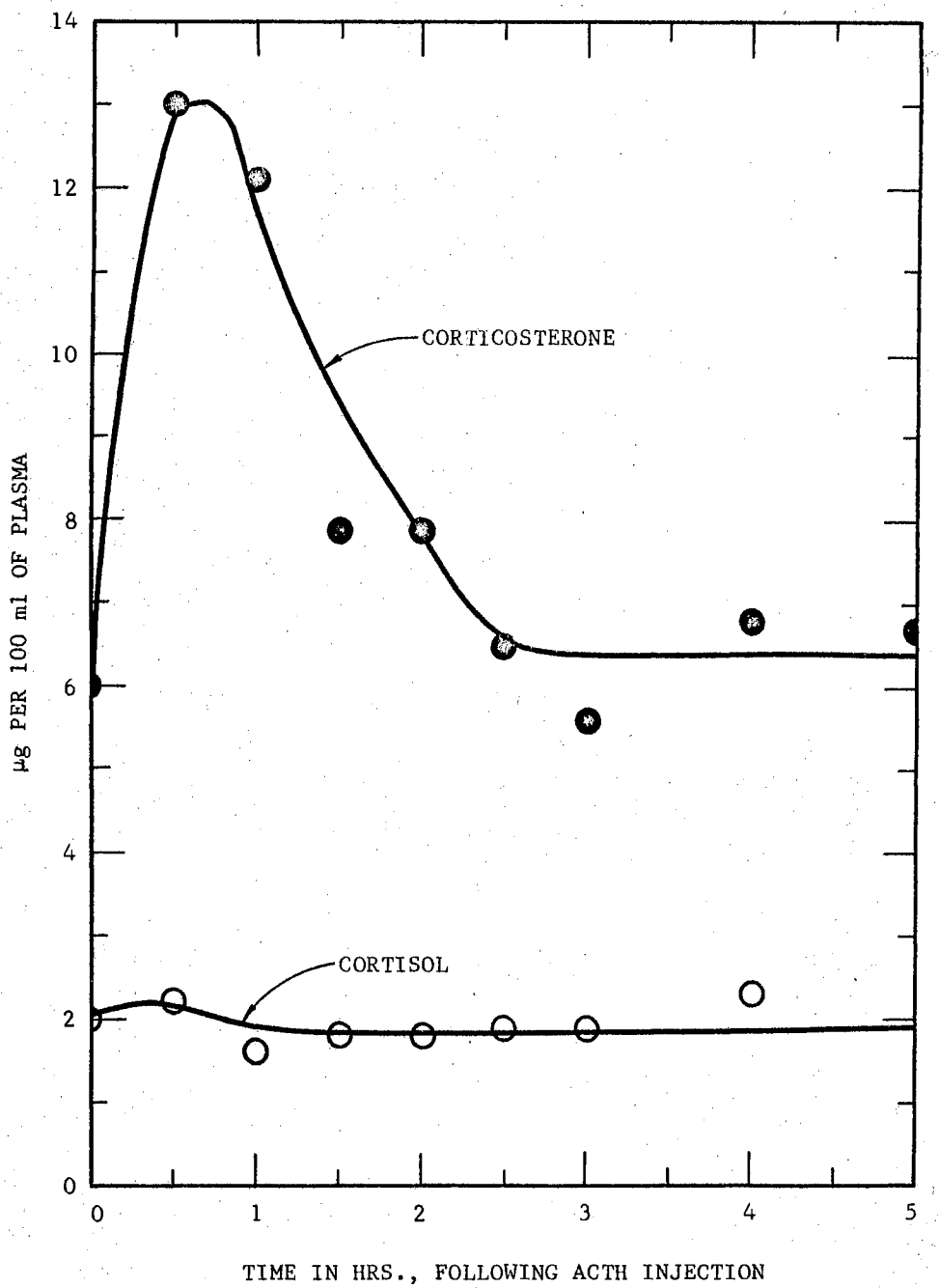


Figure 8. Temporal Changes in Corticosteroid Levels in Rabbit Peripheral Blood Plasma Following Intravenous Administration of ACTH

have essentially confirmed the initial *in vivo* findings. Morozova's (1965) investigations revealed that contrary to the *in vivo* observations, the rabbit adrenal tissue *in vitro* showed maximum cortisol production after stimulation of the rabbits for only three days. In all of these investigations rather impure ACTH preparations were used and none reported the corticosteroid levels in peripheral blood plasma.

