STUDIES ON A NOVEL STEROID RADIOIMMUNOASSAY AND ITS APPLICATION TO THE NEONATAL DETECTION OF CONGENITAL ADRENAL HYPERPLASIA

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DECLARATION

The work presented in this thesis was performed solely by the author, with the exception of areas of acknowledged collaboration.

PUBLICATIONS

1. **Thomson, S.** Development of a novel androstenedione radioimmunoassay and its application to the detection of congenital adrenal hyperplasia. Medical Laboratory Sciences 1987; **44**(2): 196.

2. Egan SM, Betts P, **Thomson S**, Wallace AM and Wood PJ. A blood spot androstenedione assay suitable for home monitoring of steroid replacement therapy in congenital adrenal hyperplasia. Annals of Clinical Biochemistry 1989; **26**: 262-267.

3. **Thomson S,** Wallace AM and Cook B. A 'blood spot ' androstenedione radioimmunoassay able to detect congenital adrenal hyperplasia. Journal of Inherited Metabolic Disease 1989; **12**(2): 318-320.

4. **Thomson S,** Wallace AM and Cook B. Development of a radioimmunoassay employing a [^{125}I] radioligand for measurement of androstenedione in both human serum and neonatal blood spot samples. Clinical Chemistry 1989; **35**/8: 1706-1712.

SUMMARY

Congenital adrenal hyperplasia (CAH) is the commonest adrenal disorder in childhood (worldwide incidence is 1:14,554) and because it is potentially fatal, early detection is necessary. Mass screening of newborn populations for other diseases, such as congenital hypothyroidism, phenylketonuria and galactosaemia, has been established based on blood samples collected onto filter paper (Guthrie cards). Elevated concentrations of the steroid, androstenedione (A4), occur in untreated, classical cases of CAH, and are modestly raised in late-onset cases. Some increase may also occur in polycystic ovarian disease. Several groups throughout the world have added screening (or pilot screening studies) for 21-hydroxylase deficiency, the most common form of congenital adrenal hyperplasia, to their existing neonatal programmes. From 1984, this was the case in Scotland but, due to the lack of central funding, screening was reluctantly stopped in 1986. The CAH screening programmes are based on the measurement of another steroid, 17-hydroxyprogesterone (17-OHP), also elevated in untreated cases. However, A4 determination offers greater potential as a diagnostic aid because it also can detect the second most common form of CAH, 11β-hydroxylase deficiency. In addition, A4 measurement may be a better indicator of the efficacy of glucocorticoid replacement therapy in CAH patients than 17-OHP measurement.

Unfortunately, because most A4 immunoassays are based on a tritiated label, they are cumbersome, expensive and too insensitive to allow measurement in filter paper blood-spot samples. This makes them unsuitable for large-scale screening and home-monitoring of replacement therapy in patients with CAH.

The aim of this investigation was to produce a sensitive, specific, precise and robust immunoassay to measure A4 in serum and blood eluted from filter paper samples. Antisera were raised in rabbits against A4 linked to bovine serum albumin (BSA) at positions 3, 6 or 11 on the steroid nucleus. Gamma-emitting labels were prepared by linking ^{125}[I]-iodohistamine at positions 3, 6 or 11 on the steroid nucleus. Linkages were through either carboxymethyloxime (CMO) or hemisuccinate (HS) bridges. A major part of the work involved the selection of the best combination of A4 antibody and A4 label. Separation of antibody bound and free fraction was achieved by centrifugation of microencapsulated antibody or centrifugation after incubation with a second antibody.

An antiserum raised against A4-CMO-BSA used with an A4-CMO-^{125}[I]-iodohistamine tracer was selected, on the basis of sensitivity and specificity, as the best combination of reagents. The performance of these reagents in an immunoassay for A4 extracted from serum or eluates of neonatal dried blood-spot samples was evaluated. A direct, non-extraction blood-spot immunnoassay was also evaluated to facilitate large-scale screening for CAH. Concentrations of A4 in serum under normal and pathological conditions, such as CAH and polycystic ovarian disease were investigated. Androstenedione was measured in blood spots from neonates born at term or prematurely, with respiratory distress syndrome, or with CAH.

For the serum assay, the range of the standard curve was 1-21 nmol/l of A4. The coefficients of variation of the within- and between-batches were <10% for low, medium and high serum pools. Mean analytical recovery of tritiated androstenedione or unlabelled androstenedione, which had been added to sera before solvent extraction, was >95% in both cases. Measurements of androstenedione in dilutions of sera from patients with increased A4 concentrations exhibited parallelism with the standard curve.

Using this immunnoassay, concentrations of A4 in patients' sera showed good agreement with concentrations found with the routine method which employs a tritiated tracer. The correlation coefficient was 0.94 with a slope of 1.02 and an intercept of 0.9 nmol/l of A4. Results for samples from the U.K. National External Quality Assessment Scheme compared well with those from the other participating laboratories. The bias was +12.2% and the variability of bias was 6.9% over a six month period.

Serum androstenedione concentrations were similar in men and women and both groups showed a reduction with age. For men and women aged 18-40, the normal range for A4 (nmol/l) was 1.6-8.4 (x=5.0; n=78) and 0.6-8.8 (x=4.7; n=70), respectively. For men and women aged 41-65, the normal range was 1.2-6.6 (x=3.9; n=21) and 0.9-6.8 (x=3.9; n=32), respectively. Diurnal variation in a group of normal male volunteers was demonstrated by a marked early morning peak. Androstenedione concentrations were raised in sera from patients with CAH. They were grossly increased in an untreated neonate and in a twelve-year-old receiving inadequate steroid replacement therapy and modestly raised in a late-onset case.

Mean analytical recovery of androstenedione from neonatal blood spots was >99% with or without solvent extraction. For the extraction and direct blood-spot assays, the range of the standard curves was 4-525 and 5-300 nmol/l of A4, respectively. For four quality control blood spots, the mean inter-assay coefficient of variation was <10 and <16% and the mean intra-assay was <10 and <13%, using the extraction and direct methods, respectively.

The normal reference interval for androstenedione for infants born at term was <4-22 and <5-88 nmol/l with or without solvent extraction, respectively. Higher concentrations of A4 were found in the direct assay than in the extraction method. Whether measuring directly or after solvent extraction, A4 concentrations were raised in some samples from infants born prematurely or who were suffering from respiratory distress syndrome and in all cases of CAH (including one case of 11β-hydroxylase deficiency).

This thesis describes reagent production and performance of a sensitve, specific and robust radioimmunnoassay of androstenedione employing a [^{125}I]-labelled ligand which is suitable for measuring A4 in both serum and neonatal blood-spot samples.

ABBREVIATIONS

A4	Androstenedione (4-Androsten-3,17-dione)
A4-3CM0	Androstenedione-3-carboxymethyloxime
A4-6βHS	Androstenedione-6β-hemisuccinate
A4-11αHS	Androstenedione-11α-hemisuccinate
A4-3CMO-BSA	Androstenedione-3-carboxymethyloxime-Bovine Serum Albumin
A4-6βHS-BSA	Androstenedione-6β-hemisuccinate-Bovine Serum Albumin
A4-11αHS-BSA	Androstenedione-11α-hemisuccinate-Bovine Serum Albumin
Ab	Antibody
Ab-2	Second antibody
ANS	δ-Anilino-1-napthalene sulphonic acid
BSA	Bovine Serum Albumin
C	Carbon
^{o} C	degrees Celcius
CAH	Congenital Adrenal Hyperplasia
Ci	Curie
cm	centimetre
CMO	Carboxymethyloxime
cpm	counts per minute
CR	Cross Reaction
CV	Coefficient of Variation
Danazol	17-pregn-4-en-20-yno(2,3-d)isoxazol-17-ol
DAR	Donkey Anti-Rabbit

ABBREVIATIONS CONTINUED

EPPS	N-(2-hydroxyethyl)-piperazine-N^1-3-propanesulphonic acid
g	gram
[^{3}H]	radioisotope of hydrogen
HLA	Histocompatibility complex
HS	Hemisuccinate
Hz	hertz
[^{125}I]	radioisotope of iodine
k	kilo- x 10^3
l	litre
m	milli- x 10^{-3}
min	minute
mm	millimetre
mol	moles
n	nano- x 10^{-9}
NRS	Normal Rabbit Serum
17-OHP	17-Hydroxyprogesterone
p	pico x 10^{-12}
PKU	Phenylketonuria
RIA	Radioimmunoassay
rpm	revolutions per minute
s	second
SD	Standard Deviation

ABBREVIATIONS CONTINUED

S.E.M.	Standard Error of Mean
SHBG	Sex Hormone Binding Globulin
TLC	Thin Layer Chromatography
μ	micro- x 10^{-6}
v/v	volume/volume
w/v	weight/volume

EQUATIONS

a) percentage cross reaction in cross reaction studies

b) percentage of total label bound in the construction of dilution curves

c) percentage of maximum label bound in the construction of dose-response curves.

$$\text{a) \%CR} = \frac{\text{Mass of steroid S required to displace 50\% of labelled S}}{\text{Mass of steroid X required to displace 50\% of labelled S}} \times 100$$

$$\text{b) \%B/T} = \frac{\text{Bound labelled ligand (cpm)}}{\text{total counts (cpm)}} \times 100$$

$$\text{C) \%B/Bo} = \frac{\text{Bound labelled ligand (cpm)}}{\text{Maximum bound labelled ligand (cpm) (zero standard)}} \times 100$$

AIM OF THIS STUDY

The aim of this study was to produce an androstenedione antibody and a γ-emitting androstenedione label suitable for a RIA of androstenedione both in serum and in eluates from neonatal dried blood spot specimens.

To achieve this, the main objectives were to:

1. Raise antisera in rabbits against a selection of androstenedione conjugates.
2. Prepare a selection of androstenedione radioligands.
3. Compare different combinations of label and antibody.
4. Test antisera for cross reactions with steroids similar to androstenedione.
5. Select the best combination of label and antibody suitable for an androstenedione radioimmunoassay.
6. Develop a radioimmunoassay to measure androstenedione in serum.
7. Develop a radioimmunoassay to measure androstenedione in dried blood spots.
8. Evaluate the developed radioimmunoassays.
9. Compare the serum androstenedione results with the 'routine' serum radioimmunoassay based on a tritium label.
10. Clinically validate the developed radioimmunoassays.

CHAPTER 1 INTRODUCTION

1.1. THE ADRENAL GLAND

1.1.1. Anatomy

The adrenal glands are small paired, endocrine organs, with a combined weight of 8 - 10 g in the adult. They are located above the kidneys (Fig. 1.), and are divided into a central portion (medulla), responsible for the production of adrenaline and nor-adrenaline, and an outer portion (cortex), the site of synthesis of mineralocortcoids, glucocorticoids and adrenal androgens. There are three histologically defined zones of the adrenal cortex, the zona glomerulosa, the zona fasciculata and the zona reticularis. In man, the outer zona glomerulosa consists of islets of cells immediately beneath the capsule, so that the underlying zona fasciculata extends in numerous places to the capsule. The zona fasciculata comprises the bulk of the cortex and consists of regular columns of large, cholesterol-laden cells which appear vacuolated under the light

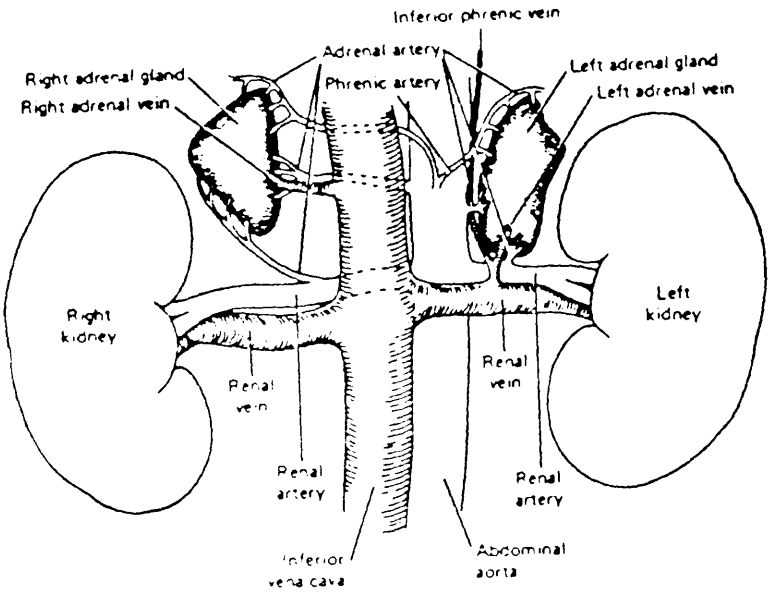


FIG. 1. Location of adrenal glands.

microscope. The zona reticularis is the zone nearest the medulla and consists of slightly smaller, more compact cells. The zona glomerulosa specifically synthesises aldosterone while the cells of both the zona fasciculata and zona reticularis synthesise glucocortoids and androgens. It has been suggested[1] that the zona fasciculata and zona reticularis represent different morphological forms of the same functional unit.

1.1.2. Physiology

The chief action of the mineralocorticoids (e.g. aldosterone and deoxycorticosterone) is on the kidney tubules, where they promote the retention of sodium (and water) and stimulate excretion of potassium. Aldosterone secretion is primarily regulated via the renin-angiotensin system, which is responsive to the blood volume (Fig.2.). Aldosterone secretion may also be stimulated by serum potassium concentration and adrenocorticotrophic hormone (ACTH).

The glucocorticoids (e.g. cortisol and 11-deoxycortisol) play a major role in carbohydrate, protein and fat metabolism. In addition, they are important in the body's response to stress, are anti-inflammatory and can inhibit the normal immunological response. Cortisol enhances hepatic gluconeogenesis, the formation of glucose from non-carbohydrate precursors such as amino acids. This results initially in increased deposition of glycogen in the liver. Any excess glucose synthesised enters the bloodstream and may produce hyperglycaemia. Cortisol also antagonises the peripheral action of insulin on glucose uptake, enhancing hyperglycaemia. The cortisol response to physical and physiological stress is extremely important and, if absent, collapse and even death may result from circulatory failure.

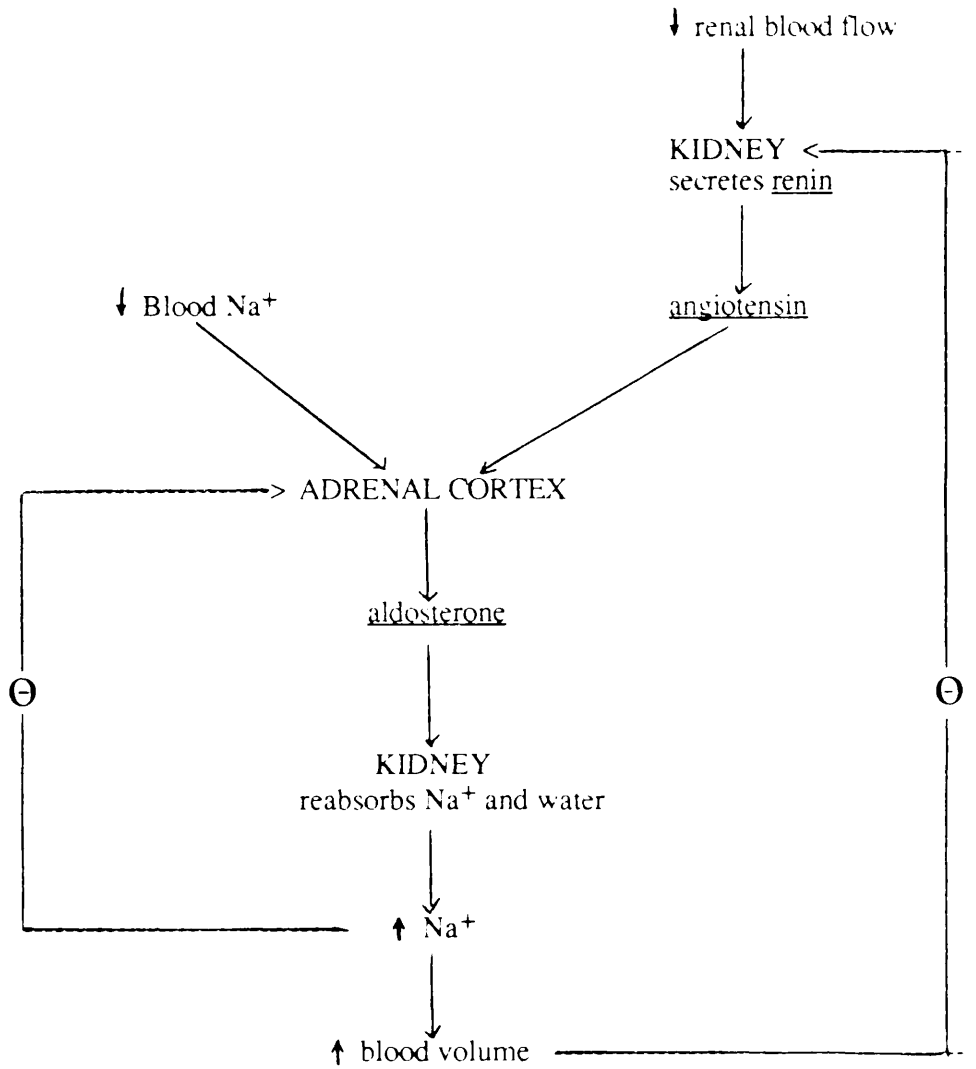


Fig. 2. Regulation of aldosterone secretion.
(↑) Increased. (↓) Decreased. (Θ) Inhibition.

The androgens are responsible for the development of phenotype and normal sexual function of the male. The most abundant circulating androgen is dehydroepiandrosterone sulphate, which is almost exclusively of adrenal origin. The testes, and to a lesser extent the ovaries, produce quantitatively more C_{19} androgens, including androstenedione (A4), and minor amounts of C_{18} oestrogens than the adrenal cortices of either sex. The adrenal androgens, dehydroepiandrosterone, dehydroepiandrosterone sulphate and androstenedione have minimal intrinsic androgenic activity. However, they contribute to androgenicity by their peripheral conversion to the more potent androgens, testosterone and dihydrotestosterone. Although, dehydroepiandrosterone and dehydroepiandrosterone sulphate are secreted in greater quantities, androstenedione is qualitatively more important, since it is more readily converted, peripherally, to testosterone. In males, conversion of androstenedione to testosterone accounts for less than five percent of the production rate of the latter hormone, and thus the physiological effect is negligible. In adult males, excess adrenal androgen secretion has no clinical consequences; however, in boys it causes premature penile enlargement and early development of secondary sexual characteristics. In females, ovarian androgen production is low, thus the adrenal substantially contributes to total androgen production by the peripheral conversion of dehydroepiandrosterone to androstenedione. Abnormal adrenal function, resulting in excess androgen, is manifested by virilisation in females.

1.1.3. Biosynthesis of Steroid Hormones

Steroid hormones are produced from cholesterol. Cholesterol storage, as cholesterol esters in lipid droplets, is controlled by the action of two opposing enzymes, cholesterol esterase and cholesterol ester synthetase. In general, trophic hormones, eg. ACTH, stimulate the esterase and inhibit the synthetase, thus increasing the availability of free cholesterol for steroid hormone synthesis.

A simplified scheme of the steroid biosynthesis is shown in Fig. 3.