Therefore, an experiment was designed to assess the effect of chronic stress on adrenocortical response in the rabbits. Following adrenal stimulation for 28 days, the daily intravenous injections of 20 IU ACTH (chromatographically purified) preparation, peripheral blood plasma levels of cortisol and corticosterone were quantitated using the EFM. The corticosteroid levels in the chronically stimulated group was compared to those of the control and the chronically suppressed groups which received only vehicle (sterile peanut oil with 5% beeswax) and dexamethasone, respectively, for the same length of time as shown in Table XVI.

The corticosteroid levels were at a minimum indicating adrenal suppression in dexamethasone suppressed animals. Cortisol level more than doubled in the chronically stimulated group compared to that of controls (an increase of 150%). No change was observed in the corticosterone levels between the two groups.

Our observations on the corticosteroid levels in the peripheral blood plasma where cortisol:corticosterone ratio was less than unity markedly differs from that of adrenal-vein blood where the ratio was far more than unity as observed by Kass *et al*. (1954), in the chronically stimulated rabbits.

TABLE XVI

EFFECT OF CHRONIC STRESS ON THE CORTICOSTEROID LEVELS IN RABBIT PERIPHERAL BLOOD PLASMA

No. of Rabbits	Group and Treatment	µg/100 ml plasma (F)	(B)	F/B
6	Controls 0.5 ml of vehicle im daily, for 28 days	2.10	8.30	0.24
5	Adrenal Stimulation 0.5 ml (20 IU) ACTH im daily, for 28 days	5.20	8.60	0.63
6	Adrenal Suppression 0.5 ml (one mg) dexamethasone im daily, for 28 days	0.95	0.52	1.10

F = Cortisol B = Corticosterone

Corticosteroid Levels in Pathological States

It appears that a variety of diseases and pathological states are associated with changes in adrenocortical function. These changes are complex and cannot be fully understood at present. It is not known whether they are primary, and thus of etiological significance, or secondary to the disease process. If adrenocortical abnormalities are primary, they would probably be reflected in the corticosteroid levels. Both cortisol and corticosterone levels were assayed in two pathological states: (1) a case of "porphyremia" in cattle utilizing an ultraviolet spectrophotometric method, and (2) in swine suspected of a "porcine stress syndrome" by EFM. The comparison as to numbers of animals

surveyed, time required and information obtained is striking and demonstrates the suitability of EFM as a clinical diagnostic tool.

Corticosteroid Levels in Porphyric Cattle

An impairment in adrenocortical function was suspected in porphyric cattle where the pathological manifestation is due to an inborn error in metabolism. Peripheral blood plasma levels of both cortisol and corticosterone were measured in this study from one porphyric cow, one porphyric bull and one normal cow immediately before and one hour after 200 IU ACTH (Adrenomone) administered intramuscularly and as shown in Table XVII. The objective in exogenous ACTH administration was to compare the adrenocortical response of porphyric bovine to that of the normal. At the time this study was made, the EFM was not yet developed and hence, a spectrophotometric method (Venkataseshu and Estergreen, 1965) was used in quantitating corticosteroids.

TABLE XVII

CORTICOSTEROID LEVELS IN PORPHYRIC CATTLE*

Description	Pre-ACTH			Post-ACTH		
	F	B	F/B	F	B	F/B
Normal Cow	6.1	1.7	3.6	7.7	1.6	5.0
Porphyric Cow	2.3	0.5	4.2	4.9	1.2	4.2
Porphyric Bull	3.4	1.5	2.2	6.1	1.4	4.5

F = Cortisol B = Corticosterone

*Values expressed on a μg % basis.

The pre-injection levels of both cortisol and corticosterone were lower in the porphyric cattle compared to the normal. Following ACTH administration, the cortisol levels almost doubled from pre-injection levels and agrees with the findings of the author from a previous study in cattle (Venkataseshu, 1965). The failure to observe the doubling effect on the cortisol level one hour post-injection in the normal could not be explained. Following ACTH injection, the level of corticosterone increased markedly from the very low pre-ACTH administration levels in the porphyric cow.

The findings in this study, because of insufficient numbers, can not be conclusive. The low levels of corticosteroids indicate the possibility of a suppressed adrenocortical function in porphyric cattle. However, the adrenocortical response to exogenous ACTH eliminated the possiblity of an impairment in the adrenal enzyme systems involved in the steroidogenic pathway.

Corticosteroid Levels in Pigs Suspected of Porcine Stress Syndrome

Topel and co-workers (1968) described a condition in swine which they termed "Porcine Stress Syndrome" (PSS). Swine which have been bred for rapid growth qualities, more efficient feed conversion, superior muscling and minimum backfat were more susceptible to development of this condition. Clinically, the disease was characterized by acute death in market-age pigs and usually occurred during or immediately following stressful conditions such as transporting, fighting or other forms of exertion. These investigators postulated a possible impairment of the pituitary-adrenal system in such animals.

In this study, cortisol and corticosterone levels were quantitated

using the EFM from two ml blood serum samples obtained from pigs that were suspected of PSS with a view to better understanding adrenal function in such animals. Corticosteroid levels were investigated from three groups of swine: the control, low-incidence and the high-incidence groups and the results are shown in Table XVIII.

The mean cortisol levels were 12.6, 13.3, and 13.5 and corticosterone 4.0, 4.5, and 5.4 in the control, low-incidence and high-incidence groups, respectively. There seems to be no apparent difference in either the cortisol or corticosterone levels between the three groups. The F/B ratio ranged from 0.33 in the controls to 0.42 in the high-incidence group. It would appear that there is no adrenal involvement in the porcine stress syndrome subjects.

TABLE XVIII

CORTICOSTEROID LEVELS IN PIGS SUSPECTED OF A PORCINE STRESS SYNDROME

Control Group				Low-Incidence Group				High-Incidence Group			
Pig #	F	B	F/B	Pig #	F	B	F/B	Pig #	F	B	F/B
C1	14.0	3.2	0.2	H6	10.5	4.1	0.39	Y23	15.8	5.5	0.35
C2	12.0	3.9	0.3	H9	10.1	3.4	0.34	Y25	9.8	4.6	0.47
C3	10.8	5.6	0.5	H12	15.2	3.0	0.2	Y27	18.0	2.6	0.15
C5	15.1	3.4	0.2	H13	12.8	6.5	0.51	Y29	12.8	5.6	0.44
C6	11.2	3.8	0.34	H15	17.4	6.8	0.39	Y30	14.6	6.5	0.45
				H16	10.6	5.8	0.55	Y39	8.9	3.5	0.39
				H18	16.4	3.3	0.20	Y40	15.7	8.0	0.51
				H19	10.3	3.8	0.47	Y41	10.5	5.7	0.54
				H20	16.2	4.2	0.26				
				H22	10.8	4.0	0.37				
Mean	12.6	4.0	0.33	Mean	13.3	4.5	0.36	Mean	13.5	5.4	0.42

F = Cortisol

B = Corticosterone

CHAPTER V

DISCUSSION

Development of an Extended Fluorometric Method for Simultaneous Determination of Cortisol and Corticosterone in Biological Samples

A fluorometric method has been developed for the simultaneous determination of cortisol and corticosterone in biological samples. This method appears to be simple, precise, sensitive, highly specific and applicable to both experimental and clinical conditions. Two important aspects of the extended fluorometric method merit closer consideration. The first concerns the separation of interfering fluorescing substances from the steroid compounds and the second the simultaneous determination of cortisol and corticosterone in the same biological sample.

Removal of Non-Specific Fluorogens

It has been known for some years that, when measuring adrenocorticoids in biological extracts by fluorescence in sulfuric acid, some type of correction is needed in order to eliminate "non-specific interference" (Moncola et al., 1959). Several simple methods for the determination of glucocorticoids in plasma have been described in which the fluorescent reagent is added directly to the plasma extract. Lack of specificity is observed in these brief techniques and some investigators have proposed a correction for non-steroidal fluorescence.

One method of correcting this is to eliminate the interfering fluorogens before they contribute to the final fluorescence reading, and the other is to estimate the relative contribution of interfering fluorogens to the total fluorescence, and eliminate them mathematically.