Mineralocorticoids

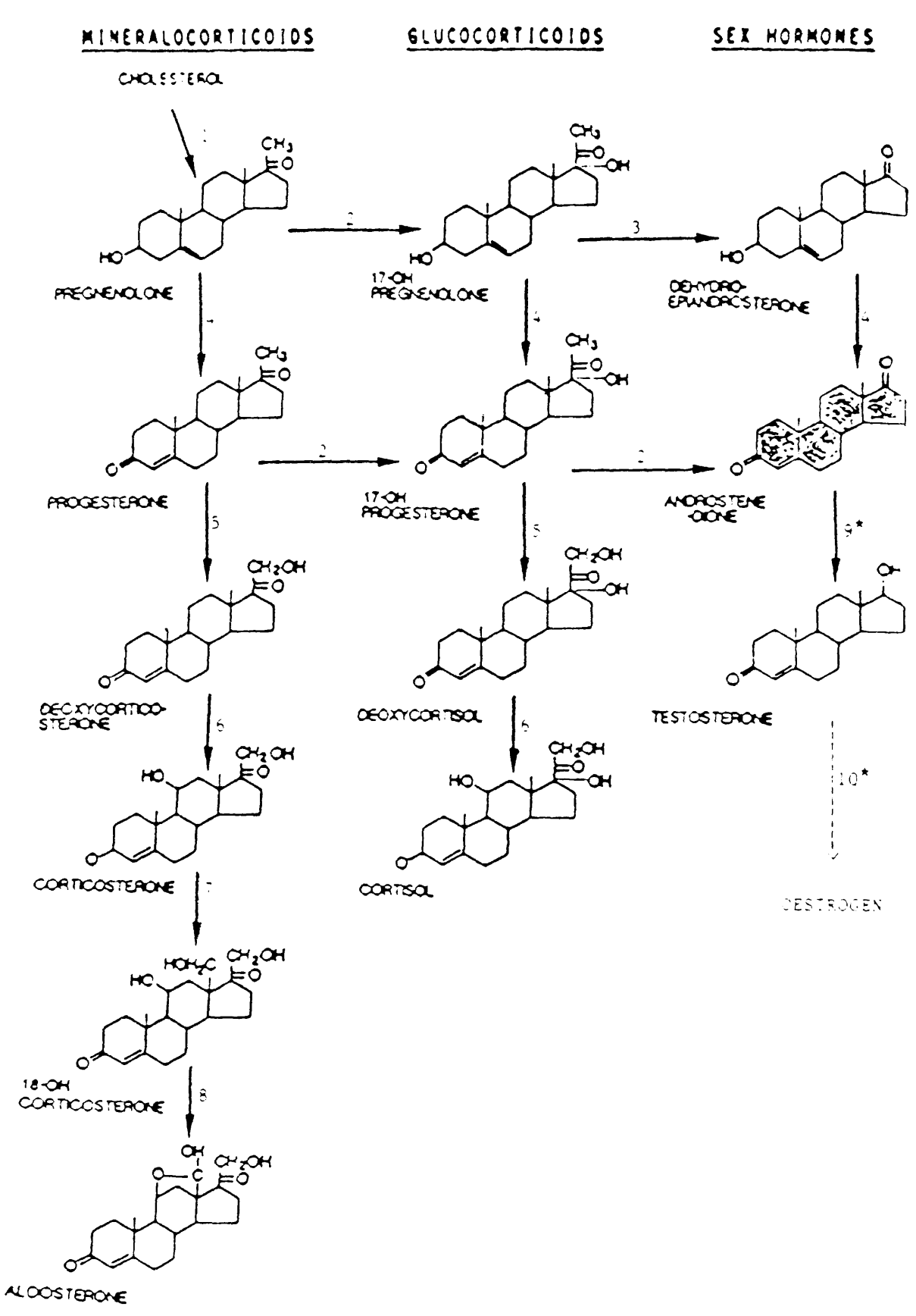
Pregnenolone, a Δ-5 steroid, is converted to a biologically active Δ-4 steroid, progesterone, by the 3β-hydroxysteroid dehydrogenase/isomerase enzyme system. The progesterone is then hydroxylated at position 21 to form deoxycorticosterone, an active sodium-retaining hormone. When deoxycorticosterone is hydroxylated at position 11α , corticosterone, a weak mineralocorticoid (but strong glucocorticoid), is formed. Corticosterone is the precursor of aldosterone, the most potent sodium-retaining hormone. Synthesis of aldosterone occurs in the zona glomerulosa, while the synthesis of corticosterone occurs further from the capsule in the zona fasciculata and zona reticularis.

Glucocorticoids

Glucocorticoid synthesis requires hydroxylation at position 17. Pregnenolone and progesterone are converted by 17-hydroxylase to 17-hydroxypregnenolone and 17-hydroxyprogesterone, respectively. Conversion of 17-hydroxypregnenolone to 17-hydroxyprogesterone is by enzymic steps similar to those that convert pregnenolone to progesterone.

Fig. 3. A simplified scheme of the traditional (and more recent) view of the biosynthesis of steroid hormones. Roman numerals indicate enzyme reactions. (*) Principally in the testes and the ovaries.

1. 17, 20 desmolase (P450scc)
2. 17α-hydroxylase (P450c17)
3. 17, 20 lyase (")
4. 3β-hydroxysteroid dehydrogenase
5. 21-hydroxylase (P450c21)
6. 11β-hydroxylase (P450c11)
7. 18-hydroxylase (")
8. 18-dehydrogenase (")
9.* 17α-steroid dehydrogenase
10.* aromatase system (P450aro)


MINERALOCORTICOIDS
GLUCOCORTICOIDS
SEX HORMONES
CHOLESTEROL
PREGNENOLONE
17-OH PREGNENOLONE
DEHYDRO-EPIANDROSTERONE
PROGESTERONE
17-OH PROGESTERONE
ANDROSTENE-DIONE
DEOXYCORTICO-STERONE
DEOXYCORTISOL
TESTOSTERONE
CORTICOSTERONE
CORTISOL
ESTROGEN
18-OH CORTICOSTERONE
ALDOSTERONE

When 17-hydroxyprogesterone undergoes 21-hydroxylation, 11-deoxcortisol is formed and this is further hydroxylated to form cortisol, the most potent glucocorticoid in man. Parallel hydroxylation steps of progesterone and 17-hydroxyprogesterone result in corticosterone and cortisol respectively.

Adrenal androgens

The main C_{19} steroid secreted by the adrenal cortex is dehydroepiandrosterone. It is produced by the side-chain cleavage of the C_{21} steroid, 17-hydroxypregnenolone, by the action of a 17,20 lyase enzyme. Dehydroepiandrosterone, a Δ-5 steroid with little androgenic activity, is converted to a Δ-4 steroid, androstenedione, a moderately active androgen, by the 3-β-hydroxysteroid dehydrogenase/isomerase enzyme system. When androstenedione is reduced at C-17, testosterone is formed.

Advances in modern protein chemistry and the application of contemporary molecular biology have combined to alter the above traditional view of steroidogenesis. Understanding of the steroidogenic enzymes was inherently limited by indirect methods of investigation eg. measurement of steroidal precursors and products in patients or in crude systems in vitro. That earlier approach indicated each steroidogenic step was mediated by multiple enzymes that differed amongst the various steroidogenic cells. The basic simplicity of the system was only appreciated when cDNAs and genes were cloned. The cloning work has now shown that the investigative emphasis in control of steroidogenesis must be shifted to the fine control of a small number of multi-purpose enzymes such as P450c11 and P450c17.

Most steroidogenic enzymes are members of the cytochrome P450 group of oxidases which contain a heme group and reduce atmospheric oxygen with electrons from NADPH. The P450 is the enzyme binding the steroidal substrate and mediating the steroidal conversion on an active site associated with the heme group. Most P450 enzymes can metabolise multiple substrates, catalysing a broad array of oxidations.

Four distinct P450 enzymes are involved in adrenal steroidogenesis[2] and their positions in the pathway are shown in Figure 2. P450scc is the side-chain cleavage enzyme mediating the series of reactions formerly termed 20,22 desmolase. P450c11 mediates 11-hydroxylase, 18-hydroxylase and 18-methyloxidase activities. P450c17 mediates both 17α–hydroxylase and 17,20 lyase activities. P450c21 mediates the 21 hydroxylations of both glucocorticoids and mineralocorticoids. In the gonads (and elsewhere) P450aro mediates aromatisation of androgens to estrogens.

1.1.4. Regulation of Adrenal Function

Glucocorticoid secretion is controlled by corticotrophin releasing factor from the hypothalamus via adrenocorticotrophic hormone from the anterior pituitary. In addition to an inbuilt circadian rhythm, there is a negative feedback control, whereby corticotrophin releasing factor, adrenocorticotrophic hormone and cortisol secretions are reduced or inhibited when plasma corticosteroid levels are too high[3] and increased when they are inappropriately low, as shown in Fig.4. With respect to regulation and secretion, the zona glomerulosa and the zona fasiculata behave as two separate glands. The zona fasciculata and zona reticularis are stimulated by adrenocorticotrophic hormone to secrete cortisol, corticosterone and adrenal androgens, by increasing the conversion of cholesterol to pregnenolone, while angiotensin stimulates aldosterone secretion by the zona glomerulosa with adrenocorticotrophic hormone probably exerting only a secondary influence on the glomerular secretion of aldosterone[4-6] (Fig.5.). The zona fasciculata lacks the enzyme necessary for the terminal step of aldosterone synthesis, whereas the zona glomerulosa lacks the 17α-hydroxylase activity required for the production of 17-hydroxycorticoids and androgens.

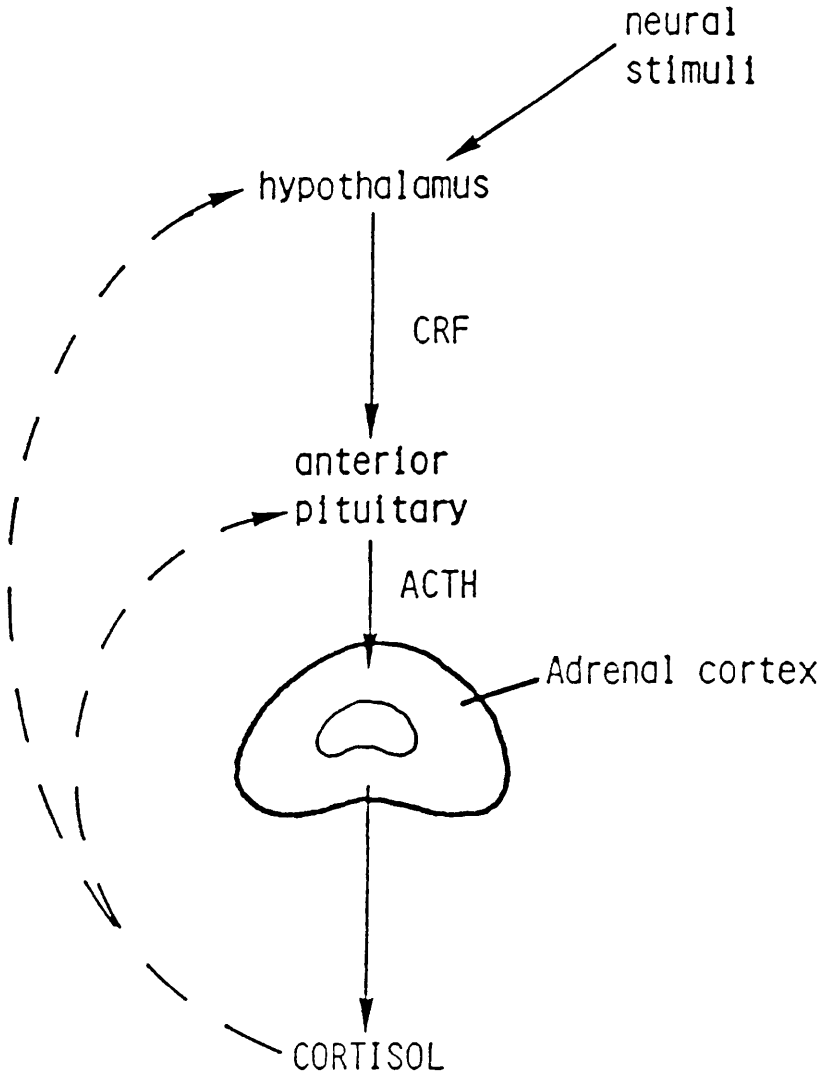
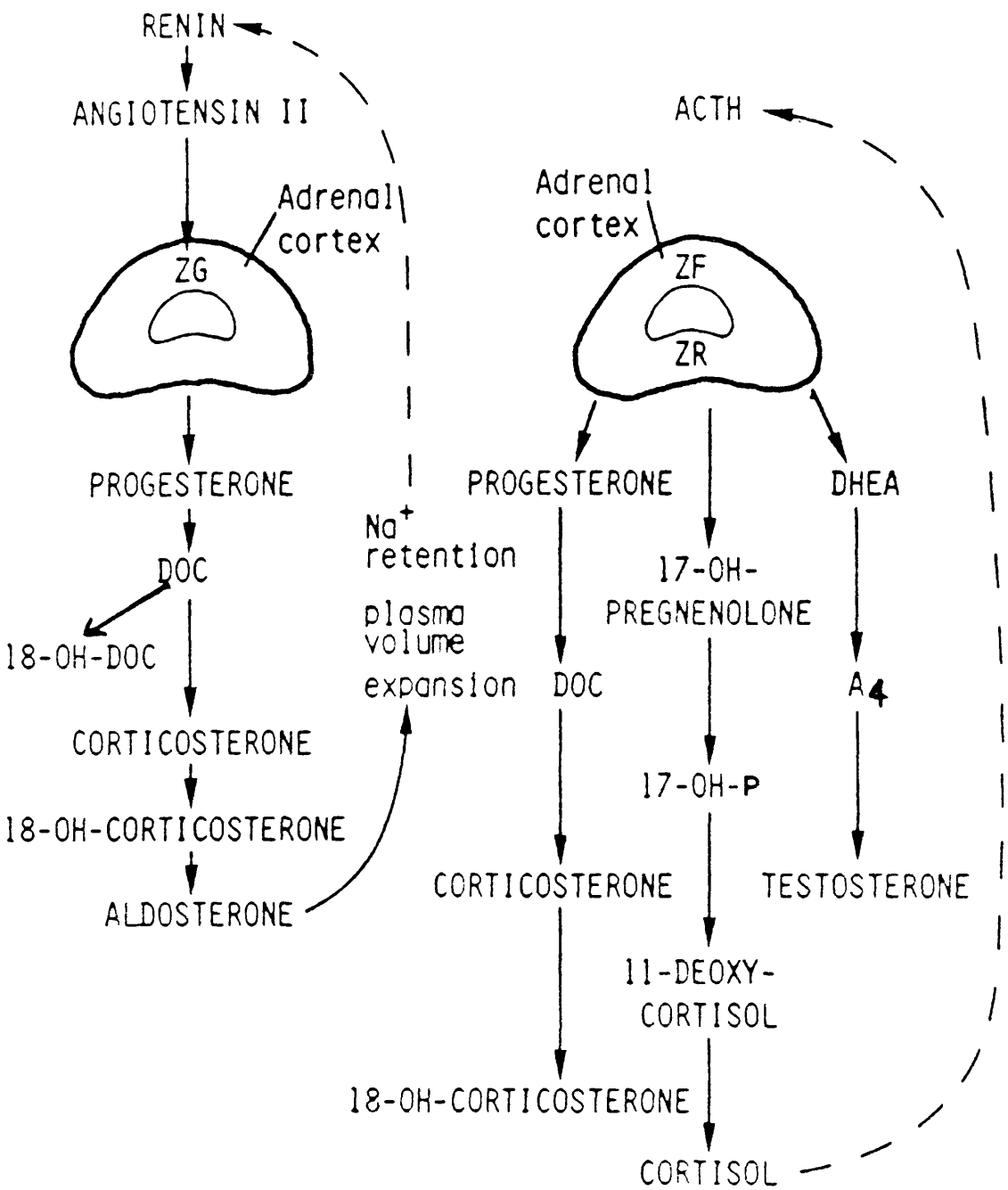


Fig. 4. Regulation of glucocorticoid secretion. (—) Stimulation. (---) Inhibition. (CRF) Corticotrophin releasing factor. (ACTH) Adrenocorticotrophic hormone.

Fig. 5. Regulation of steroid hormone biosynthesis assuming the 'two-gland' theory. (—) Stimulation. (---) Inhibition. (ZG) Zona glomerulosa. (ZF) Zona fasciculata. (ZR) Zona reticularis. (DOC) Deoxycorticosterone. (DHEA) Dehydroepiandrosterone. (17-OH-P) 17-Hydroxyprogesterone. (ACTH) Adrenocorticotrophic hormone. (A4) Adrostenedione.


RENIN
ANGIOTENSIN II
Adrenal cortex
ZG
PROGESTERONE
DOC
18-OH-DOC
CORTICOSTERONE
18-OH-CORTICOSTERONE
ALDOSTERONE
Na+ retention
plasma volume expansion
ACTH
Adrenal cortex
ZF
ZR
PROGESTERONE
DHEA
17-OH-PREGNENOLONE
DOC
A4
17-OH-P
CORTICOSTERONE
TESTOSTERONE
11-DEOXY-CORTISOL
18-OH-CORTICOSTERONE
CORTISOL

1.1.5. The Fetal Adrenal Gland

Anatomy

The adrenal glands of the fetus grow rapidly and by the end of the first trimester are equal to or greater than the size of the fetal kidney[7]. Approximately fifty percent of fetal adrenal mass consists of an inner fetal zone composed of large, oesinophilic cells producing Δ-5 steroids. The fetal zone is surrounded by the outer, definitive or adult zone which is composed of small, basophilic cells resembling those of the adult zona glomerulosa. The fetal zone atrophies within three months after birth. During the next three years, the adult adrenal cortex develops from the outer layer of the cortex and differentiates into the three adrenal zones, zona glomerulosa, zona fasciculata and zona reticularis.

Physiology

There is functional specialisation of the fetal and definitive zones of the fetal adrenal gland,[7] both of which are stimulated by adrenocorticotrophic hormone. The definitive zone produces primarily corticoids, including most fetal cortisol. The fetal zone is unable to produce cortisol due to a deficiency or block of the steroid 3β-hydroxysteroid dehydrogenase enzyme system.[9] It produces, however, dehydroepiandrosterone sulphate and other Δ-5 steroids. Fetal dehydroepiandrosterone sulphate is hydroxylated at C-16 by the fetal liver and transported to the placenta where it is desulfated before aromatisaton to oestriol. Maternal dehyroepiandrosterone sulphate either follows this route, or is transported to the placenta to be desulfated and converted by enzymic pathways common to steroid-producing tissues to androstenedione and testosterone. These androgens are aromatised by the placenta to oestrone and oestradiol, respectively.[10]

1.2. CONGENITAL ADRENAL HYPERLASIA (CAH)

The commonest adrenal disease in infancy and childhood is CAH. It was first described[11] in clinical and pathological detail in 1865, but the pathophysiology was not understood until 1950.[12,13] CAH is an inherited deficiency of one of the enzymes necessary for the biosynthesis of the principal adrenal steroid, cortisol. Decreased cortisol secretion results in uninhibited adrenocorticotrophic hormone secretion from the pituitary (Fig.6.) further stimulating the adrenal cortex leading to oversecretion of steroids before the enzyme block.

A number of inborn errors of steroid biosynthesis, characterised over the last twenty years, are shown in Table 1. Only the 21- and 11β-hydroxylase deficiencies will be discussed further, as they represent ninety-five and four percent, respectively of total CAH cases. The other deficiencies, representing one percent of total CAH cases, are rare and occur only as single case studies in the literature.

1.2.1. 21-Hydroxylase Deficiency

Impairment of 21-hydroxylation is the most common enzymatic deficiency in CAH. The most prominent feature of 21-hydroxylase deficiency in the neonate is virilisation of the female genitalia. Males with this enzyme defect may not manifest obvious genital abnormalities at birth, but without treatment will manifest progressive virilisation with precocious puberty and advanced bone age.

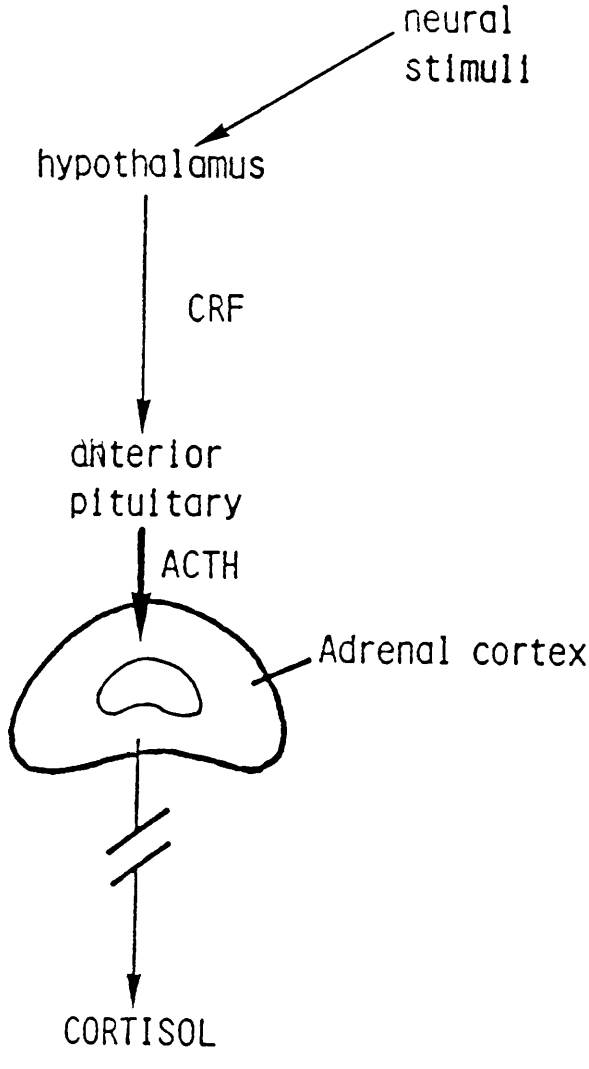


Fig. 6. Loss of cortisol feedback inhibition mechanism in congenital adrenal hyperplasia. (—) Overstimulation. (//) Enzyme block. (—) Stimulation. (CRF) Corticotrophin releasing factor. (ACTH) Adrenocorticotrophic hormone.

TABLE. 1. Clinical features and abnormal hormone levels resulting from adrenal enzyme defects.

CLINICAL FEATURES						BIOCHEMISTRY						
sexual ambiguity ♀	sexual ambiguity ♂	SW	hyper-tension	virilisation	enzyme defect	% of total CAH	ALD	17-OHP	A_4	DHEA	T	R
+	-	-	-	+	21-hydroxylase SV	95	N	▲ ▲	▲▲	N/▲	▲	N/▲
+	-	+	-	+	21-hydroxylase SW		▼	▲ ▲	▲▲	N/▲	▲	▲▲
+	-	-	+	+	11β -hydroxylase	4	▼	▲	▲▲	▲	▲	▼▼
+	+	+	-	-	3β -HSD	1	▼	N/▲	N/▲	▲▲▲	N/▼	▲
-	+	-	+	-	17α -hydroxylase		▼	▼	▼	▼	▼	▼
-	+	+	-	-	cholesterol desmolase		▼▼	▼	▼	▼	▼	▲
-	-	+	-	-	18-hydroxylase		▼▼	N	N	N	N	▲

(SW) Salt-wasting. (SV) Simple-virilising. (ALD) Aldosterone. (T) Testosterone.