The first method involves either partitioning prior to addition of fluorescent reagent or quantitation at an early time after extraction of the steroid into the fluorescent reagent. Many different partitioning procedures have been used. The use of sodium hydroxide to extract interfering estrogens was incorporated into almost every brief method in the literature. All the fluorometric methods and the ultraviolet spectrophotometric method used in this study involved washing with sodium hydroxide. The use of petroleum ether or iso-octane to extract fluorescing contaminants are used in several brief methods. The pre-extraction of plasma with iso-octane was incorporated in the EFM for the same reason. The partition of the plasma steroids between carbon tetrachloride (Rudd et al., 1963; van der Vies, 1961) or benzene (Braunsberg and James, 1962) and the aqueous phase was designed to isolate the corticosterone from the cortisol component, as well as to eliminate interfering fluorogens.

A second way to improve the accuracy of brief fluorometric methods is by means of a correction factor. Several correction factors have been suggested including fluorescence development in two different strengths of sulfuric acid by Silber et al. (1958) or subtraction of the Y intercept of a graph of fluorescence vs volume of sample from the total fluorescence of the plasma sample by Moncola et al. (1959). In order to keep the interference of non-specific fluorescence material in the plasma as low as possible, it is recommended that the

fluorometric measurement is carried out 5 minutes after the purified sample has been exposed to the fluorescent reagent (De Moor and Steno, 1963). Recently Spencer-Peet et al. (1965) have observed that extracts of human plasma contain a component with constant fluorescence between 8-30 minutes after being exposed to the ethanolic sulfuric acid and another component exhibiting linear increase of fluorescence with time. This observation was used to develop a correction factor to calculate the "true" level of steroids in human plasma. These correction factors were carefully tested in brief methods evaluated by Frankel and co-workers (1967) in measuring corticosterone in avian blood plasma. They used criteria such as volume dependence, time dependence, chromatographic elimination and isolation and proved the inaccuracy of those brief methods involving correction factors.

In the EFM, the background fluorescence was almost completely eliminated as follows: (1) by pre-extraction of plasma with iso-octane removing lipids less polar than C-21 and C-19 steroids; (2) by alkali wash eliminating estrogens, as well as phenolic and acidic steroids; (3) by thin-layer chromatograph resulting in chromatographic elimination of polar and non-polar non-steroidal fluorogens; (4) by using minimal volumes of plasma; (5) by extensive washing of silica gel and glassware as well as careful checks on purity of reagents; (6) by using tritiated standards with higher specific activities compared to C^{14} labelled standards for monitoring losses through the method; (7) by using an acid-alcohol mixture with a 65:35 ratio; (8) by using a primary and secondary filter combination that was less sensitive but more specific; and (9) by using 10% potassium oxalate instead of heparin as an anti-coagulant since the latter interfered with fluorescence measurement

(Werk, Jr. et al., 1967).

The elimination of almost all interfering fluorogenic contaminants in the EFM was demonstrated by the near zero fluorescence values in the plasma samples from the bilaterally adrenalectomized and orchidectomized animals.

Specificity of the Extended Fluorometric Methods

The specificity of the EFM has been thoroughly validated by using both physiological as well as physio-chemical criteria since specificity is the most important requirement of any quantitative method. The fluorometric methods reported in the literature failed to positively identify the cortisol and corticosterone that were being quantitated and so their specificity is subject to question.

The specificity of the EFM regarding quantitation of both cortisol and corticosterone was established as follows: (1) inability to quantitate any measurable levels of corticosteroids (near blank values) in the plasma samples from adrenalectomized and adrenal suppressed animals; (2) the expected increase in corticosteroid levels (corticosterone component in rabbits) following intravenous administration of ACTH being observed in the rabbit blood plasma in acutely stressed animals; (3) chromatographic elimination of non-specific fluorogens and fractionation of cortisol and corticosterone components of the rabbit blood plasma; (4) a different technique of elution being used for cortisol component from that of corticosterone from the TLP; (5) the R_f values of the isolated cortisol and corticosterone being identical with that of authentic standards in different TLC solvent systems; (6) chromatography to a constant specific activity of the isolated cortisol

and corticosterone by the EFM; and (7) confirmation of proof of structure of the isolated compounds by EFM as to cortisol and corticosterone by mass-spectrometry.

The mean cortisol and corticosterone levels in rabbit peripheral blood plasma were 1.80 and 7.70 μg%, respectively, as determined by EFM. The brief method of Guillemin et al. and that of van der Vies used in comparison with EFM regarding precision and accuracy, demonstrated an error of 92% and 36%, respectively, in corticosterone levels. The method of Guillemin and co-workers could not differentiate between cortisol and corticosterone components of rabbit blood plasma. The precision and accuracy of the brief method of van der Vies was improved considerably by a modification introduced by us, namely addition of internal standards to monitor for losses through the method. Failure to incorporate a chromatographic purification step to eliminate non-steroidal fluorogens as in our EFM, could explain the upward bias observed in the corticosteroid levels obtained in the brief methods.

Application to Experimental Conditions

The concentration of free steroids in peripheral blood plasma is influenced by three factors: (1) rate of steroid production and secretion; (2) rate of distribution into the tissues; and (3) rate of metabolism and excretion (Eik-Nes, 1960). Therefore, any difference in the observed levels of steroids could be a result of change in any of these factors. On the other hand, a lack of difference between two samplings could be due to two or more alterations that balance each other out. The corticosteroid concentration in peripheral blood plasma besides being a function of supply, distribution and disposal are also affected

indirectly by the following factors: (1) diurnal variation; (2) age; (3) thyroid function; (4) renal disease; (5) pregnancy; (6) medication; and (7) blood collection methods.

Effect of Blood Collection Procedures on Corticosteroid Levels

It was important to know the effects of various blood collection procedures on the corticosteroid levels in rabbit peripheral blood plasma to procure meaningful information about adrenocortical function. Therefore, we studied the effect of two anesthetics namely ether and sodium pentabarbitol and two methods of blood collection procedures either decapitation or mild vacuum suction from the ear vein, on corticosteroid levels in the rabbit peripheral blood plasma. A significant increase ($P < 0.01$) in corticosterone levels following ether anesthesia was observed. None of the other treatments were significantly different from each other ($P > 0.25$) in corticosterone levels. Ether seems to act directly on the hypothalamic median eminence to (presumably) release corticotrophin-releasing factor (CRF) into the hypophysial portal system to activate ACTH secretion by the adenohypophysis (Greer and Rockie, 1968). This could explain our observations on increased corticosteroid levels following only ether anesthesia.

Effect of Exogenous ACTH on Corticosteroid Levels

It appears as though a distinction has to be made between two apparently different effects of corticotrophin on corticosteroid production. These are (1) a rapid effect which is evident within a few minutes (acute effects); and (2) a slow action which is evident only after several days or weeks of treatment (chronic effects).

Acute Effects of ACTH. In any condition of stress, ACTH is brought into play which in turn accelerates the steps between cholesterol and pregnenolone in the steroid biosynthetic pathway (Grant et al., 1967). We simulated "acute stress effects" on corticosteroid levels in this study by intravenous administration of ACTH.

A "doubling effect" in corticosterone level was observed at the end of half-hour post-injection. However, in humans (Grant et al., 1967) and cattle (Venkataseshu, 1965), this "doubling effect" was observed only in cortisol levels. In rabbits, corticosterone is primarily secreted by the adrenal cortex whereas both in humans and cattle, it is cortisol. The acute effects of exogenous ACTH could be due to the following factors: (1) increased rate of corticosteroid release from the cell; (2) synthesis of new enzyme protein; (3) stimulation of an enzyme reaction or reactions in the biogenic sequence; (4) regulation of rate of formation of a necessary co-factor; and (5) integration of the activities of the corticosteroid biosynthetic enzymes which are distributed between mitochondria and microsomes.

It is noteworthy that the effects of ether anesthesia in rabbits resemble that of an acute stress in that only corticosterone is elevated and not cortisol.

Chronic Effects of ACTH on Corticosteroid Levels

In our study, the chronic effects of ACTH on corticosteroid levels in rabbit peripheral blood plasma differed markedly from that of acute effects in that the "doubling effect" was observed only in the level of cortisol, but not corticosterone.