(R) Renin. (17-OH-P) 17-Hydroxyprogesterone. (N) Normal. (▲) Increased levels.

(▼) Decreased levels. (A_4) Androstenedione.

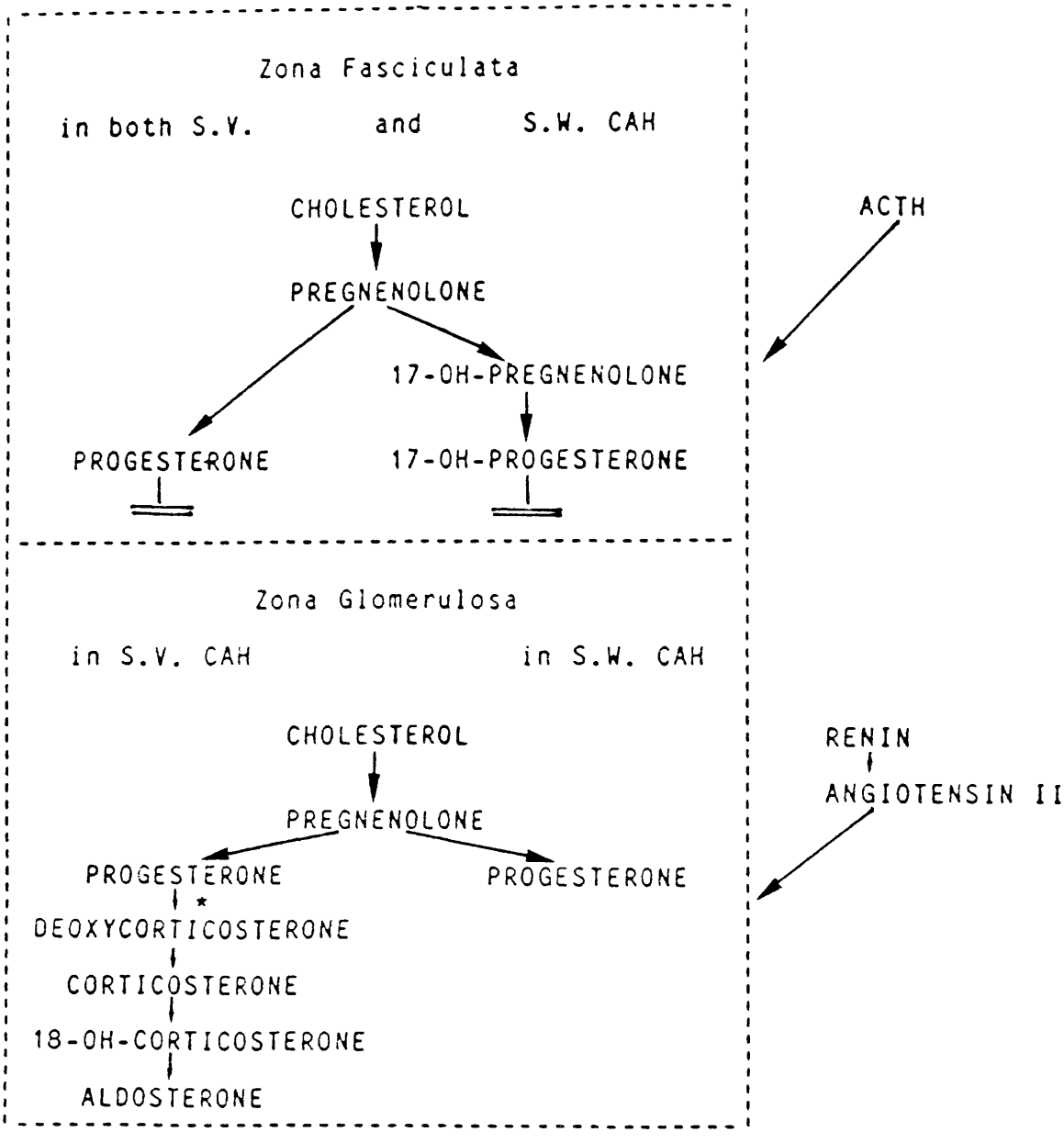
Adrenocortical function begins in the third month of gestation, a critical time for sexual differentiation. If at this time, female fetuses are exposed to high levels of androgen, as in CAH, they will manifest virilisation of the external genitalia but have a normal reproductive tract.[14] In the male, the normal source of androgen is the fetal testis, but androgen from the adrenal or exogenous sources can cause subtle changes of the external genitalia eg. pigmentation of the scrotum or slight enlargement of the penis.

A salt-wasting form of CAH is found in approximately fifty to seventy-five percent of the cases of 21-hydroxylase deficiency. In addition to virilisation, there is profound aldosterone deficiency. This salt-wasting form of 21-hydroxylase CAH may present with a life-threatening crisis within the first few weeks of life and, therefore, must be recognised soon after birth to allow replacement therapy to be initiated.

A hypothesis to explain why some cases of CAH are salt-wasting while others are only virilising has been recently suggested.[15] It states that in both simple virilisers and salt-wasters, there is a fasciculata defect of 21-hydroxylation in both the 17-hydroxy and 17-deoxy pathways. However, in the salt-waster there is a defect in 21-hydroxylation in the glomerulosa, while in the simple viriliser the glomerulosa is spared this defect (Fig.7.), thus allowing normal mineralocorticoid production.

Fig. 7. Pathway of steroid biosynthesis in the simple-virilising (S.V.) and salt-wasting (S.W.) forms of congenital adrenal hyperplasia due to 21-hydroxylase deficiency.

(=) Enzyme block. (*) 21-hyroxylase enzyme.
(ACTH) Adrenocorticotrophic hormone.


Zona Fasciculata
in both S.V. and S.W. CAH
CHOLESTEROL
PREGNENOLONE
17-OH-PREGNENOLONE
PROGESTERONE
17-OH-PROGESTERONE
ACTH
Zona Glomerulosa
in S.V. CAH
in S.W. CAH
CHOLESTEROL
PREGNENOLONE
PROGESTERONE
PROGESTERONE
DEOXYCORTICOSTERONE
CORTICOSTERONE
18-OH-CORTICOSTERONE
ALDOSTERONE
RENIN
ANGIOTENSIN II

Non classical symptomatic (late-onset) CAH is a symptom characterised by virilisation, menstrual disturbances and endocrinological features consistent with 21-hydroxylase deficiency that presents in later childhood or adolesence[16-18]. Unlike females born with classical 21-hydroxylase deficiency, females with late-onset 21-hydroxylase deficiency demonstrate no evidence of virilisation in utero. Both types of patients respond similarly to glucocorticoid treatment.

The late presentation of a biochemical defect has raised the question as to whether this is the same inherited disorder as CAH with delayed presentation, or is an aquired disorder distinct from CAH. There is a close genetic linkage between the gene responsible for 21-hydroxylase deficiency and HLA, the major histocompatability complex in humans[19,20]. Reports have provided evidence that late-onset CAH is in fact also genetically linked to HLA[21-23] and it has been proposed that classical and late-onset 21-hydroxylase deficiencies are allelic variants.[18,22,23] In patients with cryptic 21-hydroxylase deficiency, hormonal abnormalities are similar to late-onset but without clinical stigmata.

1.2.2. 11β-Hydroxylase Deficiency

CAH due to 11β-hydroxylase deficiency is a much rarer disorder than 21-hydroxylase deficiency, accounting for approximately four percent of all CAH cases diagnosed.[24-26] This form of CAH was recognised in the early 1950's by the presence of hypertension in addition to virilisation.[27,28] The defect in 11β-hydroxylation in an affected individual was demonstrated[29,30] several years later. Subsequent reports[31-34] have further documented the clinical and biochemical abnormalities in such patients.

An 11β-hydroxylase defect in the zona fasiculata results in decreased cortisol biosynthesis and induction of adrenocorticotrophic hormone hypersecretion, which in turn, causes overproduction of androgens and steroid precursors prior to the enzyme block, notably 11-deoxycortisol and deoxycorticosterone. The latter steroid is reponsible for the hypertension feature.[35-37] The enzyme is also necessary in the zona glomerulosa to convert deoxycorticosterone to corticosterone and hypoaldosteronism can usually be documented biochemically but is asymptomatic.[38] The cause of hypoaldosteronism cannot be attributed exclusively to a biosynthetic block, but is also secondary to the suppression of the renin-angiotensin system by the mineralocorticoid effect of deoxycorticosterone.[39,40]

Virilisation is found in affected individuals and results from an overproduction of androstenedione with enhanced conversion to testosterone.[41,42] The levels of these androgens are similar to those found in 21-hydroxylase deficiency.[43] As with 21-hydroxylase deficiency, masculinisation of the female genitalia before birth is due to the effect of increased androgens early in gestation, when sexual differentiation occurs.

CAH due to 11β–hydroxylase deficiency is a heterogeneous disorder and consists of a number of aberrations with different clinical expressions. The range is wide and at one end of the spectrum is the classical congenital defect which is usually detectable very early in life by pronounced virilisation with[44-48] or without hypertension.[42,49,50] At the other extreme exists the late-onset forms of the disorder, described in post-menarchal females whose only complaints have been hirsutism and menstrual disturbances.[51-53]

Such clinical variability may be the expression of a single genetic defect in which different alleles express varying degrees of enzymic deficiency, as is the case in 21-hydroxylase deficiency.[24,25,54] However, the 11β–hydroxylation step is quite complicated, requiring several enzymes and cofactors; thus several mutations are possible.[25,55] These may account for the clinical heterogeneity observed in patients with this defect.

1.2.3. Treatment of CAH

Treatment of CAH is simple, effective and cheap. Since 1949, when the efficacy of cortisone therapy in CAH was discovered,[12,56] glucocorticoid replacement has been the keystone of treatment. Salt-retaining steroids have been used in addition to glucocorticoids in the salt-wasting form of the disorder.

Deficiency of both cortisol and aldosterone results in hyponatraemia/hyperkalaemia leading to feeding difficulties, vomiting, dehydration and peripheral circulatory collapse. This salt-losing crisis requires immediate treatment with intravenous saline and dextrose. Cortisol and mineralocorticoid replacement therapy is required. Plasma administration may be necessary if there is peripheral circulatory failure.[57] The amount of mineralocorticoid, and the concentration and amount of saline solution are adjusted according to the results of frequent electrolyte determination and assessment of the state of hydration and blood pressure measurements.[58] Long term replacement therapy is required to maintain sufficient glucocorticoid and mineralocorticoid (if necessary) to allow normal growth during infancy and childhood, prepubertal development, and adult reproductive potential.

Therapuetic control is monitored by clinical and biochemical parameters. The former includes serial measurements of growth velocity and skeletal age, signs of cortisolism, age at onset of pubertal stages, and regularity of menses in the post menarchal female. Several monitors have been assessed in an attempt to determine the correct level of replacement therapy. Measurements of adrenal steroid precursors in plasma, advocated as an index of therapeutic control, include 17-hydroxprogesterone[59] and androstenedione.[60] This aspect is discussed later in relation to an androstenedione blood spot assay.

Surgical correction of the virilised female genitalia is usually performed between six and twelve months of age. In patients with 21-hydroxylase deficiency, fertility in males, and feminisation, menstruation and fertility in females can be expected with adequate treatment. Long-term psychological guidance and support for the patient and family by the physician is essential.

1.2.4. Incidence of CAH

The prevalence of CAH appears to vary considerably throughout the world. Since 1956, estimations of the incidence of CAH based on case surveys have varied between 1:490 in the Yupik Eskimos of south western Alaska[61] to 1:67,000 in the Maryland, USA.[62] High incidences were reported in the area around Rome (1:5,580)[63], Zurich (1:5,041)[64], Munich (1:9,831)[65] and the Austrian Tyrol (1:8,991)[66]. The incidence of CAH in Birmingham was estimated,[67] over a ten year period, to be 1:7,255.

From 1978 neonatal screening programmes or pilot studies have been established nationally or regionally. The incidences of CAH, as determined by most screening programmes, were higher than those reported by case study.[61,68,69] In 1988 Pang *et al* reported a worldwide incidence of 1:14,554 for classical CAH detected by screening programmes in several countries[70] (> 1million neonates were screened between 1980 and 1988). Excluded from this figure were two populations the occurrence of CAH is very much greater. These are the Yupik Eskimos and the people of La Reunion, France where the incidence of CAH is 1:282[71] and 1:2,141,[69] respectively. In Scotland, between 1984 an 1986, a national neonatal screening programme for CAH revealed a prevalence of 1:17,098[68] This was higher than 1:20,907 obtained in a retrospective Scottish study.[68] In the USA, screening for CAH is undertaken in several states. In June 1989, Texas began screening its newborn population (2,500 samples/day) for CAH.[72] These figures, summarised in Table 2, apply almost exclusively to the 21-hydroxylase deficiency; the other enzyme defects are very much rarer, and appear only as single case reports. Surveys[85] in Israel have revealed an unusually high incidence of CAH in general, but particularly of the 11β-hydroxylase defect among the Jews of North African origin. As with the Eskimo population, this high incidence may be related to consanguinity.

Several population studies have established that the 21-hydroxylase deficiency is transmitted as an autosomal recessive trait,[62,64,86] with males and females equally at risk.[87] This was borne out by the worldwide CAH screening experience[70] and a case survey of 80 children with CAH, conducted between 1968 and 1988, by Donaldson *et al* who found the male to female ratio to be 1:1.[88]

Table 2. Mean incidence of congenital adrenal hyperplasia.

AREA	INCIDENCE (*) CASE STUDY	SCREENING
Worldwide	1:23,147 (88)[70]	1:14,554 (88)[70]
Salt-Wasting		1:18,921 (88)[70]
Simple-Virilising		1:63,068 (88)[70]
Scotland	1:20,907 (86)[68]	1:17,098 (86)[68]
England,		
Birmingham	1: 7,255 (66)[67]	
South West	1:13,433 (90)+	
Switzerland,	1:15,472 (80)[26]	
"	1:18,445 (58)[64]	
Zurich	1: 5,041 (58)[64]	
West Germany, Munich	1: 9,831 (77)[65]	
Austria, Tyrol	1: 8,991 (70)[66]	
Italy,		
Emilia-Romagna		1: 8,586 (82)[73]
"		1:14,600 (85)[74]
Rome		1: 5,580 (83)[63]
France,		
Lille/Lyon	1:23,000 (87)[69]	1:12,000 (87)[69]
La Reunion		1: 2,141 (87)[69]
New Zealand		1:16,258 (87)[75]
Japan	1:43,674 (81)[76]	1:15,000 (87)[77]
		1: 7,550 (82)[78]
United States,		
Alaska, Yupik Eskimo	1: 490 (69)[61]	1: 282 (82)[71]
Native	1: 1,481 (69)[61]	
Caucasian		1:13,773 (82)[71]
Washington		1:19,192 (88)[79]
		1:17,942 (88)[70]
Maryland	1:67,000 (56)[62]	
Illinois		
	1:40,000 (72)[80]	1:12,190 (89)[81]
	1:26,792 (65)[44]	1:13,333 (88)[70]
		1:15,000 (88)[82]
North Carolina		1:13,147 (89)[83]
Wisconsin	1:15,000 (66)[84]	
Canada,		
Toronto	1:26,292 (72)[79]	

(*) Year of publication. (+) Donaldson, M D C 1990-personal communication.

Either the simple virilising or salt-wasting form is found almost consistently within one family, with the salt-wasting form occurring in about a third to a half of the patients with 21-hydroxylase deficiency.[89] However, more recently, in determining the worldwide prevalence of CAH by screening, a 77% relative incidence is shown for salt-wasting hyperplasia and 23% for simple virilising.[70]

1.2.5. Screening for CAH

As a result of electrolyte imbalance and circulatory failure, CAH may have fatal consequences if it is not treated. Children with partial deficiencies may not be identified for several years.[90] The treatment of CAH by oral administration of glucocorticoids and mineralocorticoid, if required, is simple, cheap and effective. These factors, in conjunction with the relatively high incidence, meet the criteria proposed for the institution of a screening programme.[91] Several groups have proposed[92-93] that all newborn infants should be screened for 21-hydroxylase deficiency, since both classical and non-classical types have gone unrecognised. More recently, Pang et al confirmed that newborn screening improved overall case detection of CAH worldwide and improved case detection of newborns with the salt-wasting form of CAH whose diagnosis previously might have been missed, resulting in death.[70]

Early diagnosis by clinical observation is confirmed by the measurement of adrenal steroid precursors in plasma and/or adrenal steroid metabolites in urine. Detailed urinary profiles of steroid metabolites by capillary gas column chromatography provide definitive information as to the site of enzyme defects in

neonates with CAH.[95,96] Such measurements are superior to those of urinary steroid metabolites by conventional gas liquid chromatography and calculation of the 11-oxygenation index.[97] The most widely accepted marker of the 21-hydroxylase deficiency is an elevated plasma 17-hydroxyprogesterone level.[57] Early methods[98,99] for measuring 17-hydroxyprogesterone relied on the extraction of the steroid from the plasma followed by a competitive protein binding assay. Radioimmunoassay has now replaced competitive protein binding methods because of its better sensitivity, precision and specificity.[100,101] The development of a micro-filter paper method[92] for measuring 17-hydroxyprogesterone and the introduction of ^{125}[I]-label made possible mass neonatal screening for CAH. This method utilises a heel prick for obtaining a capillary blood specimen to spot onto filter paper which is then analysed for 17-hydroxyprogesterone by RIA. The hormone is stable on filter paper and the filter paper specimens can be sent to the screening laboratory through the postal system.

Although 17-hydroxyprogesterone levels are grossly elevated in 21-hydroxylase deficiency, they may be only modestly raised in the less common 11β-hydroxylase block.[102] Therefore, measurement of 17-hydroxyprogesterone may not select cases of CAH due to 11β-hydroxylase deficiency. Androstenedione levels have been shown to be grossly elevated in both 11β-hydroxylase and 21-hydroxylase enzyme deficiencies[103,104] and may therefore offer greater potential as a diagnostic aid.

Prenatal Diagnosis

The 21-hydroxylase gene is located on chromosome 6, between the HLA-A locus and the centromere.[105] This observation has been used to advantage in the detection of heterozygotes[106] for 21-hydroxylase deficiency, clinically unrecognised homozygotes and for prenatal diagnosis.[107]

HLA genotyping of amniotic cells collected from pregnancies at risk has been successful in the prenatal diagnosis of CAH due to 21-hydroxylase deficiency.[108] However, this is a complex and time-consuming method and does not detect CAH due to 11β-hydroxylase deficiency because close genetic linkage between the gene responsible for 11β-hydroxylase deficiency and HLA has not been established in this form of CAH.[109] At present, the definitive test for the prenatal detection of this disorder is the measurement of 17-hydroxyprogesterone concentrations in mid-trimester amniotic fluid.[110-112]

1.3. RADIOIMMUNOASSAY

In 1977, a reliable screening test for CAH became available.[92] This was based on a micro filter paper method of RIA for 17-hydroxyprogesterone. RIA is based on saturation analysis and has made possible the measurement of very low levels (pg) of hormones with a high degree of sensitivity and specificity. This elegant procedure was first reported in 1960 when details of a thyroxine assay using thyroxine-binding globulin were published[113] and a plasma insulin RIA was described.[114] The basis of saturation analysis is the competition between labelled and unlabelled forms of the ligand for specific sites on the antibody. The amount of labelled ligand bound to antibody is inversely proportional to the weight of unlabelled ligand present.

The requirements for any RIA procedure are a specific antibody, a labelled antigen and a means of separation of antibody bound and free fractions. These requirements are discussed below with special reference to the measurement of steroids by RIA.

1.3.1. Antibody Production

The ability of the immune system to respond to an antigen is dependent on molecular size. Molecules with molecular weights below 6,000 are poor antigens. Steroids are small (molecular weights approximately 300), non-immunogenic molecules which can fortunately be made immunogenic by covalent

attachment to larger molecules such as proteins.[115,116] Due to the lack of suitable functional groups in the steroid molecule itself, derivatives are required which can then be linked to the ε-amino group of lysine residues on the protein carrier. The derivative provides a "bridge" of four to six carbon atoms between carrier and hapten which improves specificity by allowing the hapten to project above the hydrated surface of the protein. The importance of the "bridge" in relation to the performance of antibodies with iodinated steroid labels will be discussed later. Stable steroid/bovine serum albumin conjugates capable of eliciting antibody formation were first produced by Lieberman and Erlanger [117,118] and in 1969 the first practical steroid RIA was reported.[119]

The antibody response to injected immunogen can be increased by the inclusion of adjuvants - the most widely used was introduced by Freund[120] in 1951. Freund's adjuvant consists of neutral detergent, paraffin oil and (in the complete form) killed mycobacterium. The detergent binds both the oil and an aqueous solution of the immunogen, allowing the formation of a stable emulsion. Freund's adjuvant enables the very slow release of immunogen, avoiding rapid uptake into the circulation leading to degradation by proteolytic enzymes. It also aids phagocytosis of the immunogen by macrophages, causes the formation of a local granulomatous lesion which may act as a focus for antibody formation and causes local and general stimulation of the reticulo-endothelial system.