Bush (1953) has shown that for any one species the ratio

hydrocortisone/corticosterone (F/B) in the adrenal vein is relatively constant, although the variation between species was large, e.g. F/B in the sheep is greater than 20, and in the rabbit is less than 0.05. Stimulation of the adrenal by corticotrophin produces a rapid increase in steroid output which is detectable within 2-10 minutes (Hechter et al., 1951; Bush, 1953) without altering the F/B ratio. However, Kass et. al., (1954) found that in rabbits prolonged treatment with corticotrophin resulted in considerable alteration of the F/B ratio from 0.05 to 4.0. Their data clearly showed that prolonged corticotrophin treatment produced a considerable increase of hydrocortisone and decrease of corticosterone. The observation that during this corticotrophin treatment there was a two- to three-fold increase in the adrenal weight would suggest that the alteration in corticosteroid pattern may be due to synthesis of new enzyme protein and, in particular, the 17-hydroxylating system. The observations of Grant et al., (1957) on the 11β-hydroxylating activity of human adrenal homogenates would seem to fall into the group of long-term corticotrophin effects.

Grant et al. (1967) have developed a concept of zonation of steroid hormones in the adrenal cortex. Briefly, aldosterone is formed by Zona glomerulosa, while Zona fasciculata and Zona reticularis constitute a single functioning unit in which the "clear cells" of Zona fasciculata act as a storage depot for steroid precursors. The "compact cells" of Zona reticularis form mainly corticosteroids from immediate precursors brought to the cells by the blood.

In conditions of acute stress or immediately after ACTH injections, steroid precursors in the "clear cells" are utilized for hormone production and the cells become "compact" in type. ACTH had little effect

in increasing steroid production in vitro by adrenal slices composed of "compact cells", whereas it had a marked effect on "clear cell" slices (Grant et al., 1967). The short term steroidogenic effect of the trophic hormone is directed to steroid precursors in the interface clear cells and results in immediate productions and liberation of steroid hormones, mainly corticosteroids. The main effect of ACTH on the "compact cell" is long term. It is associated with an increase in protein synthesis and results in the cell having hydroxylating enzyme systems required to synthesize steroid hormones from precursors.

Fevold (1967) confirmed the existence of a Δ^5-pregnenolone-17α-hydroxylase pathway which is preferentially stimulated by long term ACTH administration for cortisol formation in rabbits. A concomitant decrease in corticosterone level was not observed in our study with the increased cortisol level in the rabbit peripheral blood plasma and agrees with the findings of Yudeav and Anfinogenova (1960) and Krum and Glen (1965). Neither reported that there was more cortisol than corticosterone secreted as originally found by Kass and co-workers (1954).

Application to Clinical Conditions

The studies on the corticosteroid levels in pathological states were primarily carried out to check the applicability of our extended fluorometric method in routine clinical investigation. Using a spectrophotometric method which involved paper chromatography and required large volumes (one-half to one liter) of blood for analysis, we could quantitate corticosteroid levels only in three cattle in three weeks in the porphyric study. Whereas, applying our extended fluorometric method

we were able to quantitate both cortisol and corticosterone from two ml of blood serum from 23 animals in a study involving PSS suspected swine in less than two weeks.

As pointed out earlier in the review of literature, any method used for the estimation of corticosteroids in clinical investigation, regardless of their simplicity, must satisfy the requirements of specificity, reproducibility and sensitivity. Other points to be considered in the application of a method in routine work are rapidity, and cost. In most clinical investigations, the amount of biological material that is available for corticosteroid quantitation is very small. The drawback of ultraviolet spectrophotometric methods can be readily seen in our study with porphyric cattle where one-half to one liter of blood had to be collected from each animal to enable us to quantitate corticosteroid levels. If the concentration of corticosteroids have to be determined in blood from subjects with low adrenal function, the brief fluorometric methods can be used, but, as discussed, the higher blank values due to the non-specific fluorogens in these methods may give erroneous results.

It appears that a variety of chronic diseases are associated with changes in adrenocortical function. If adrenocortical abnormalities are primary, several potential sites of disturbed function might be predicted. These include the hypothalamic driving mechanism and its feedback control; pathways of steroidogenesis, with alternative routes leading to the production of abnormal and potentially harmful metabolies; altered protein binding of steroids; altered catabolism; altered peripheral metabolism; and a change in renal handling of corticosteroids. The overall picture of adrenocortical function could be obtained with fair amount of accuracy by investigating the

corticosteroid levels in the peripheral blood which is the sum of supply, distribution, and disposal.

Our simple, precise, sensitive and specific method for the simultaneous determination of cortisol and corticosterone should be applicable to many species and will allow the routine investigation of adrenocortical function in many experimental and clinical conditions.

CHAPTER VI

SUMMARY AND CONCLUSIONS

In this study, a fluorometric method has been developed for the simultaneous determination of cortisol and corticosterone in biological samples. This method appears to be simple, precise, sensitive, highly specific and applicable to both experimental and clinical conditions. Two important aspects of the EFM merit closer consideration. The first concerns the separation of interfering fluorescing substances from the steroid compounds and the second, simultaneous determination of cortisol and corticosterone in the same biological sample.

The background fluorescence due to the non-specific interfering fluorogens were completely eliminated in the EFM as follows: (1) by pre-extraction of plasma with iso-octane removing lipids less polar than C-21 and C-19 steroids; (2) by alkali wash eliminating estrogens, as well as phenolic and acidic steroids; (3) by thin-layer chromatography resulting in chromatographic elimination of polar and non-polar, non-steroidal fluorogens; (4) by using minimal volumes of plasma; (5) by extensive washing of silica gel and glassware as well as careful checks on purity of reagents; (6) by using tritiated standards with higher specific activities compared to C^{14} labelled standards for monitoring losses through the method; (7) by using an acid-alcohol mixture with a 65:35 ratio; (8) by using a primary and secondary filter combination that was less sensitive but more specific; and (9) by using 10%

potassium oxalate instead of heparin as an anti-coagulant since the latter interfered with fluorescence measurement (Werk, Jr. and co-workers, 1967).

The mean cortisol and corticosterone levels in rabbit peripheral blood plasma were 1.80 and 7.70 μg%, respectively, as determined by EFM. The brief method of Guillemin et al. and that of van der Vies used in comparison with EFM demonstrated an error of 92% and 36%, respectively, in precision of estimating corticosterone levels. Failure to incorporate a chromatographic purification step to eliminate non-steroidal fluorogens as in our EFM, could explain the upward bias observed in the corticosteroid levels obtained in the brief methods.

An experiment designed to test the effects of various blood collection procedures in the corticosteroid levels in rabbit peripheral blood plasma applying EFM, demonstrated a significant increase ($P < 0.01$) in corticosterone levels following ether anesthesia and this agrees with the observations of Greer and Rockie (1968) in rats. Further, applicability of the EFM was demonstrated in experiments conducted to see the response to acute and chronic effects of exogenous ACTH on corticosteroid levels in rabbit peripheral blood plasma. Our results demonstrate conclusively that ACTH results in the elevation of corticosterone, but not cortisol, in peripheral circulation of rabbits within 30 minutes after injection.

In response to chronic effects of ACTH, we observed a marked increase in cortisol level (3.10 μg% more compared to that of controls) in the peripheral blood plasma of rabbits. We failed to observe a concomitant decrease in corticosterone level, along with the increase in cortisol level, in the rabbit peripheral blood plasma unlike in the

adrenal vein blood of these animals as observed by Kass and co-workers (1954).

EFM was utilized to assess the corticosteroid levels in our studies with swine suspected of a porcine stress syndrome, while an ultraviolet spectrophotometric method was utilized in a study with porphyric bovine.

The comparison as to the number of animals surveyed, time required and information obtained is striking and demonstrates the suitability of EFM as a clinical diagnostic tool.

The EFM not only separates the interfering substances from the steroid compounds, thus increasing the specificity and precision, but also allows a simultaneous fractionated determination of cortisol and corticosterone. Besides having a high degree of precision, sensitivity and specificity, the present method has been thoroughly validated both physiologically as well as physico-chemically.