1.3.2. Characterisation of Antibody

Antisera are characterised by titre and specificity.

Titre

Titre is the dilution of antiserum used in the assay tube to ensure a binding of labelled antigen of fifty percent in the absence of unlabelled hormone and is found by performing an antibody dilution curve. Steroid antibodies normally have titres between 1/1,000 and 1/100,000.[121]

Specificity

Because of the natural occurrence of many steroids with closely related chemical structures, there is a high risk that an antibody produced in response to a steroid immunogen may not be absolutely specific for the selected steroid. This means that assessment of the specificity of steroid antisera is mandatory. Antibody specificity can be assessed by cross reaction studies. An index of specificity can be obtained from displacement studies and is calculated from the mass of immunogenic steroid required to displace fifty percent of the radiolabelled immunogenic steroid to the mass of cross reacting steroid to displace the same percentage of labelled steroid.[119] This cross reation equation is given in the equations section.

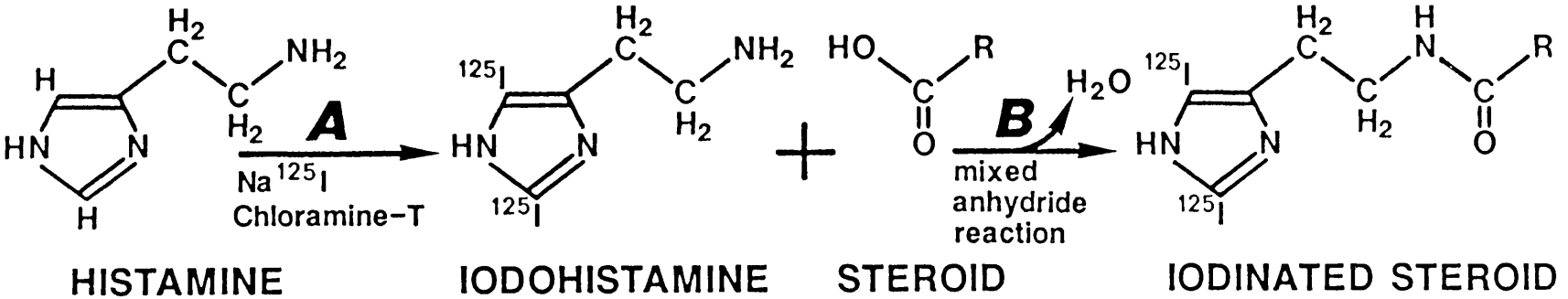
The specificity of a particular antiserum may be strongly influenced by the position on the steroid nucleus used for conjugation. Several workers have shown,[122,123] for example, that antisera tend to be specific for that part of the steroid molecule furthest from the site of conjugation. Hence, an antiserum may be more likely to recognise changes in the D ring if the site of conjugation is at the 3-position.

1.4. LABEL

Essential to any binding assay is a means for determining the distribution between the antibody bound and free fractions. This is achieved by incoroporating a small amount of highly purified labelled ligand into the assay system which should behave similarly to the unlabelled ligand and be detectable by accurate, direct and simple procedures. Currently, radioactive labels are the most common type in hormone assays but non-isotopic labels such as enzymes and luminescent reagents are becoming increasingly popular.

Gamma-emitting isotopes of iodine e.g. ^{125}I can be used to label steroids.[124-127] Although this isotope has the disadvantage of a relatively short half-life (60 days), it has a high specific activity (up to 2,200 Ci/mmol) and is easier and cheaper to detect than tritium. For ^{125}I, expensive scintillation fluid is not required and multihead counters allow fast sample throughput.

The iodine atom is comparable in size to the A ring of the steroid nucleus and would cause considerable distortion of the molecule if it was incorporated directly. Iodine is, therefore, first bound to an aromatic amine which is then linked to an activated steroid derivative. Details of the conjugation labelling method employing Chloramine-T[128] are illustrated in Fig. 8. Sodium [^{125}I]-iodide is initially converted to a more reactive form (free iodine or I^+) by the strong oxidising agent, Chloramine-T, which causes a direct iodination of the aromatic amine (tyramine or histamine). The reaction is stopped by the addition of the reducing agent, sodium metabisulphite. The halogenated aromatic amine



A SUBSTITUTION OF ^{125}I ON THE IMIDAZOLE SIDE CHAIN

B COUPLING OF IODOHISTAMINE TO STEROID DERIVATIVE

FOR -3CMO DERIVATIVE

$R = \begin{matrix}\text{STEROID}\\ \text{NUCLEUS}\end{matrix} = N\text{-}O\text{-}CH_2\text{-}$

FOR -11αHS DERIVATIVE

$R = \begin{matrix}\text{STEROID}\\ \text{NUCLEUS}\end{matrix} \text{- - -} O\text{-}\overset{\overset{O}{\|}}{C}\text{-}(CH_2)_2\text{-}$

FIG. 8. Conjugation method of iodination using Chloramine-T.

is then linked to the steroid nucleus through a mixed anhydride reaction to a carboxymethyloxime or hemisuccinate bridge.[117] The iodinated steroid is finally purified[129] before use in RIA by paper, thin layer or high pressure liquid chromatography.

1.5. Separation Methods

Once the primary antibody-hapten reaction is complete, it is necessary to determine the distribution of the hapten between the bound and the free forms. Usually, this requires that the bound fraction be physically separated from the free fraction. A good separation method should be quick, efficient, cheap and uncomplicated to enable large sample capacity to be achieved.

1.5.1. Dextran-Coated Charcoal

The use of adsorption of the unbound steroid fraction by charcoal modified by coating with dextran was proposed in 1965.[130] This method of separation was used extensively in early steroid RIA's with adequate precision. Temperature and time of exposure to dextran coated charcoal suspensions, however, require careful optimisation to avoid "stripping" of antigen from antibody. "Magnetisable charcoal" has been developed in which charcoal and magnetisable ferric oxide trapped in a polyacrylamide gel permit separation by a magnet rather than centrifugation.[131]

1.5.2. Second Antibody

In 1962, a separation procedure was introduced[132] in RIA systems in which a second antibody (Ab-2) specific to the γ-globulin of the species in which the first antibody was raised, is used to precipitate the bound complex. Double antibody separations are highly specific and are capable of almost complete separation of bound and free forms of antigen. Optimisation of this method requires careful selection of the second antibody concentration to allow total precipitation of the bound fraction.

The major disadvantages of the double antibody method are the reagent cost involved in selecting and supplying large volumes of antiserum (second antibodies have lower affinities and must, therefore, be used at lower titres), the prolonged incubation time required to achieve complete separation and the interference they can suffer from a number of non-specific factors.[133] The addition of a protein precipitant such as polyethylene glycol can facilitate the formation of the first antibody-second antibody complex and so reduce the incubation time.[134]

1.5.3. Solid Phase

In solid phase separation systems, the antibody (either first or second) can be coupled to an insoluble support,[135] such as particles of cellulose, dextran, latex polymer, micron sized glass or continuous surfaces of polystyrene or polypropylene discs or the walls of plastic tubes. Because of the adsorptive properties of plastic surfaces, tubes and discs can be directly coated with antibody. The adsorptive interaction is, however, less stable than covalent linkage, and hence the major drawback is lack of reproducibility. When the

antiserum is covalently linked to particulate solid phase, any loss of antibody affinity (and therefore assay sensitivity) due to coupling can be compensated by improved precision resulting from the efficiency of separation.[136]

An alternative approach is the use of antibody covalently bound to magnetic particles (polymer-coated iron oxide),[137] a system where mixing and separation are simply achieved by the application of a magnetic field.

1.5.4. Microencapsulated Antibody

A reagent ideal for use in hapten RIA is encapsulated antibody. The large antibody molecule is surrounded by a polyamide membrane, permeable only to small molecular mass molecules such as steroid hormones. Centrifugation of the microcapsules containing antibody separates the antibody bound from the free fraction. This method of separation stemmed from early work on microcapsules[138] and artificial cells[139] containing proteins,[140] such as erythrocyte haemolysates and enzymes.

The semi-permeable membrane isolates and protects the encapsulated protein, while allowing free transfer of low molecular mass compounds between external and internal environments. Hence, the suitability of microcapsules to hapten and free hormone RIA's. Use of microcapsules in RIA has been reported for digoxin,[141] free thyroxine[142-144] and cortisol.[145]

The preparation of microcapules involves polymerisation at the interface of a water-in-oil emulsion. This is accomplished by forming an emulsion of an alkaline aqueous solution, containing a diamine plus the protein to be encapsulated, in an organic solvent system. Then a diacid chloride, which is soluble only in the organic solvent, is added to the emulsion. The diamine,

which has some solubility in the organic solvent, diffuses into the organic phase and forms a polymer membrane on the organic solvent side of the interface according to the reaction:

$$H_2\text{-}R_1\text{-}NH_2 + ClOC\text{-}R_2\text{-}COCl \longrightarrow$$

$$[\text{-}HN\text{-}R_1\text{-}NH\text{-}CO\text{-}R_2\text{-}CO\text{-}]_n + HCl$$

The encapsulated droplets are separated from the organic solvent, washed with detergent and resuspended in an aqueous medium.

A comparison of three separation methods of RIA for 17-hydroxprogesterone using the same antiserum microencapsulated, linked to cellulose, or in solution has been carried out.[146] Microencapsulated antibody compared well with solid phase antibody and performed better than a "soluble" antibody system with charcoal separation. Work has been also been carried out on lyophylised capsules.[147] The microcapsules containing antibody are sufficiently robust to withstand repeat dispensing and are of similar density to the medium and remain in suspension during incubation. Preparation of microencapsulated antibody is quick, cheap and reproducible and was, consequently, used in this study for initial cross-reaction experiments. Cross-reaction studies with a soluble second antibody yielded similar information.

1.6. EXTRACTION VERSUS DIRECT RIA

In biologicial fluids, steroids may occur in forms other than as free steroids. They can bind to proteins other than antibodies[148] or can be present in the form of conjugates. Albumin binds steroids weakly but has a high capacity for them. Certain steroids bind with high affinity to cortisol-binding globulin (e.g. cortisol and progesterone) and sex-hormone-binding globulin (e.g. testosterone and androstenedione).[149] Steroids also occur as conjugates mainly as sulphates or glucosiduronidates. The presence of these other forms of steroids creates problems when a steroid is being measured by RIA in whole blood.

The extraction of steroids by organic solvent prior to RIA is a means of separating them from water-soluble steroid conjugates and serum proteins and thus increases assay specificity. On the other hand, an extraction step is a source of imprecision and error.[150]

The elimination of the extraction step and development of a direct RIA for the measurement of steroids offers considerable potential advantages, including practical convenience, improved precision, avoidance of hazardous solvents and larger batch sizes.[151] In 1982, direct non extraction RIA's for progesterone were reported which compared favourably with those having conventional extraction procedures.[152] RIA incorporating an extraction step is impractical for use in large-scale screening because of the high sample through-put (e.g. 200-500 samples/day for the Scottish neonatal population).

CHAPTER 2 MATERIALS AND METHODS

APPARATUS

Gamma radiation was measured on a NE-1600 Gamma Counter, Nuclear Enterprises, Edinburgh, U.K. and beta radiation with a "Prias" scintillation counter from Canberra Packard, Pangbourne, Berkshire, U.K. Purification of radioactive labels involved the use of T.L.C. plates (pre-coated with silica gel 60 F_{254}) from Merck Clinical Research Division, 267 Church St., Blackpool, Lancashire, U.K., which were scanned on a Radiochromatogram Scanner from Packard Instruments Ltd., 13-17 Church Rd., Caversham, Berkshire, U.K. Solvent extraction of steroids involved the use of a Zippette Repeating Pipette from Jencons Scientific Ltd., Cherry Court Industrial Estate, Stanbridge Rd., Leighton Buzzard, U.K., an S.M.I. Multi-Tube Vortexer from Alpha Laboratories, Easteigh, Hampshire, U.K. and a Buchler Vortex Evaporator from Arnold Horwell, London, U.K. Immunogen was dissolved with the aid of a sonicating water bath from Q.H. Kerry, Kerry Ultrasonics Ltd., Hunting Gate, Wilkbury Way, Hitchin, Hertfordshire, U.K. or an Ultrasonic Disintigrator Probe from M.S.E. Scientific Instruments, Manor Rd., Crawley, West Sussex, U.K. An Ultra-Turrax Disperser was obtained from Janke and Kungel, Wroclaw, Poland. U.K. agents:- Semat Ltd., St. Albans, Hertfordshire, U.K. and was employed in the microencapsulation of antibody.

Unless otherwise stated, centrifugation was carried out using a M.S.E. "Coolspin" centrifuge (as above). In addition, an ultracentrifuge and "Centaur" centrifuge, both M.S.E. (as above) were employed. In the preparation of charcoal-stripped serum, Whatman No.1 filter paper was used from Whatman Lab. Sales Ltd., Unit 1, Caldred Rd., Parkwood Industrial Estate, Maidstone, Kent, U.K. Glass test tubes, 10 ml (100x16 mm) and 5ml (75x12 mm), were from S. Murray and Co., Holburn House, Old Woking, Surrey, U.K. Repeat dispensing of reagents was performed with a 200 µl adjustable Microdispenser Pipette from Socorex, ISBA Ltd., Remiens, Switzerland. Plastic test tubes, 5 ml (plastic, 75x12 mm), were from Sarstedt Ltd., Beaumont Leys, Leiscester, U.K.

MATERIALS

All common reagents and solvents were of 'AnalaR' grade and were from either B.D.H. Chemicals Ltd. or Sigma Chemical Co. both Dorset, U.K. Steroids were purchased from Steraloids Ltd., 31 Radcliffe Rd., Croydon, U.K. Freund's complete adjuvant was from Difco Laboratories, P.O. Box 1058 A, Detroit, Michigan, U.S.A. Precipitating antibody (donkey anti-rabbit and donkey anti-sheep) was supplied by the Scottish Antibody Production Unit, Law Hospital, Carluke, U.K. Antisera were raised in New Zealand white rabbits. Tritiated androstenedione ([1,2 6,7-^{3}H]-androst-4-ene-3,17-dione) and Na^{125}I were supplied from Amersham International, Buckinghamshire, U.K. The antiserum used with the tritiated tracer was supplied by Guildhay Antisera, University of Surrey, U.K., and was raised against androstenedione-17α-carboxythio-ether ovalbumin (ref. no. HP/S/673-1A). Hexane-1,6-diamine, terephthaloyl chloride and Span 85 (sorbitan trioleate) were obtained from Koch-Light Laboratories, Slough, Berkshire, U.K. The following reagents were from Sigma (as above): Tween 20 (polyoxyethylene sorbitan monolaurate), isobutylchloroformate, N-(2-hydroxyethyl)-piperazine-N^{1}-3-propanesulphonic acid (EPPS), bovine serum albumin (fraction V) and carbodiimadazole. Sepharose-Cl4B was obtained from Pharmacia Fine Chemicals, A B, Box 175, 45-751 04 Upsala, Sweden. Tri-N-butylamine was from Eastman-Kodak Co., Rochester, New York, U.S.A. and absolute alcohol was from James Burroughs Ltd., 60 Montford Pl., London,U.K. Newly- expired packs of erythrocytes were obtained from Blood Transfusion, Stobhill Hospital, Glasgow, U.K. Filter paper cards (Schleiser and Schuell, grade 2992 cellulose) for blood spots were obtained from Anderman & Co., 145 London Rd., Kingston-upon-Thames, Surrey, U.K. Dioxan was purified before use by running it through a glass column packed with chromatographic standard alumina.

2.1. Preparation of Solutions and Buffers

Phosphate Buffer (0.05 M, pH 7.4)

Unless otherwise stated, a phosphate buffer (0.5 mol/l pH7.4) was used and was prepared as follows: A 0.25 M sodium dihydrogen orthophosphate solution (A) was prepared by dissolving 70.98 g $NaH_2PO_4.2H_20$ in distilled water and making up to 250 ml. A 0.5 M disodium hydrogen orthophosphate solution (B) was prepared by dissolving 35.5 g Na_2HP0_4 in distilled water and making up to 500 ml. Sodium chloride (17.5 g) and merthiolate (0.5 g) were dissolved in distilled water (250 ml). Solution A (30 ml) and solution B (120 ml) were added to this and mixed. The pH was checked and adjusted to 7.4 and the solution diluted to 2 1 with distilled water. The solution was stored at 4 ^{o}C for up to 6 months.

RIA Buffer

RIA buffer was freshly prepared for each assay by dissolving BSA (0.25% w/v) in phosphate buffer.

Citrate Buffer (Na Citrate/HCl, pH 4.0)

A disodium citrate solution was prepared by dissolving citric acid monohydrate (21.01 g) in a volume of distilled water (500 ml). Sodium hydroxide (200 ml of a 1M solution) was added and the solution was made up to 1 1 with distilled water. Hydrochloric acid (8.77 ml of 0.1 M) was diluted to 1 1 with distilled water. Citrate buffer (pH 4.0) was prepared by mixing disodium citrate solution (55.1 ml) with hydrochloric acid (44.9 ml of 0.1M).

EPPS Coupling Buffer (EPPS 0.1 M, Na azide 0.05% w/v, pH 8.0)

EPPS (25.23 g) was dissolved in distilled water (750 ml) and titrated to pH 8.0 with sodium hydroxide (1.0 M). Sodium azide (500 mg) was dissolved in this solution and the volume made up to 1 l with distilled water. EPPS buffer was stored at 4 °C for up to one month.

Bicarbonate Solution (0.5 M, pH 8.0)

Sodium bicarbonate (42 g) was dissolved in distilled water and made up to l l.

Acetate Buffer (0.1 M, pH 4.0)

Anhydrous sodium acetate (1.74 g) was dissolved in distilled water (750 ml). Glacial acetic acid (9 ml) was added and the volume made up to 1 l with distilled water.

Isotonic Saline (0.154 M)

Sodium chloride (9 g) was dissolved in distilled water and made up to 1 l.

Wash Solution (0.9% Na/C1 w/v, 0.2% Tween 20 v/v)

A solution of isotonic saline was prepared (5 l) to which Tween 20 (10 ml) was added and mixed before storing at 4 °C. The supernatant was removed and discarded by a multi-head aspirator linked to a vacuum pump.

Organic Phase

Chloroform/cyclohexane (1:4,v/v) containing 1% sorbitan trioleate.

Diamine Reagent

Sodium bicarbonate (0.8 g) and hexane-1,6-diamine (1 g) were dissolved in 10 ml distilled water and the pH adjusted to 9 with 5 mol/l hydrochloric acid. Polyethyleneimine (2 g of a 50% solution) was added, mixed for 10 min and the final volume adjusted to 20 ml with distilled water.

Organic Diacid Chloride Reagent

Powdered terephthaloyl chloride (1 g) was added to 180 ml of organic phase and mixed at 4 ^{o}C for 1 h. Undissolved terepthaloyl chloride was removed by centrifugation.

2.2. PREPARATION OF STANDARD MATERIAL FOR RIA

2.2.1. Androstenedione Standard Solutions for RIA Development

Androstenedione (1 mg) was dissolved in ethanol (10 ml), giving a stock standard solution of 100 μg/ml. The stock standard solution (1 ml) was diluted with ethanol (99 ml), giving an intermediate solution of 1μg/ml. Intermediate solution (500 μl) was evaporated to dryness in a 100 ml volumetric flask. RIA buffer (100 ml) was added to the dried residue to give a working solution of 5,000 pg/ml. Working standards were prepared, as shown in Table 3. Aliquots (200 μl) of each standard solution were pipetted into appropriately labelled plastic test tubes (5 ml, 75x12 mm), sealed and stored at -20 °C for up to 6 months.

2.2.2. Steroid Standard Solutions for Cross Reaction Studies

Steroid (1 mg) was dissolved in ethanol (10 ml), giving a stock standard solution of 100 μg/ml, which was stored at 4 °C. Stock standard solution (1 ml) was diluted with ethanol (99 ml) giving an intermediate solution of 1 μg/ml which was also stored at 4 °C. Intermediate solution (1 ml) was evaporated to dryness under a stream of air. RIA buffer (6.7 ml) was added and well mixed, giving a working solution of 30,000 pg/200 μl, which was freshly prepared for each RIA.

Table 3. Serial dilutions of the working solution (5,000 pg/ml) of androstenedione (A4) used to prepare standards.

	Volume (ml)		
Standard	Working Solution	RIA Buffer	Concentration A4 (pg/200 µl)
0	0.0	10.0	0.0
1	0.1	9.9	10
2	0.2	9.8	20
3	0.5	9.5	50
4	1.0	9.0	100
5	2.0	8.0	200
6	4.0	6.0	400
7	8.0	2.0	800

2.2.3. Androstenedione Standard Solutions for Serum RIA

Androstenedione standards were prepared by dissolving A4 in ethanol to give a final concentration of 100 mg/l. This solution was further diluted in ethanol to give concentrations of 0.7, 1.4, 3.5, 7.0, 10.5, 17.5, and 21 nmol/l.

2.2.4. Androstenedione Blood Spot Standards

A suitable material was provided by the addition of charcoal-stripped serum to saline-washed, packed erythrocytes. This was then spiked with androstenedione and spotted onto filter paper cards.

A) Preparation of agarose-coated charcoal: Agarose (12.5 g) was added to distilled water (250 ml) and heated to 70 ^{o}C with constant stirring. Activated charcoal (50 g) was then added and mixed to form a thick slurry. This was allowed to cool to about 50 ^{o}C before adding to acetone (500 ml) with vigorous stirring in a Fume cupboard. A granular precipitate of agarose-coated charcoal was separated by filtration through Whatman No.1 filter paper. This was spread on filter paper, covered and left overnight to dry.

B) Preparation of charcoal-stripped serum: Agarose-coated charcoal (10 g) was added to pooled female serum (100 ml) and placed on a rotary mixer overnight at room temperature. The mixture was centrifuged at 3,000 rpm for 15 minutes in polypropylene centrifuge tubes (2x50 ml). The supernatant was decanted into tubes and further sedimented in an ultracentrifuge at 10,000 rpm for 30 min.

C) Construction of whole blood matrix: Newly-expired packed erythrocytes (40 ml) were added to graduated polypropylene centrifuge tubes (4x50 ml) and spun at 3,000 rpm for 20 min. The top layer of serum and erythrocytes was aspirated leaving 25 ml packed erythrocytes. Isotonic saline (25 ml) was added, well mixed and centrifuged as above. The supernatant was aspirated, discarded and the packed erythrocytes rewashed with saline. The supernatant and the top layer of packed erythrocytes were aspirated leaving 15 ml of packed erythrocytes in each centrifuge tube. The packed, erythrocyte aliquots were pooled and mixed. Charcoal-stripped serum (50 ml) was added to an equivalent volume of saline-washed packed erythrocytes and was well mixed to produce a whole blood matrix for the construction of androstenedione dried blood spot standards.

D) Construction of dried blood spot standards: Intermediate androstenedione standard solution (300 µl of 1 µg/ml) was evaporated to dryness in each of two small conical flasks. For the top standard, I, 4.5 ml of blood was added to one flask (giving 525 nmol/l androstenedione in serum, assuming a 50% haematocrit). To the other flask was added 6 ml of blood (giving 350 nmol/l androstenedione in serum). After 10 min, both flasks were well mixed and the contents of the latter (standard H) was double diluted with blood, as shown in Table 4, to give a standard curve.

Filter paper cards were labelled with identifcation for each standard and arranged horizontally, raised up off the bench. After mixing, aliquots (25 µl) of each standard were spotted onto the cards which were kept flat for a minimum of 15 min. The cards were allowed to dry at room temperature for at least 6 h and stored in an air-tight container at 4 ^{o}C for up to 8 weeks.

Table 4. Serial dilutions of androstenedione (A4) in blood used to prepare blood-spot standards. The resulting A4 concentration+ in a blood-spot disc is given.

Standard	A4 (nmol/l serum)	A4 (pg/5 mm disc)
A*	0	0
B	5.5	4.5
C	11.0	9.0
D	22.0	18.75
E	43.7	37.5
F	87.5	75.0
G	175.0	150.0
H	350.0	300.0
I	525.0	450.0

(+) Given that a 5 mm blood spot disc contains 6 µl of whole blood (or 3 µl of serum).

(*) Blood (3 ml) containing no A4 was used as the zero standard which gives maximum binding of labelled A4 by antibodies.

2.3. PRODUCTION OF REAGENTS FOR RIA

2.3.1. Production of Androstenedione Antiserum

Antibodies were raised against the immunogens. A4-3CMO-BSA, A4-6βHS-BSA and A_4-11αHS-BSA in rabbits A/B/C; D/E/F and G/H/I/J respectively. Rabbit G (for the A_4-11αHS-BSA immunogen) died 14 days after the first immunisation, and was replaced by rabbit J. Rabbits E and I died after 7 and 9 months respectively. In all other cases, the rabbits survived for more than one year. The immunisation schedule is outlined in Fig.9.

The procedure involves three stages :

A) Immunogen preparation: Immunogen (1 mg) was dissolved in isotonic saline (4 ml) by gentle sonication in a sonicating water bath for 3 min. An equal volume of Freund's complete adjuvant was added and the tube placed in a beaker of crushed ice. More severe sonication continued with an ultrasonic disintegrator probe (set at frequency 15 kHz) until an emulsion was produced.

B) Injection procedure: The immunogen/Freund's emulsion was injected intramuscularly into each of 3 rabbits as follows:

(i) left thigh - 1 x 0.5 ml injection
(ii) right thigh - 1 x 0.5 ml injection
(iii) left side of spine - 2 x 0.25 ml injection
(iv) right side of spine - 2 x 0.25 ml injection

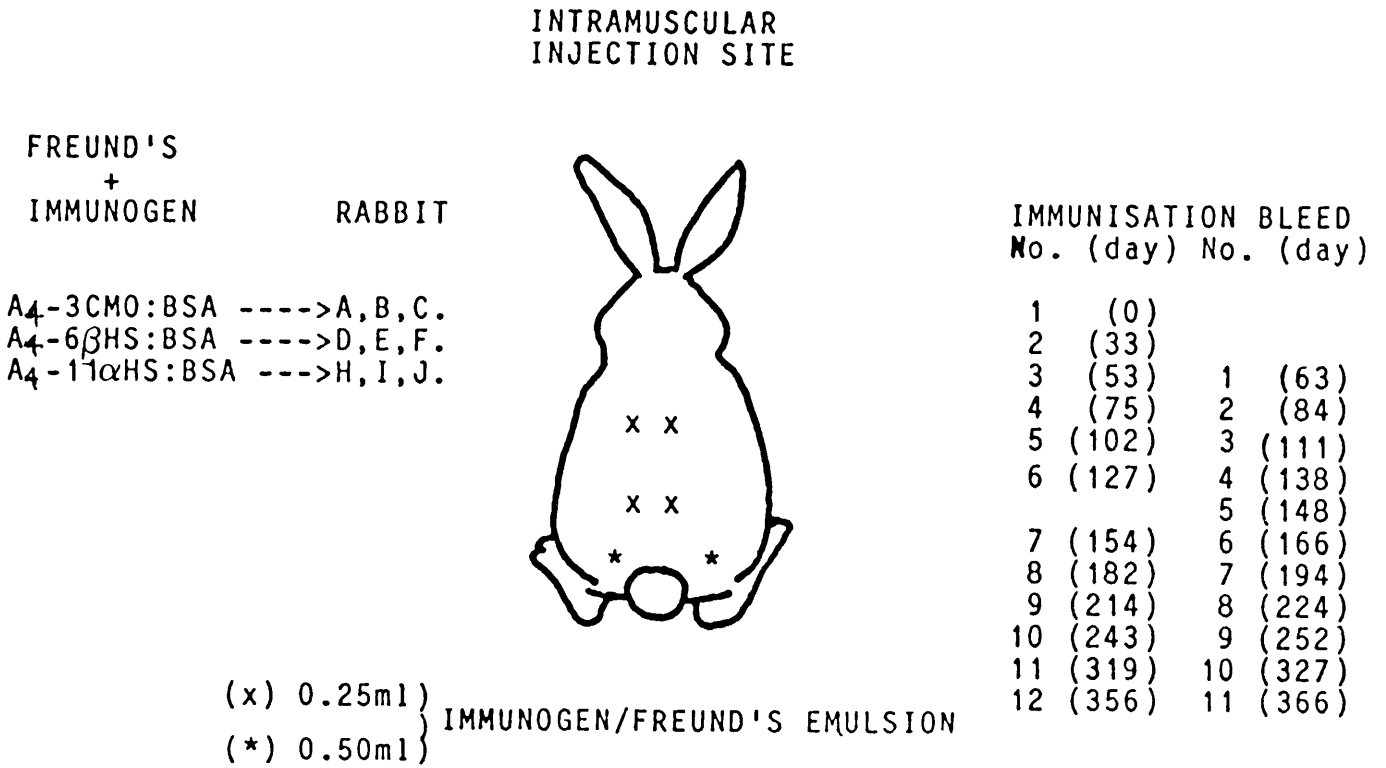


FIG. 9. Outline of immunisation schedule to produce antiserum in rabbits against androstenedione.

This procedure was repeated at approximately monthly intervals for 1 year.

C) Bleeding procedure: Ten days after the third and subsequent boosts, a test bleed (1 ml blood) was taken from an ear vein. The sample was allowed to clot overnight and the serum stored at -20 ^{o}C in 100 µl aliquots. If the test bleed obtained a high titre antibody, a larger bleed (40-50 ml blood) was collected from an ear vein. The serum (1 ml) from this bleed was stored as above and the remaining serum was diluted 1:1 (v/v) with phosphate buffer and stored at -20 ^{o}C in 20 ml aliquots.

2.3.2. Preparation of Solid Phase Second Antibody

All rabbit antisera were assessed by titration studies using a solid phase second antibody (Ab-2) separation method. Donkey anti-rabbit antiserum was linked to Sepharose beads to provide this reagent.

The following solutions were freshly prepared:

EPPS Coupling Buffer (EPPS 0.1 M, Na azide 0.05% w/v, pH 8.0).
Bicarbonate Solution (0.5 M, pH 8.0)
Acetate Buffer (0.1 M, pH 4.0).
Isotonic Saline (0.154 M)

A) Activation of Sepharose: Sepharose-CL-4B stock slurry (500 ml) was poured into a measuring cylinder and allowed to settle overnight. Isotonic saline was then added to double the settled gel volume giving a calibrated Sepharose-CL-4B solution. This Sepharose-CL-4B solution (200 ml) was added to a

sintered glass funnel (porosity 3) which was linked to a Buchner flask (2 1 capacity) and filter-pump. The gel was dehydrated by washing successively with 500 ml of: (a) distilled water (b) acetone in distilled water 30% v/v (c) acetone 50% v/v (d) acetone 70% v/v (e) acetone 100% without allowing the gel to dry out. The vacuum filtration was switched off between washes and the gel was resuspended by gently stirring while adding the fresh wash solutions. The dehydrated gel slurry was further resuspended in acetone and transferred to a pre-calibrated conical flask (200 ml). The volume was made up to the 200 ml calibration mark with acetone. Carbonyldiimidazole (4.87 g) was added to activate the Sepharose, the flask sealed with a ground glass stopper and stirred on a magnetic stirrer for 60 min. The activated gel was transferred to the sintered glass funnel. The flask was rinsed with acetone which was also added to the sintered glass funnel. The gel was rehydrated by washing successively with 500 ml of: (a) acetone 100% (b) acetone 70% v/v (c) acetone 50% v/v (d) acetone 30% v/v (e) distilled water (f) EPPS coupling buffer again, without allowing the gel to dry out and resuspending gently between washes.

B) Coupling of antibody to Sepharose: Donkey anti-rabbit antibody (25 ml) was added to a 250 ml polycarbonate centrifuge tube (pre-calibrated at 200 ml). The activated gel was transferred to the polycarbonate bottle, made up to the 200 ml calibration mark with EPPS coupling buffer and shaken gently overnight. This was centrifuged at 1200 g for 20 min. The precipitate was resuspended by the addition of bicarbonate solution to the calibration mark and shaken for 20 min. This was recentrifuged, the supernatant discarded and the bicarbonate wash repeated. The precipitate was then resuspended in acetate buffer (0.1 M, pH 4), made up to the calibration mark and shaken for 60 min, recentrifuged, then the supernatant discarded and the acetate wash repeated but with overnight shaking.

This was again recentrifuged, the supernatant discarded, the precipitate resuspended up to 200 ml with isotonic saline and shaken for 20 min. Recentrifugation and the saline wash were repeated. Final centrifugation was followed by resuspending the precipitate in isotonic saline up to the calibration mark and storing at 4 ^{o}C.

C) Working solution for RIA: The stock solution of solid phase second antibody, Ab-2, was diluted with RIA buffer to give a 1% working solution.

2.3.3. Preparation of Microencapsulated Antibody

The method was based on that of Wallace and Wood.[146]

An antibody solution was prepared by adding androstenedione antiserum (200 µl) to 1.8 ml 1% w/v BSA and Coomassie brilliant blue (10 mg). Coomassie brilliant blue is a protein dye, enabling detection of any antibody which may have leaked from the microcapsules. The following reagents were freshly prepared and stored in ice:

Organic Phase
Diamine Reagent
Organic Diacid Chloride Reagent

Organic phase (30 ml) was added to a 100 ml conical flask on ice and mixed with an Ultra-Turrax Disperser operated with an 18N stainless steel shaft and an I.K.I. Thyristor speed controller set at 11/2. (The Ultra-Turrax Disperser

head had been pre-cooled in 100 ml conical flask containing organic phase on ice). Diamine reagent (3 ml) was added to the antibody solution and the mixture immediately added to the mixing organic phase. Emulsification proceeded for 90 s and interfacial polymerisation was initiated by the drop-wise addition of diacid chloride reagent (30 ml) added over 1 min. The mixing continued for a further 2 min. Mixing was stopped and the reaction terminated by adding the flask contents to a beaker containing organic phase (50 ml).

At this stage, it is necessary to transfer the microcapsules to an aqueous environment. The capsules were first sedimented by centrifuging (500xg for 5 min) in 2x60 ml polypropylene centrifuge tubes. The organic phase was carefully decanted and discarded and the microcapsule precipitate gently suspended in a 40% v/v aqueous ethanol containing 10% v/v Tween 20 (10 ml per centrifuge tube). The final volume was increased to 50 ml per tube with 40% v/v aqueous ethanol and centrifuged at 2,000 x g for 30 min. The resulting pellet was washed several times to a total volume of 4 1 with 0.9% w/v saline and finally suspended in phosphate buffer (20 ml) and stored at 4 ^{o}C.

Working solution for RIA: The required volume of stock solution was removed and RIA buffer added to give the working dilution (i.e. that which gave B/T of 40% in microencapsulated Ab dilution experiments).

2.3.4. Preparation of Androstenedione- ^{125}Iodohistamine Labels

Three androstenedione tracers were prepared by coupling each androstenedione derivative (A_4-3CMO, A_4-6βHS and A_4-11αHS) to histamine which had firstly been radiolabelled with ^{125}I sodium iodine.

A) Preparation of androstenedione derivatives: Each androstenedione derivative (2.7 mg) was placed in a glass tube (5 ml, 75x12 mm) and dissolved in dioxan (500 µl). A4-3CMO did not dissolve easily and an additional 500 µl was required, followed by sonication in a water bath. These androstenedione/dioxan solutions were stored at -20 °C in 50 µl aliquots.

B) Activation of androstenedione: Tri-N-butylamine/dioxan (10 µl of 1:50 v/v) was added to an aliquot of androstenedione derivative, followed by isobutylchloroformate/ dioxan (10 µl of 1:100 v/v), mixing after each addition. This was incubated for 1 h at 10 °C and the reaction then quenched by the addition of pre-cooled dioxan (280 µl).

C) Iodination of histamine: The following reagents were added to a 10 ml glass tube (100x16 mm): histamine/phosphate buffer 0.5 M, pH 8.0 (10 µl of 1.1 mg/5 ml); sodium^{125} iodide (1 mCi in 10 µl); chloramine-T/distilled water (10 µl of 15 mg/ml). The tube contents were mixed gently for 20 s and the reaction terminated by the addition of sodium metabisulphite/distilled water (10 µl of 15 mg/500 µl). The tube was placed in an ice bath.

D) Coupling of activated androstenedione to iodinated histamine: Sodium hydroxide 0.2 M (10 µl) was added to the iodination mix followed by the activated androstenedione (50 µl). The tube contents were vortexed prior to incubation at 10 ^{o}C for at least 1 h.

E) Purification of iodinated steroid: Iodinated androstenedione was purified by adding 1 ml of each of two reagents, hydrochloric acid 0.1 M and ethyl acetate, with vortexing after each addition. When the tube contents settled into 2 phases, the upper organic layer was removed and discarded. After each 1 ml addition of the following reagents, sodium hydroxide 0.1 M, phosphate buffer pH 7.0 and ethyl acetate, the tubes were vortex mixed. The tube contents were allowed to settle and the upper organic layer was removed into another glass tube containing anhydrous sodium sulphate (5 mg). The ethyl acetate addition was repeated twice and the organic layers pooled. After mixing with anhydrous sodium sulphate, the pooled organic extracts were removed into another glass tube. The extract was evaporated to 300 µl under a stream of air, by placing the tube on a hot plate set at 37 ^{o}C. Using a glass capillary tube, [^{125} I]-androstenedione (100 µl) was streaked across each of 3 T.L.C. plates, approximately 2 cm from the bottom. The 3 plates (upright, origin at the bottom) were developed in a solvent mixture, chloroform: methanol: acetic acid (90:10:1 by volume) for 90 min. The plates were removed, air dried, and placed (face up, origin first) on a radiochromatogram scanner at speed setting 1; range 100 K; gasflow 6.5 and time K set at 10. Each T.L.C. plate was matched with the resulting chart recorder tracing (Fig.10.) and the silica corresponding to the radioactive peak(s) scraped onto tin foil.

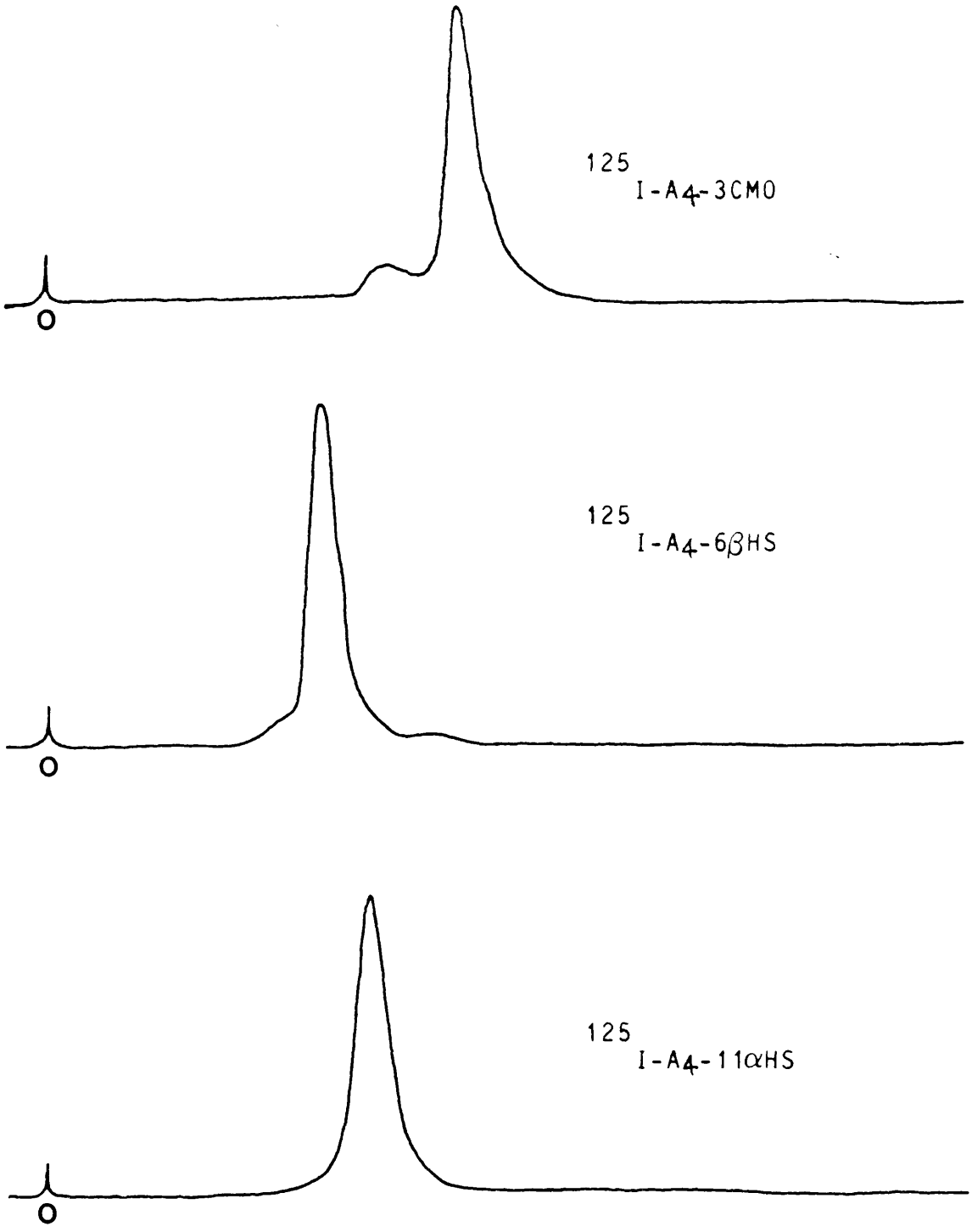


FIG. 10. Radiochromatographic scans of T.L.C. plates after development of ^{125}I-histamine-androstenedione-tracers.

(O) Origin.