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A P P E N D I X

TABLE XIX

ANALYSIS OF VARIANCE OF EFFECT OF ANESTHETIC ON PLASMA CORTICOSTERONE LEVELS

Source of Variance	df	ss	ms	F ratio
Mean	1	1,528.96	1,528.96	
Treatments	3	129.66	43.22	10.69
Error	20	80.73	4.04	
TOTAL	24	210.39	8.77	

TABLE XX

ANALYSIS OF VARIANCE OF EFFECT OF ANESTHETIC ON PLASMA CORTISOL LEVELS

Source of Variance	df	ss	ms	F ratio
Mean	1	102.13	102.13	
Treatments	3	2.30	0.76	2.92
Error	20	5.28	0.26	
TOTAL	24	7.58	0.31	

TABLE XXI

ANALYSIS OF VARIANCE OF EFFECT OF ANESTHETIC ON PLASMA F/B RATIO

Source of Variance	df	ss	ms	F ratio
Mean	1	2.01	2.01	
Treatments	3	0.06	0.02	2.35
Error	20	0.17	0.0085	
TOTAL	24	0.23	0.0095	

TABLE XXII

COMPARISON OF RANKED MEANS OF DIFFERENT TREATMENTS BY DUNCAN MULTIPLE RANGE TEST

Ranked Means		P=2	P=3	P=4
Treatment	rp	2.95	3.10	3.18
	Mean	2.42	2.54	2.61
Ether	11.87	4.24*	5.49*	5.83*
Vacuum**	7.63	1.25	1.59	
Sod. Pentabarb.**	6.38	0.34		
Decapitation**	6.04			

*Ether is significantly different from all other treatments ($P<0.01$).

**Vacuum, sod. pentabarb., and decapitation were not significantly different from each other ($P>0.25$).

TABLE XXIII

REGRESSION EQUATION FOR THE CORTICOSTERONE STANDARD CURVE

$\Sigma Y = 0.684$	$\Sigma X = 431.0$
$\Sigma Y^2 = 0.044$	$\Sigma X^2 = 17,815.0$
$(\Sigma Y)^2 = 0.468$	$(\Sigma X)^2 = 185,761.0$

$\Sigma xy = 27.9$

$bxy = 0.0015$

$\hat{Y} = 0.0456 + 0.0015\ (X - 28.73)$

$\hat{Y} = 0.0456 + 0.0015X - 0.043$

$\hat{Y} = 1.5X + 2$

TABLE XXIV

REGRESSION EQUATION FOR THE CORTISOL STANDARD CURVE

$\Sigma Y = 0.684$	$\Sigma X = 243$
$\Sigma Y^2 = 0.044$	$\Sigma X^2 = 5,783$
$(\Sigma Y)^2 = 0.468$	$(\Sigma X)^2 = 59,049$

$\Sigma xy = 15.9$
$bxy = 0.001$

$\hat{Y} = 0.045 + 0.001\ (X - 16.20)$

$\hat{Y} = 0.045 + 0.001X - 0.0162$

$\hat{Y} = 0.001X + 0.029$

$\hat{Y} = 1X + 2.9$

VITA

Ganjam Kuppuswamy Venkataseshu

Candidate for the Degree of

Doctor of Philosophy

Thesis: A MICROMETHOD FOR THE SIMULTANEOUS DETERMINATION OF CORTISOL AND CORTICOSTERONE AND ITS APPLICATION TO EXPERIMENTAL AND CLINICAL CONDITIONS

Major Field: Physiology

Biographical:

Personal Data: Born in Madras, India, July 6, 1940, the son of Ganjam Kuppuswamy and Padmavathi Kuppuswamy.

Education: Attended elementary and secondary school in Tirupati, India; graduated from S. V. High School, Tirupati, India, in 1954; received Fellow of Arts and Science degree from Andhra University, India, in 1956; received the degree of Bachelor of Science in Zoology, in 1958; and the degree of Bachelor of Veterinary Science, in 1962, from Sri Venkateswara University, Tirupati, India; received the degree of Master of Science in Dairy Science from Washington State University, Pullman, Washington, in 1965; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1970.

Professional Experience: Held the positions of Diagnostic Assistant in Pathology, and Assistant Lecturer in Surgery at the School of Veterinary Medicine, Sri Venkateswara University, Tirupati, India, from June, 1962 to November, 1962; worked as a graduate research assistant from November, 1962 to December, 1965 at the Department of Dairy Science, Washington State University, Pullman, Washington; and from January, 1966 to September, 1969, worked as a graduate research assistant at the Department of Physiology and Pharmacology, Oklahoma State University, Stillwater, Oklahoma.