The scraping of silica was carried out in a polythene bag within a designated Fume cupboard for safety reasons. The silica was placed in a glass tube and eluted 3 times with ethanol (1 ml). The combined eluates were placed in a screw cap glass tube and stored at 4 ^{o}C. This purified, iodinated androstenedione preparation was stable for at least 8 weeks.

F) Working solution for RIA: The volume of stock solution containing the required counts was evaporated to dryness in a 50 ml conical flask under a stream of air. The volume of RIA buffer required to give 10,000 cpm/200 µl (i.e. cpm per RIA tube) was added. Total count tubes (TC), containing only labelled androstenedione, were included in each RIA to quantitate the amounts of labelled androstenedione added to each assay tube. They did not undergo the wash procedure.

2.3.5. Solvent Extraction of Androstenedione from Samples

A) Extraction of A4 from serum samples: Sample (50 µl) plus distilled water (50 µl) was added to a 10 ml glass tube (100x16 mm). Hexane:ether (3 ml of 4:1 by volume) was added using a Zippette Repeating Pipette. The tubes were mixed on a Multi-Tube Vortexer operated at speed 4 for 4 min. The lower, aqueous phase was frozen by immersing the lower part of the tube in a bath of solid carbon dioxide in methanol. The upper hexane: ether phase, containing extracted steroid, was decanted carefully into a glass tube (5 ml, 75x12 mm). For each ethanolic standard, 20 µl was added to a glass tube followed by solvent (3 ml). Solvent from all tubes was evaporated on a Buchler Vortex-Evaporator at speed setting 4 under a vacuum at 45 ^{o}C.

B) Extraction of A_4 from dried blood spots: A disc (5 mm diameter) was punched from a dried blood spot into a 10 ml glass tube (100x16 mm) and eluted overnight with saline (100 µl) at 22 °C. Solvent was added and the tubes mixed at speed 2 for 8 min prior to centrifugation on a Centaur centrifuge for 2 min at 1,000 r.p.m. The aqueous phase was frozen and the method continued as above yielding glass tubes containing dried extracts.

CHAPTER 3. EVALUATION OF REAGENTS: EXPERIMENTS AND RESULTS

Antisera were assessed by titre and specificity. Initial Ab titre experiments (section 3.1.) utilised a solid phase second Ab separation method. A more convenient method of separation was achieved by encapsulating antibodies in semi-permeable spheres. However, since microencapsulation of serum from all bleeds was not practical, only antisera with a titre >1/4,000 were selected for microencapsulation. Combinations of microencapsulated Ab and label, which produced sensitive standard curves, were used in cross reaction studies with related steroids to assess specificity (section 3.2.).

3.1. ANTIBODY TITRE.

Antiserum was diluted 1/100 to 1/80,000 with RIA buffer. Antibody dilution curves were derived using a solid phase second antibody separation, Table 5. The antiserum dilution which gave a B/T of 50% was calculated from the antibody dilution curve and this titre determined the volume of antiserum to be encapsulated. Antibody titres for all combinations of antibody and label were monitored in each animal during one year. In animals that produced measurable antibody titres, the titres increased during the first six months and remained fairly constant thereafter (Table 6).

Table 5. Protocol for the construction of androstenedione (A4) antibody dilution curves.

Tubes	Volume (µl)	
	For Total Counts	For Ab Dilution Curve
Ab	-	100
^{125}I-A4 (10,000 cpm)	100	100
Incubated overnight at 22 °C		
Solid phase Ab-2	-	200
*Incubated 60 min at 22 °C		
Wash solution	-	3,000

Centrifuged at 2,000 rpm for 3 min at 4 °C. Supernatant aspirated, discarded and wash repeated twice. Precipitates were counted and Ab dilution curves constructed.

(Ab-2) Second antibody. (*) With shaking.

Table 6. Antibody titres* (x 10^{-3}) as determined by incubating antibodies raised against immunogens linked to androstenedione (A4) at positions 3, 6 and 11 with similarly linked androstenedione iodohistamine labels.

		^{125}I-A4 label		
Immunogen	**Antibody**	**-3CMO**	**-6βHS**	**-11αHS**
	A	400	-	3.7
-3CMO	B	65	-	-
	C	130	-	4.2
	D	-	-	-
-6βHS	E	----------died----------		
	F		11	-
	H	-	-	40
-11αHS	I	-	-	85
	J	-	-	280

(*) Titre is the dilution required to ensure a binding of 50 percent of label.
(-) Binding < 4 x 10^{-3} (-3CMO) -3-Carboxymethyloxime.
(-6βHS) -6β-Hemisuccinate. (-11αHS) -11α-Hemisuccinate.

Rabbit D failed to produce antiserum able to bind to any of the iodhistamine A4 radioligands, and rabbit E died from unknown causes; both are omitted from Table 6. Rabbits A, B and C, immunised with A4-3CMO-BSA, produced antisera of high titre with ^{125}I-A4-3CMO label. These antisera produced no binding with ^{125}I-A4-6βHS label and only Ab C bound ^{125}I-A4-11αHS label with a moderate titre. Rabbits D and F, immunised with A4-6βHS-BSA, produced antisera which did not bind ^{125}I-A4-3CMO or ^{125}I-A4-11αHS labels, and only antibody F had a high titre with the ^{125}I-A4-6βHS label. Rabbits H, I and J, immunised with A4-11αHS-BSA, produced antisera of high titre with ^{125}I-A4-11αHS label but no binding with ^{125}I-A4-3CMO or ^{125}I-A4-6βHS labels. Results illustrate that titre is variable and depends on the position of the iodohistamine linkage to A4 and the position of linkage of BSA in the immunogen.

3.1.1. Suitable Antisera for Microencapsulation

In cases where titre was >1/4,000, antisera were encapsulated and possible displacement of the radioligand by unlabelled A4 was investigated. Ab's A, B, C; F; and H, I, J were microencapsulated for evaluation with ^{125}I-A4-3CMO; ^{125}I-A4-6βHS or ^{125}I-A4-11αHS labels.

3.1.2. Selection of Microencapsulated Ab (Mic.Ab) Dilution

Microencapsulated Ab preparation was diluted 1/5 to 1/160 with RIA buffer. Dilutions of microencapsulated Ab's A, B, and C were incubated with ^{125}I-A4-3CMO label. Dilutions of microencapsulated Ab F were incubated with ^{125}I-A4-6βHS label. Dilutions of microencapsulated Ab's H, I, J and C were incubated with ^{125}I-A4-11αHS label. A microcapsule dilution curve was produced, Table 7, and separation of bound and free fractions was achieved by centrifugal precipitation of microcapsules. The dilutions of microencapsulated Ab's giving a B/T of 40%, shown in Table 8, were calculated from the dilution curves and were used in the following dose-response and cross reaction experiments.

3.1.3. Comparison of Standard Curves for Combinations of Ab and Label

Dose-response curves (Table 9) were obtained by incubating thawed androstenedione standard solutions (200 µl) with microencapsulated Ab's A, B, C; F; and C, H, I, J with the labels, ^{125}I-A4-3CMO; ^{125}I-A4-6βHS; and ^{125}I-A4-11αHS respectively. The resulting standard curves are shown in Fig.11. Microencapsulated Ab's A, B and C with the ^{125}I-A4-3CMO label gave the most sensitive standard curves. Sensitive standard curves were also obtained with microencapsulated AB's C and I and the ^{125}I-A4-11αHS label. A less sensitive standard curve was produced by microencapsulated Ab F with the ^{125}I-A4-6βHS label.

Table 7. Protocol for the construction of dilution curves with antibody-containing microcapsules.

Tubes	Volume (µl)	
	For Total Counts	For Mic.Ab Dilution Curve
Mic.Ab	-	200
^{125}I-A4 (10,000 cpm)	200	200
Buffer		200
*Incubated 45 min		
Wash solution	-	3,000

Centrifuged at 2,000 rpm for 3 min at 4 °C. Precipitates were rewashed and counted. Mic.Ab dilution curves were constructed.

(*) In a 37 °C water bath. (Mic.) Microencapsulated.

Table 8. Summary of dilutions of microcapsules at which androstenedione (A4) antibodies bind 40 percent of the label.

		^{125}I-A4 label		
Immunogen	**Mic.Ab**	**-3CMO**	**-6βHS**	**-11αHS**
	A	1/40	-	-
-3CMO	B	1/100	-	-
	C	1/80	-	1/10
-6βHS	F	-	1/150	-
	H	-	-	1/40
-11αHS	I	-	-	1/110
	J	-	-	1/10

(Mic.) Microencapsulated. (-3CMO) -3-Carboxymethyloxime.
(-6βHS) -6β-Hemisuccinate. (-11αHS) -11α-Hemisuccinate.

Table 9. Protocol for the construction of androstenedione (A4) dose-response curves.

Tubes	Volume (µl)	
	For Total Counts	For A4 Standard Curve
Standard	-	200
^{125}I-A4	200	200
Mic.Ab	-	200
	*Incubated 45 min	
Wash solution	-	3,000

Centrifuged at 2,000 rpm for 3 min at 4 °C. Precipitates were rewashed and counted. Dose-response curves were constructed.

(*) In a 37 °C water bath. (Mic.) Microencapsulated.

Fig. 11. Comparison of dose-response curves for different combinations of [^{125}I]-radiolabels and microencapsulated antibodies. Separation of antibody-bound from -free fractions was achieved by centrifugation of microcapsules.

Androstenedione standard solutions were incubated with:

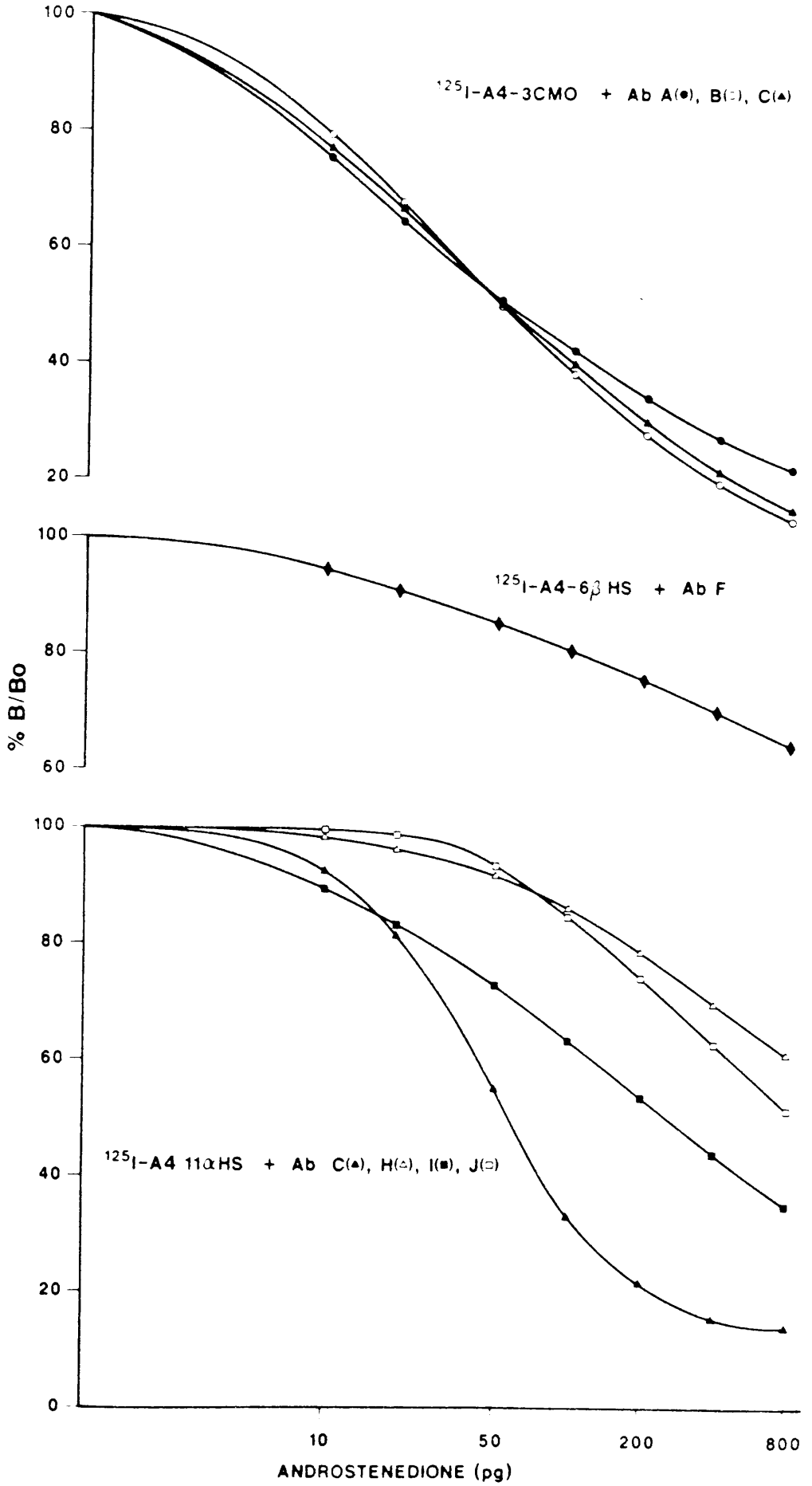
-3CMO label and -3CMO antibodies A, B and C (top panel);

-6βHS label and -6βHS antibody F (middle panel); and

-11αHS label and -11αHS antibodies H, I and J

or -3CMO antibody C (bottom panel).

(Mic.) Microencapsulated.


100
80
60
40
20
^{125}I-A4-3CMO + Ab A(●), B(□), C(▲)
100
80
60
^{125}I-A4-6β HS + Ab F
% B/Bo
100
80
60
40
20
0
^{125}I-A4 11αHS + Ab C(▲), H(△), I(■), J(□)
10
50
200
800
ANDROSTENEDIONE (pg)

Insensitive standard curves were produced by microencapsulated Ab's H and J with the ^{125}I-A4-11αHS label. No further experiments were carried out which involved Ab's F, H and J because of the insensitive standard curves which resulted. A summary of sensitivities is given in Table 10.

3.2. ANTIBODY SPECIFICITY.

Cross reaction with 21 related steroids was studied in systems where sensitivity was <15 pg (defined as a 10% decrease in binding of the zero standard) by the method outlined by Abraham *et al.*[119]

i) Initial cross reaction study: Each competing steroid (30,000 pg/200 µl) and androstenedione standard was assayed as shown in Table 9 and the value B/Bo was calculated. Those competing steroids which had a B/Bo > 50% and hence a cross reaction < 0.5% were not investigated further. Competing steroids which had a B/Bo < 50% and, therefore, a cross reaction > 0.5% were investigated in more detail to determine the exact cross reaction.

ii) Final cross reaction study: As steroids cross react to different degrees e.g. with modest or high competition, it was necessary to construct a standard curve covering the appropriate range. A range of steroid concentrations (A or B) was selected (Table 11): Range A for steroids which showed modest competition (cross reaction > 0.5%), and Range B for steroids which showed high competition (cross reaction >> 0.5%).

Table 10. Summary of androstenedione standard curve sensitivities* (pg).

		^{125}I-A4 label		
Immunogen	**Mic.Ab**	**-3CMO**	**-6βHS**	**-11αHS**
	A	3.6	-	-
-3CMO	B	4.8	-	-
	C	3.6	-	13
-6βHS	F	-	26	-
	H	-	-	70
-11αHS	I	-	-	9
	J	-	-	68

(*) Sensitivity is the amount of unlabelled antigen required to produce a 10 percent drop in the binding of the zero standard. (Mic.) Microencapsulated. (-3CMO) -3-Carboxymethyloxime. (-6βHS) -6β-Hemisuccinate. (-11αHS) -11α-Hemisuccinate.

Table 11. Serial doubling dilutions of standard solutions of steroids which cross react with modest competition (type A; 30,000 pg/200 µl) or with high competition (type B; 20,000 pg/200 µl).

	Steroid Type (pg)	
Standard	**A**	**B**
0	0	0
1	469	156
2	938	312
3	1,875	625
4	3,750	1,250
5	7,500	2,500
6	15,000	5,000
7	30,000	10,000
8	-	20,000

Dose-response curves were derived with the standard or competing steroid by the method outlined in Table 9. The standard curves were constructed and the mass of each competing steroid needed to displace 50% of the bound labelled androstenedione was determined. Cross reactions were calculated for the competing steroids and the results are shown in Table 12.

Steroids which exhibited high cross reaction were 5α-androstan-3, 17-dione and 1,4-androstadien-3,17-dione for all antibodies tested. Andrenosterone showed high cross reaction with microencapsulated Ab's C and I with ^{125}I-A4-11αHS label and moderate interference with microencapsulated Ab C and ^{125}I-A4-3CMO label. This illustrates the importance of the linkage position of iodohistamine to the steroid nucleus, which is the only difference between these two systems and yet, there is a marked difference in specificity. High cross reactivity was shown by 5β-androstan-3,17-dione with microencapsulated Ab B and ^{125}I-A4-3CMO label and moderate cross reactivity with microencapsulated Ab C with both labels. These findings are shown in Fig.12. Minor interference was shown by etiocholanolone with microencapsulated Ab C and ^{125}I-A4-3CMO. These findings show that microencapsulated Ab A has the least interference from competing steroids. Antibody I with ^{125}I-A4-11αHS system was not evaluated further because of poor displacement of label over the range of concentrations of A4 likely to occur in patients' samples.

Table 12 . Cross Reaction* Studies

	Assay System+				
	A,3/3	B,3/3	C,3/3	C,3/11	I,11/11
Androstenedione	100	100	100	100	100
5α-androstane-3,17-dione	37	42	40	26	17
1,4-androstadiene-3,17-dione	18	56	43	27	25
4-androstene-3,11,17-trione	1	3	5	38	31
5β-androstane-3,17-dione	2	21	6	6	2
5α-androstane-3α,17α-diol,	<0.5 in all systems				
5α-androstane-3α,17β-diol,					
5α-androstane-3β,17α-diol,					
5-androstene-3β,17β-diol, androsterone,					
dehydroepiandrosterone, 5α-dihydrotestosterone, testosterone, epitestosterone, etiocholanolone, 11-deoxycorticosterone, cortisol, progesterone,					
17-hydroxyprogesterone, 17β-oestradiol					

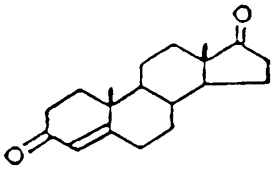
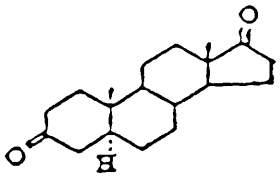
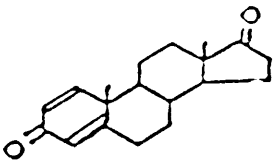
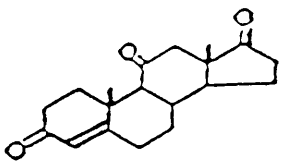
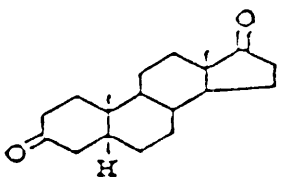
(*) All results expressed as % cross reactivity= [(mass of A_4 required to displace 50% of bound labeled A_4 / mass of steroid under test required to displace 50% of bound labeled A_4)] x 100.

(+) Rabbit, and position of attachment to steroid nucleus of immunogen/ label.

Fig. 12. Steroids showing major cross reacton with androstenedione (A4) antibodies with ^{125}I-A4-3CMO or ^{125}I-A4-3CMO label.

A4-3CMO antibodies A and B with a ^{125}I-A4-3CMO label;
A4-3CMO antibody C with ^{125}I-A4-3CMO or ^{125}I-A4-11αHS label and;
A4-11αHS antibody I with ^{125}I-A4-11αHS label.

Results are expressed as percent cross reacton on the basis that androstenedone cross reacts 100% with androstenedione antibody.

IMMUNOGEN	A	B	C	C	I
BSA linked to A_4 at position	3	3	3	3	11
IODOHISTAMINE					
linked to A_4 at position	3	3	3	11	11
 ANDROSTENEDIONE	100	100	100	100	100
 5α-ANDROSTANEDIONE	36.6	42.0	40.4	26.3	17.1
 1,4-ANDROSTADIENE-3,17-DIONE	17.9	56.2	42.7	27.4	24.8
 4-ANDROSTEN-3,11,17-TRIONE (Adrenosterone)	0.7	2.7	5.4	38.4	31.1
 5β-ANDROSTAN-3,17-DIONE	2.1	21.3	5.7	6.2	2.1

3.3. AN ALTERNATIVE (SECOND ANTIBODY) SEPARATION METHOD

An alternative method of separating bound and free fractions using a soluble second antibody (Ab-2), donkey anti-rabbit IgG, was investigated because the microcapsule dilution of Ab C used with ^{125}I-A$_4$-11αHS was low and wasteful of antibody. Donkey anti-rabbit IgG and normal rabbit serum (DAR/NRS) dilutions were optimised and antibody titres were reassessed for each antiserum pool.

3.3.1. Determination of the Soluble Second Antibody Dilution

DAR and NRS were diluted with RIA buffer 1/2 to 1/640, respectively. A dilution curve for DAR at each NRS dilution was obtained, Table 13. Dilutions of DAR and NRS were optimised and second antibody solution (400 µl of a mixture of 1/40 DAR and 1/500 NRS in assay buffer) was used in all following experiments to separate bound and free fractions.

This was prepared by mixing RIA buffer with donkey anti-rabbit antiserum and adding normal rabbit serum immediately before use. Volumes were chosen so that 1 l of reagent would contain 25 ml of antiserum and 2 ml of normal rabbit serum. The amount of reagent prepared was set by the number of samples to be determined.

Table 13. Protocol for the construction of a dilution curve with soluble second antibody.

Tubes	Volume (µl)	
	For Total Counts	For Dilution Curve
Ab C (1/20,000)	-	200
^{125}I-A4-11αHS (10,000 cpm)	200	200
* Incubated 45 min		
DAR^{+} IgG	-	200
NRS^{++}	-	200
Incubated overnight at 4 °C		

Centrifuged at 3,000 rpm for 60 min at 4 °C. Supernatants aspirated carefully and discarded. The precipitates were counted and DAR/NRS dilution chequerboard constructed.

(*) In a 37 °C water bath. (+) Donkey anti-rabbit. (++) Normal rabbit serum.

3.3.2. Reassesment of Antibody Titre Using a Soluble Second Antibody Separation

Three pools of antisera (A, B and C) were prepared by mixing a volume of serum (1 ml) from each high titre bleed. Each pool was diluted 1/100 to 1/160,000 with RIA buffer. Antibody dilution curves were derived by the method shown in Table 14. The titres for antisera pools were 100, 50, 60 and 20×10^{-3} for Ab's A, B and C each with ^{125}I-A4-3CMO label and Ab C with ^{125}I-A4-11αHS, respectively.

3.4. Measurement of Androstenedione in Serum

To obtain more information on specificity, patients' serum samples were analysed and the results were compared to the routine method, which is based on a tritiated radioligand.

3.4.1. Comparison of RIA Systems

Androstenedione was extracted from serum samples then measured by four different RIA systems, Ab's A, B, and C each with ^{125}I-A4-3CMO and Ab C with ^{125}I-A4-11αHS. The RIA procedure is shown in Table 15. Concentrations of A4 obtained vary greatly depending on the reagents used, although sensitivity and specificity studies of the reagents are similar.

Table 14. Protocol for the construction of dilution curves for androstenedione (A4) antibody pools using a soluble second antibody separation.

Tubes	Volume (µl)	
	For Total Counts	For Ab Dilution Curve
Ab	-	200
^{125}I-A4 (10,000 cpm)	200	200
	*Incubated 45 min	
Soluble Ab-2	-	400
	Incubated overnight at 4 °C	

Centrifuged at 3,000 rpm for 60 min at 4 °C. Supernatant was carefully aspirated and discarded. The precipitates were counted and Ab dilution curves constructed.

(*) In a 37 °C water bath. (Ab-2) Second antibody.

Table 15. Protocol for the measurement of androstenedione (A4) in serum samples by radioimmunassay following solvent extraction.

Tubes	Volume (µl) For Total Counts	For A4 Standard Curve	For Serum Sample
Serum sample in 10 ml tube	-	-	50
Ethanolic standard in 5 ml tube	-	50	-
Hexane:ether (4:1 by vol.)	-	3,000	3,000
Vortex sample tubes for 4 min. Decant solvent phase into 5 ml tubes following immersion in a bath of solid carbon dioxide in methanol. Evaporate solvent+ from all tubes.			
Ab	-	200	200
^{125}I-A4 (10,000 cpm)	200	200	200
* Incubated 45 min			
Soluble Ab-2	-	400	400
Incubated overnight at 4 °C			
Centrifuged at 3,000 rpm for 60 min at 4 °C. Supernatants aspirated carefully and discarded. The precipitates counted and dose-response curve constructed.			

(+) Under a vacuum at 45°C. (*) In a 37 °C water bath.

(Ab-2) Second antibody.

The results, shown in Fig.13, are compared against the routine method. Androstenedione was measured again in samples by two systems Ab C with ^{125}I-A4-3CMO or ^{125}I-A4-11αHS. However, to reduce sensitivity in the system employing ^{125}I-A4-3CMO, the volume of serum was decreased to 20 µl. The results are similar when Ab C is used with either label.

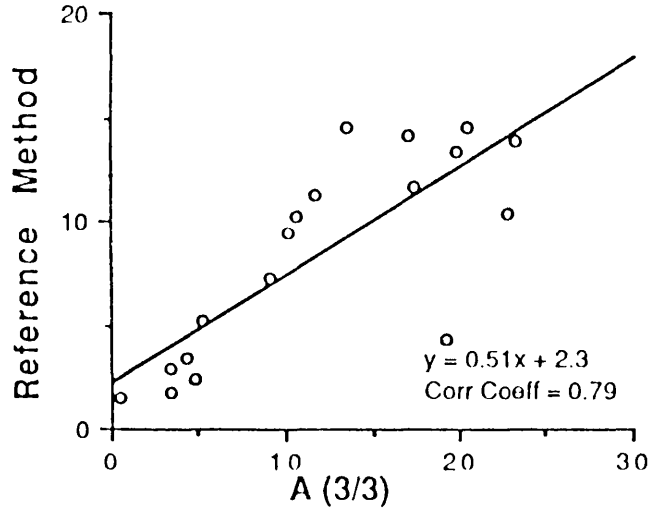
3.4.2. Final Selection of RIA System

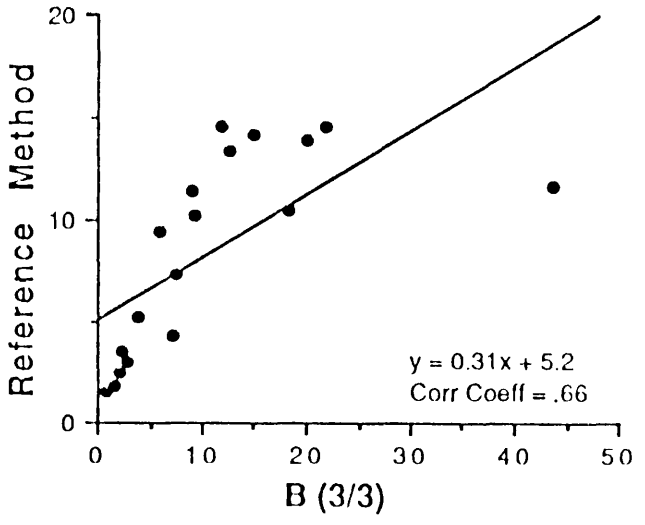
^{125}I-A4-3CMO was selected over ^{125}I-A4-11αHS when used with Ab C because of better sensitivity (requiring only 20 µl of sample) and a better correlation with the routine method. A larger comparison between the selected method and the "in house" method, conducted with 58 patients' samples, confirmed preliminary findings. The correlation coefficient was 0.94 with a slope of 1.02 and an intercept of 0.9 nmol/l, compared with 0.96, 1.02, and 1.1 nmol/l, respectively, for the preliminary study. Good comparison was obtained only if hexane:ether (4:1 by vol.) was used as the extraction solvent for the method with radioiodine label. If ether alone was used results obtained were considerably higher.

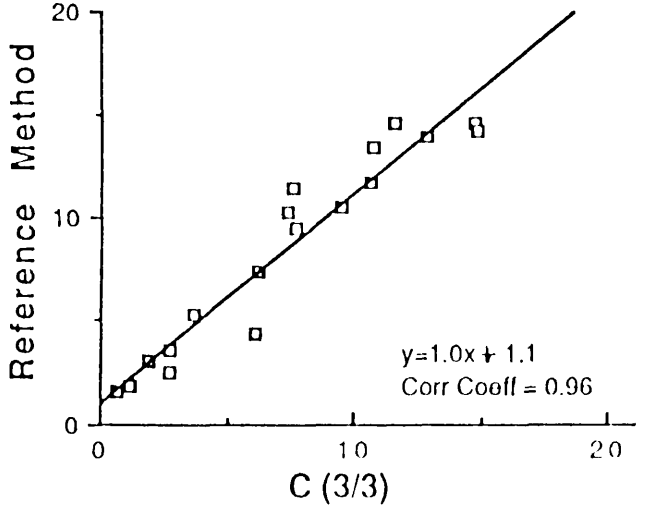
3.4.3. Measurement of A4 in Serum Samples by Direct Means

The selected method, Ab C in combination with ^{125}I-A4-3CMO using a soluble second antibody separation stage, was applied to the measurement of A4 directly in serum without prior solvent extraction by the procedure shown in Table 16. All A4 levels were greater than 28 nmol/l. This experiment was repeated using the selected reagents at low pH (Citrate buffer pH 4.0) to avoid

Fig. 13. Comparison of serum androstenedione results obtained by a reference method with a tritium label and by radioimmunnoassays with four different combinations of antiserum and iodinated radioligands. Androstenedione (A4) was extracted from serum samples and measured by a reference method and four different RIA systems. The latter employed a soluble second antibody to separate antibody-bound from the -free fracton. The four RIA systems were based on antibodies A, B and C each with ^{125}I-A4-3CMO label, coded A(3/3), B(3/3) and C(3/3), respectively and antibody C with ^{125}I-A4-11αHS, coded C(3/11). Serum A4 results of individual systems were compared with results of the reference method.


Reference Method
A (3/3)
y = 0.51x + 2.3
Corr Coeff = 0.79
0
10
20
30


Reference Method
B (3/3)
y = 0.31x + 5.2
Corr Coeff = .66
0
10
20
30
40
50


Reference Method
C (3/3)
y=1.0x + 1.1
Corr Coeff = 0.96
0
10
20

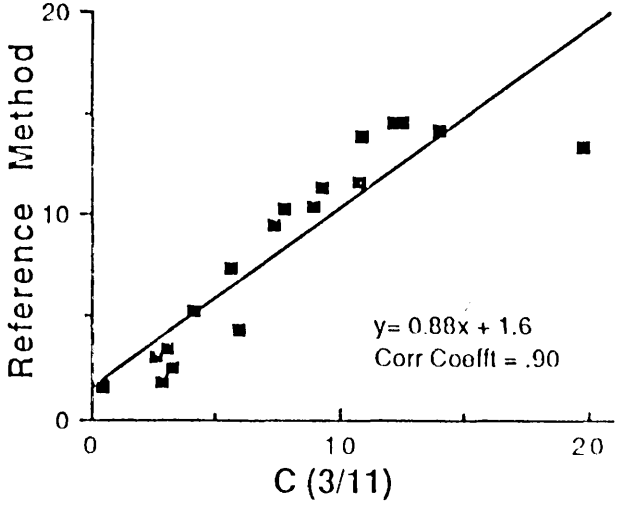

Reference Method
C (3/11)
y= 0.88x + 1.6
Corr Coefft = .90
0
10
20

Table 16. Protocol for the direct measurement of androstenedione (A4) in serum samples by radioimmunoassay without solvent extraction.

Tubes	Volume (µl) For Total Counts	For A4 Standard Curve	For Serum Sample
Ethanolic standard	-	5	-
	Evaporate to dryness		
Charcoal stripped serum	-	5	-
Serum sample	-	-	5
RIA buffer	-	50	50
	Incubated overnight at 22 °C		
^{125}I-A4-3CMO (10,000 cpm)	200	200	200
	Incubated 2 hours at 22 °C		
Soluble Ab-2	-	400	400
	Incubated overnight at 4 °C		

Centrifuged at 3,000 rpm for 60 min at 4 °C. Supernatants carefully aspirated and discarded. The precipitates were counted.

(Ab-2) Second antibody.

problems with specific steroid binding proteins. Again , all concentrations were greater than 28 nmol/l. Results showed that it was not possible to measure A4 directly in serum using this method and hence no further study was attempted in this area.

3.5. MEASUREMENT OF ANDROSTENEDIONE IN DRIED BLOOD SPOTS

Although the selected system was sensitive for a serum RIA, further improvement in sensitivity was sought to enable the system to be applied to the measurement of A4 in dried blood spots. A disc (5 mm) punched from a dried blood spot contains the equivalent of only 3 µl of serum and therefore it was necessary to obtain the most sensitive RIA possible. To achieve this, antisera from individual bleeds of rabbit C were reassessed to determine if one would provide the basis for an even more sensitive RIA. Once a fully optimised system was selected, measurement of A4 in dried blood spots following solvent extraction and by direct means was investigated.

3.5.1. Comparison of Antisera from Individual Bleeds of Ab C

An antibody dilution curve was obtained for the serum from each of seven bleeds of Ab C (denoted by Ab C-1, Ab C-2 etc., to Ab C-7) by the method shown in Table 14. Antibody titres were calculated and dose-response curves were derived as shown in Table 17, using each antibody at the appropriate dilution.

Table 17. Protocol for the construction of an androstenedione (A4) dose-response curve for each bleed from rabbit C.

Tubes	Volume (µl) For Total Counts	Volume (µl) For A4 Standard Curve
Ethanolic standard	-	20
	Evaporated to dryness	
Ab C-n	-	200
^{125}I-A4-3CMO	200	200
	Incubated 2 hours at 22 °C	
Soluble Ab-2	-	400
	Incubated overnight at 4 °C	

Centrifuged at 3,000 rpm for 60 min at 4 °C. Supernatants aspirated carefully and discarded. The precipitates counted and dose-response curves were constructed.

(Ab-2) Second antibody.

(n) Number 1 to 7 denoting antisera from each of seven bleeds.

The sensitivity of each resulting standard curve was calculated and the most sensitive standard curve was selected for use with ^{125}I-A4-3CMO in measuring A4 in dried blood spots by RIA. Antibody titres were in the range 12-17 x 10^{-5}. The sensitivity of each resulting standard curve was in the range: 0.2 - 0.5 nmol/l. The most sensitive standard curve was produced by Ab C-7 and hence this was used with ^{125}I-A4-3CMO to measure A4 in dried blood spots by RIA.

3.5.2. RIA for A4 Following Solvent Extraction from Dried Blood Spots

Androstenedione was extracted into hexane:ether (3 ml of 4:1 v/v) from a disc (5 mm) punched out from a series of blood spot standards and samples. Using the selected reagents (Ab C-7 with ^{125}I-A4-3CMO) a RIA was applied to blood-spot extracts, as shown in Table 18.

3.5.3. Measurement of Androstenedione in Dried Blood Spots by Direct Means

A disc (5 mm) was punched out from a series of blood-spot standards into a test tube and eluted overnight in saline (50 µl) at 22 °C. Using the optimised RIA, A4 was measured directly in blood-spot eluates by the method shown in Table 19.

Table 18. Protocol for a radioimmunoassay for androstenedione (A4) in dried blood-spot extracts.

Tubes	Volume (µl) For Total Counts	For A4 Standard Curve	For Unknown Sample
A4 extract	-	+	+
Ab C-7	-	200	200
^{125}I-A4-3CMO (10,000 cpm)	200	200	200
Incubated 2 hours at 22 °C			
Soluble Ab-2	-	400	400
Incubated overnight at 4 °C			

Centrifuged at 3,000 rpm for 60 min at 4 °C. Supernatants were aspirated, discarded and the precipitates counted. The standard curve was constructed and the results for samples were calculated.

(Ab-2) Second antibody.

Table 19. Protocol for the construction of an androstenedione (A4) dose-response curve in dried blood spots by direct means.

	Volume (µl)	
Tubes	For Total Counts	For A4 Standard Curve
Blood Spot Disc	-	+
Saline	-	50
Eluted overnight at 22 °C		
Ab C-7	-	200
^{125}I-A4-3CMO (10,000 cpm)	200	200
Incubated 2 hours at 22 °C		
Soluble Ab-2	-	400
Incubated overnight at 4 °C		

Centrifuged at 3,000 rpm for 60 min at 4 °C. Supernatants were carefully aspirated then discarded and the precipitates counted. The dose-response curve was constructed and the results for samples were interpolated.

(Ab-2) Second antibody.

CHAPTER 4. EVALUATION OF SELECTED A4 RIA SYSTEM: EXPERIMENTS AND RESULTS

Following the above preliminary evaluation of reagents, Ab C with ^{125}I-A4-3CMO label, a more detailed investigation was carried out. The parameters, accuracy, precision, and parallelism were investigated for the serum and blood spot RIAs.

4.1. ACCURACY

The extraction efficiency was determined by measuring the analytical recovery of A4 from serum. A volume of stock [^{3}H]-A4 was evaporated in two glass tubes. A volume (5 ml) of serum or ethanol was added to each tube and the tubes incubated overnight at 22 ^{o}C. Serum (20 µl) was extracted into hexane:ether (3 ml of 4:1 by volume) and transferred into scintillation vials. Solvent (3 ml) was also added to vials containing [^{3}H]-A4 in ethanol (20 µl). Solvent was evaporated from vials under a stream of air and scintillation fluid was added (4 ml) then the contents mixed. Beta-radiation was detected on a scintillation counter. Extraction with hexane:ether gave a mean analytical recovery of 96% (n=10).

The recovery of unlabelled A4 added to serum extracted by solvent was investigated. Androstenedione in ethanol was evaporated in a series of tubes to give a range of 25-125 nmol/l in increments of 25. To one series was added female serum (100 µl) and to another male serum. Androstenedione was measured in a volume (20 µl) of neat and spiked samples following solvent extraction. Results for the recovery of added unlabelled A4 extracted by solvent are shown in Table 20. Mean recoveries of A4 added, in increasing amounts, to serum samples from a woman and a man were 98% and 95.5%, respectively.

4.2. PRECISION

Figure 14 illustrates a typical standard curve and precision profile for the serum RIA. The range of the standard curve was 1 to 21 nmol of A4 per litre, as interpolated from the precision profile by the method of McConway et al,[153] i.e. the points at which the CV was $\leq$22%. Within-batch and between-batch precision were assessed. For quality control pools, androstenedione was added to normal male serum to give concentrations of approximately 3, 9, and 16 nmol of A4 per litre. Aliquots were stored frozen for up to 6 months. To determine within-batch precision, each pool was assayed ten times on one occasion, the results were averaged and the standard deviation (SD) and coefficient of variation (CV) were calculated. To determine between-batch precision each pool was assayed on 10 separate occasions the results averaged and the SD and CV were calculated. The within- and the between-batch CVs were consistently <10% for low-, medium-, and high-concentration serum pools.

Table 20. Analytical recovery of androstenedione added to serum obtained from two normal adult volunteers.

	Androstenedione (nmol/l)			
	Added	Determined	Expected	Recovery (%)
Female	0	2.9	-	-
	5	7.8	7.9	99
	10	13.1	12.9	101
	15	18.1	17.9	101
	20	20.8	22.9	91
Male	0	1.9	-	-
	5	6.3	6.9	91
	10	10.7	11.9	90
	15	18.0	16.9	107
	20	20.8	21.9	95

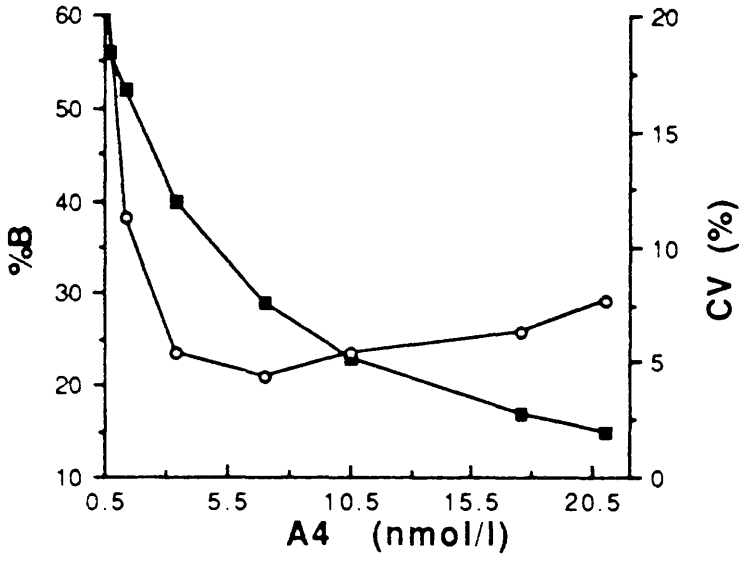
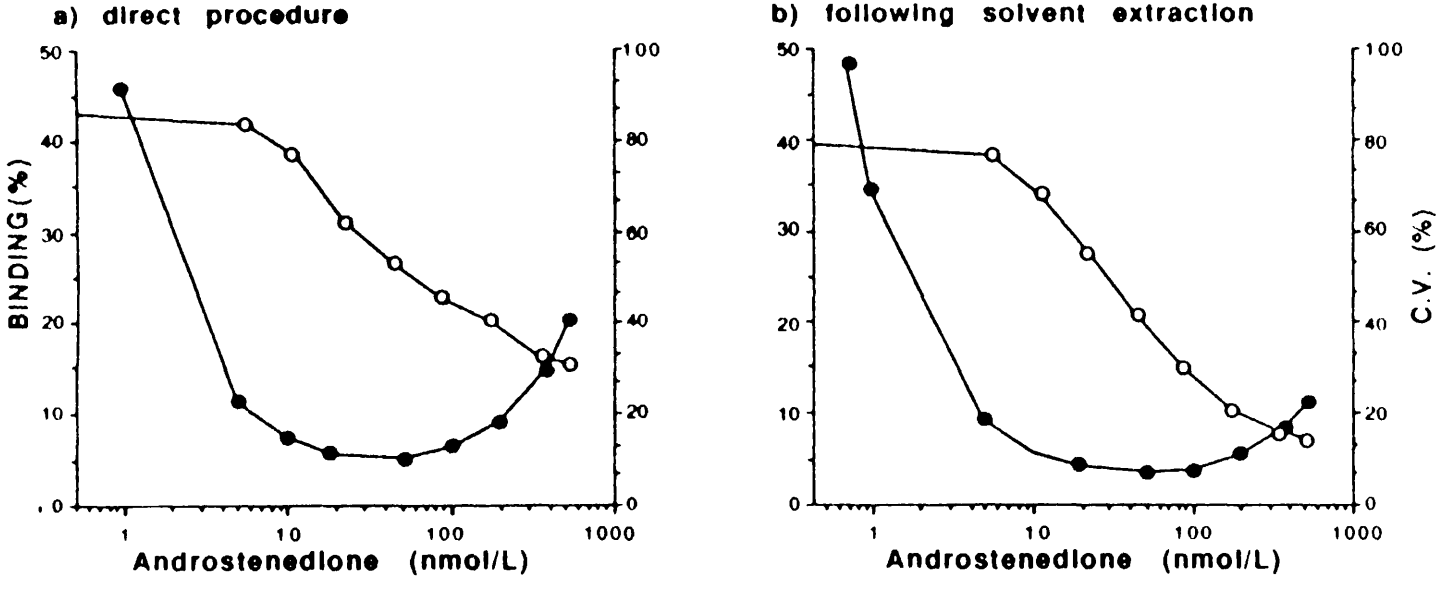


Fig. 14. A typical standard curve (-■-) and precision profile (-o-) for a serum RIA for androstenedione (A4). A4 was extracted from serum samples or evaporated from ethanolic standard solutions and incubated with antibody C and ^{125}I-A4-3CMO label for 45 min at 37 °C. After an overnight incubation with soluble second antibody at 4 °C, separation of bound and antibody-free A4 was obtained by centrifuging for 60 min at 4 °C and discarding the supernatants. The percentages bound (B) and coefficient of variation (CV) were plotted against A4 concentration

Measurement of samples from the U.K. National External Quality Assessment Scheme gave results that compared well with those from the other 15 participating laboratories. During six months the bias was +12.2% and the variability of bias was 6.9%.

A typical standard curve and precision profile for the direct and extraction blood spot assays are illustrated by Fig. 15. From the precision profiles, the ranges of the standard curves for A4, calculated as above, were 5-300 and 4-525 nmol/l, respectively. Blood spot quality controls were prepared by spiking a whole blood matrix with androstenedione to give concentrations of 25, 50, 100, and 200 nmol of A4 per litre. To determine within-batch precision, each pool was assayed twenty times on one occasion, the results were averaged and the SD and CV were calculated. Between-batch precision was determined by assaying each pool in duplicate on ten separate occasions, the results averaged and the SD and CV were calculated. The mean interassay CVs were <16% and <10%, and the mean intra-assay CVs were <13% and <10% for the direct and extraction methods, respectively, for blood spot controls.

Fig. 15. A precision profile (-●-) with a typical standard curve (-o-) for blood-spot A4 radioimmunnoassay measured a) directly in dried blood spots eluted from filter paper or b) after solvent extraction of the eluted blood. Dried blood-spot discs (5 mm) were eluted overnight into saline. Ab C-7 and ^{125}I-A4-3CMO label were incubated for 2 h at 22 ^{o}C a) directly with the eluates and b) with A4 extracted from the eluates. After an overnight incubation at 4 ^{o}C with soluble second antibody, separation of antibody-bound and -free was obtained by centrifugation for 60 min at 4 ^{o}C and discarding the supernatants. The percentages binding and coefficient of variation (CV) were plotted against A4 concentration.


a) direct procedure
BINDING (%)
50
40
30
20
10
0
100
80
60
40
20
0
1
10
100
1000
Androstenedione (nmol/L)
b) following solvent extraction
C.V. (%)
Androstenedione (nmol/L)

4.3. PARALLELISM

Parallelism of the dose-response relationship for the standard and the unknown indicates that a sample responds in the same way over the working range as analyte added to an analyte-free matrix.

To assess parallelism, A4 was measured in serum from four patients with increased A4 concentrations at various dilutions. Serum samples were diluted 1/2, 1/4, 1/8 and 1/16 with RIA buffer. A volume of neat serum (20 µl) and each dilution (20 µl) was extracted. By diluting the sample, the actual volume of serum extracted was 10, 5, 2.5 and 1.25 µl. Similar values for all dilutions were obtained if the concentrations fell within the limits of the standard curve, as shown in Table 21. These results suggest lack of interference from unknown compounds in serum samples.

It was not possible to obtain blood in sufficient volume from an infant with grossly increased A4 (i.e. untreated CAH) to assess parallelism in the blood spot RIAs.

Table 21. Parallelism Study: Measurement at different dilutions of sera from four patients with increased androstenedione concentrations.

Sample dilution	Patient A	B	C	D
Undiluted	>21*	>21	>21	12.3
1:2	22.8	26.6	19.6	9.5
1:4	22.2	26.0	21.9	11.3
1:8	23.1	23.5	21.5	12.6
1:16	21.1	26.1	24.5	9.8

(*) All results expressed as nmol/l.

CHAPTER 5. CLINICAL EVALUATION OF THE SELECTED SYSTEM USED TO MEASURE A4 IN HUMAN SERUM AND BLOOD-SPOT SAMPLES

Concentrations of A4 were measured in serum and in blood spots under normal and pathological conditions.

5.1. CONCENTRATIONS OF A4 IN HUMAN SERUM

Concentrations of A4 in serum under normal and pathological conditions are shown in Table 22. The samples from normal healthy adult volunteers were collected between 0900 and 1700 hours. There is no significant difference in A4 values for males and females, but both groups show a significant reduction with age ($P = 0.032$ and 0.009 for males and females, respectively). Diurnal variation in a group of six normal adult male volunteers was demonstrated by a marked early morning peak in serum A4 (Fig. 16). Significantly increased concentrations ($P<0.001$) were found in women with polycystic ovarian disease (Table 22). Three patients with congenital adrenal hyperplasia were investigated who had urinary steroid metabolite profiles consistent with 21-hydroxylase deficiency, and results from serum 17-hydroxyprogesterone are included for

Table 22. Serum androstenedione concentrations in healthy adults and in patients with polycystic ovarian disease (PCO) or congenital adrenal hyperplasia (CAH).

		A_4 (nmol/l)	
Subject	**n**	**Mean**	**Range ($\bar{X} \pm 2SD$)**
Normal females			
18 - 40 years old	70	4.7	0.6 - 8.8
41 - 65 years old	32	3.9	0.9 - 6.8
Normal males			
18 - 40 years old	78	5.0	1.6 - 8.4
41 - 65 years old	21	3.9	1.2 - 6.6
PCO females	14	10.8	5.0 -16.7
CAH patients			
Untreated neonate		254 (3000)*	
Late-onset adult (untreated)		19 (12)	
12-year-old (inadequate replacement)			
pre-replacement		104 (313)	
2-h post dose		82 (149)	

(*) Numbers in parentheses indicate concentrations of 17-hydroxyprogesterone, nmol/l.

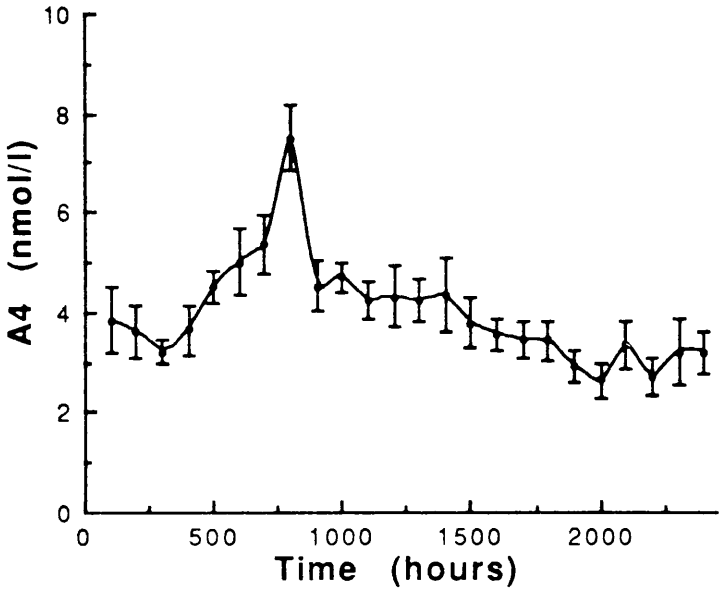


Fig. 16. Twenty-four-hour serum androstenedione profile: Androstenedione measured at hourly intervals in a group of six adult male volunteers. Results are represented as mean $\pm$ S.E.M.

comparison (Table 22). Grossly increased A4 concentrations were found in both the untreated neonate and in the 12-year-old who was receiving steroid replacement therapy. The late-onset patient was a 36-year-old woman who presented with increasing hirsutism.

5.2. CONCENTRATIONS OF A4 IN BLOOD-SPOT SAMPLES

Direct analysis of A4 was performed on blood-spot samples collected on filter-paper Guthrie cards from one infant with CAH, from infants born at term (n=106) or prematurely (n=110) or with respiratory distress syndrome (n=11). Also carried out was analysis of A4 after solvent extraction of blood spots from patients with CAH (n=9), and from infants born at term (n=85) or preterm (n=90) or with respiratory distress syndrome (n=18) (Table 23). The normal reference intervals for A4 for infants born at term were <4-22 and <5-88 nmol/l (99% confidence limits), respectively, with or without solvent extraction. Higher concentrations of A4 were found in the direct assay than in the extraction assay, which indicates possible interference from steroid conjugates that are not solvent extractable. Increased A4 concentrations were found in some samples from infants born prematurely or who were suffering respiratory distress syndrome, in all cases of 21-hydroxylase deficiency, and in one case of 11β-hydroxylase deficiency. The 11β-hydroxylase deficiency remained undiagnosed until the child reached the age of four, when signs of precocious puberty occurred. In this case A4 was measured in a filter paper blood-spot sample collected soon after birth 12 years previously.

Table 23. Androstenedione concentrations (nmol/l) in neonatal blood-spot samples measured by direct RIA and after solvent extraction.

	RIA Method	
	Extraction	Direct
Term		
n	85	106
$\bar{x}$ (range)	7.2 (<4-22)	15.7 (<5-88)
99% conf. limits	<18	<61
Premature		
n	90	110
$\bar{x}$ (range)	12.9 (<4-117)	30.3 (<5-175)
99% conf. limits	<84	<152
Stressed		
n	18	11
$\bar{x}$ (range)	26.4 (5-70)	60.9 (18-157)
99% conf. limits	<55	ID[a]
21-hydroxylase deficiency		
n	9[b]	1[c]
$\bar{x}$ (range)	234 (80->525)	107
11ß-hydroxylase deficiency		
n[d]	1[b]	0
	91	ID

[a]ID, insufficient data for statistical analysis.

[b]Retrospective analysis. [c]Prospective analysis.

[d]This boy was diagnosed at age 4 y due to the consequences of excessive androgens. Blood-spot determination was performed retrospectively 12 years after sampling.

CHAPTER 6. DISCUSSION and CONCLUSIONS

The purpose of this project was to develop a radioimmunoassay for the measurement of androstenedione in samples of human serum and, in particular, neonatal dried blood spots to permit the detection and home-monitoring of infants with congenital adrenal hyperplasia due to 21- and 11β-hydroxylase deficiencies.

Initially, the aim was to produce suitable antisera and ^{125}[I]-radiolabels. Antisera were raised in rabbits against three androstenedione derivatives, each conjugated to position 3, 6 or 11 on the steroid nucleus. Then three androstenedione radiolabels were prepared by linking ^{125}[I]-iodohistamine to position 3, 6 or 11 on the steroid nucleus. In addition, reagents eg. second antibody (soluble and solid-phase) and microencapsulated antibody were produced to separate the antibody-bound from the unbound fraction of androstenedione.

Preparation of specific steroid-protein conjugates were beyond the scope of this study and linkage positions were limited to 3, 6 and 11 by the availability of commercial material. Similarly, for the preparation of iodinated steroid labels, linkage positions were also limited to 3, 6 and 11 by the availability of steroid derivatives suitable for conjugation with iodohistamine.

Following antibody titre experiments, combinations of antisera and radiolabels were investigated to determine which systems yielded acceptable dose-response curves. Those which did were tested for sensitivity and specificity. Then, using each useful system, androstenedione was measured in serum samples and the results compared with the 'in-house' method employing a tritiated tracer. The best immunnoassay was developed further to measure androstenedione in dried blood spots from neonates, with or without solvent extraction. The latter method makes possible large-scale screening for and home-monitoring of CAH due to 21- and 11β-hydroxylase deficiencies.

The following is a discussion of the results found in this study.

A number of reports in the literature clearly indicate that the point of linkage of BSA to the steroid nucleus for the immunogen and the linkage position of iodohistamine to the steroid label are important considerations. Antisera have been raised against steroids linked to protein through many positions on the steroid nucleus. Ideally the position of linkage should lack a functional group, expose both ends of the molecule[154] and be such that the protein does not shield part of the steroid molecule.[155]

In this study it has been clearly illustrated that linkage positions of both label and immunogen can have a dramatic effect on assay performance. Both homologous (similar linkage positions for label and immunogen) and heterologous (different linkage positions for label and immunogen) systems were investigated. Linkage through position 6 provided the least immunogenic conjugate.

The combinations of antisera and tracers studied, demonstrated that, with one exception (Ab C), antisera did not bind a tracer which was linked at a different position from that of the immunogen i.e. heterologous systems. When antibody is directed against the bridge, as well as the steroid molecule in the immunogen, and the same bridge is used in the tracer, the antiserum may have a higher affinity for labelled steroid than unlabelled steroid. This problem is known as bridge recognition and results in the failure of unlabelled steroid to displace labelled steroid from the antibody binding site. For homologous systems, insensitive standard curves, possibly related to bridge recognition,[156] were found in the A4-6βHS and two of the three A2-11αHS systems, rendering such combinations impractical for development of blood spot assays. Bridge recognition did not occur in any of the -3CMO homologous systems because the A4-3CMO-iodohistamine radioligand was displaced from antisera at low A4 concentrations. These findings support the suggestion of Webb *et al* [157] that bridge binding may depend on the position of attachment to the steroid; interference is minimal when position 3 is used. In fact, homologous systems have been described that are sensitive enough to measure steroid hormone concentrations in serum, including cortisol,[158] 11-deoxycortisol,[159] aldosterone[160] and testosterone.[161] However, in this study, little success was obtained with homologous systems based on an 11-linkage, which yielded only one suitable combination of label and antibody (Ab I).

In cross-reaction studies, 5α-androstane-3,17-dione and 1,4-androstadien-3,17-dione interfered significantly for all systems tested. However high concentrations of these steroids are unlikely to occur in serum as they are present only as transitory metabolic intermediates.[162] For example, even in the human testis, where high concentrations of androgen precursors are present, these two steroids were not detected by gas chromatography-mass

spectrometry.[163] Significant cross reaction with 4-androsten-3,11,17-trione occured only when an ^{125}I-A$_4$-11αHS tracer was used.

All antisera tested were able to discriminate well between a double bond and any other group at position 17 and cross reacted strongly only with those steroids which, like androstenedione, have double bonds at positions 3 and 17 i.e. 5α-androstan-3,17-dione, 1,4-androstadien 3,17-dione and 4-androsten-3,11,17-trione. The last steroid showed high cross reaction with the microencapsulated Ab C : ^{125}I-A$_4$-11αHS heterologous system and the microencapsulated Ab I : ^{125}I-A$_4$-11αHS homologous system, but not with the A4-3CMO homologous system. It was expected that a high cross reaction may occur with the A4-11αHS homologous system as the bond at position-11 is the position of linkage in the conjugate, and so yields antisera which are not specific for that site.

Low cross reaction was exhibited by 5β-androstan-3,17-dione to all but one (microencapsulated Ab B) antiserum. This steroid differs in shape from 5α-androstan-3,17-dione which exhibits high cross reaction. The hydrogen atom in the β-position results in a steroid molecule which is no longer planar and hence prevents binding to the antibody. Whereas when the hydrogen atom is in the α-position, the plane of the steroid molecule is maintained and binding is unhindered. A very small difference in the spatial configuration of a molecule can give vastly different results with respect to antibody binding. These studies clearly illustrate that the linkage position of both the label and antibody plays an important part in the specificity of the assay system.

The most specific assay, as gauged by cross reaction studies, was obtained with antiserum raised in rabbit A against A_4-3-CMO-BSA and used with an A_4-3-CMO-iodohistamine label. In practice, however, when patients' serum samples were measured with this combination, they were positively biased.

The development of direct, non-extraction assays for A_4 may be impeded by the presence of interfering substances in samples. Goodall *et al* have demonstrated a high concentration of a circulating androstenedione conjugate.[164] In a preliminary study of a direct A_4 assay, using the selected reagents, all serum androstenedione concentrations, determined without solvent extraction, were gross over estimates. This suggests the presence of high concentrations of cross-reacting, polar conjugates of steroids. Direct steroid RIA's have been reported where results obtained are higher than those obtained after extraction. In a serum progesterone assay, the interference is thought to be due to cross reaction with steroid metabolites and conjugates.[165] Similarly, in the direct 17-hydroxyprogesterone blood spot screening assay, high cross reaction with a steroid conjugate (i.e. 17-hydroxypregnenolone sulphate) was apparent.[68]

Another source of interference can be the presence of specific steroid binding proteins in samples. Sex hormone binding globulin is a β-globulin capable of binding a variety of androgens, including androstenedione, with high affinity. Table 24 shows the relative binding affinities and association constants of some steroids binding to SHBG in blood plasma. Ways of blocking steroid binding to specific binding proteins have been described. These include the addition of excess testosterone[166] and oestradiol[167] to displace oestradiol and testosterone, respectively from SHBG, and the use of Danazol,[168] 17-pregn-4-en-20yno(2,3-d)isoxazol-17-ol, and cortisol[169] to displace progesterone from

Table 24. Relative binding affinities (RBA) of some steroids to sex hormone binding globulin.

STEROID	RBA ($x10^3$)	Ka (106 mol/l)
progesterone	7.1	8.8
androstenedione	23	29
5-androstenediol	970	1500
testosterone	1000	1600
dihydrostestosterone	2200	5500

(Ka) Affinity constant.

corticosteroid binding globulin. Various methods, including heat treatment,[170] low pH,[171] low pH with ANS[172] and salicylate[173] have been used to displace cortisol from endogenous binding proteins. Using the selected reagents at a low pH to avoid the possible problem of specific binding proteins, direct measurement of A_4 in serum was attempted again. As before, all serum A_4 concentrations were gross overestimates. Lowering the pH did not improve the depressed binding, which suggests that interference was not caused by specific binding proteins but cross-reacting polar conjugates of steroids.

The selected procedure for serum A_4 determination gave normal and pathological values in keeping with earlier reports.[162] A reduction in circulating A_4 after the age of 40, as reported by Hummer *et al*,[174] was confirmed. Whether gonads or adrenals are the source of this material is undecided, but Hummer *et al* reported a similar reduction in age matched pre- and post-menopausal women,[174] which suggests a reduced adrenal activity. Our finding that reduction is similar in males and females would support this view. Dehydroepiandrosterone sulphate, an androgen of exclusively adrenal origin, also shows a similar age related reduction.[175] Furthermore, the large adrenal contribution to circulating A_4 concentrations is indicated by the marked diurnal variation. Androgen concentrations in males have been reported to show a diurnal pattern[176] but the difference of over 100% for A_4 found in this study is more marked than the 40 - 50% change previously reported.[177]

A major practical problem with blood spot assays is the identification of suitable material to use for standards. Obtaining a large volume of healthy neonatal blood is impossible. Initially, blood from an adult female volunteer was used and endogenous adrenal androstenedione production was suppressed after dexamethasone. When such standards were used, however, maximum binding of label was obtained not by the zero standard but by neonatal samples.

The situation was rectified by using charcoal-stripped serum as the base for blood spot standards. Charcoal-stripped serum has been widely used for the preparation of standards for steroid assays, e.g. cortisol and oestradiol assays. The disadvantage of this type of matrix is that not only are steroids removed, but serum proteins are partially denatured which produces a marked reduction in electrolyte, protein and lipid concentrations.

Unlike measurement of A_4 in serum, direct measurement was possible with the blood-spot assay, using the selected reagents. The range of A_4 concentrations in eluates of blood spots collected from normal infants born at term and determined by RIA after solvent extraction, was slightly higher than that found recently by Egan *et al* i.e. 0.6-2.7 nmol/l whole blood (equivalent to 1.2 - 5.4 nmol/l serum).[178] Androstenedione concentrations were raised in some infants born prematurely or with respiratory distress syndrome whether they were measured directly or after solvent extraction. Similarly, previous studies have found elevated concentrations of 17-hydroxyprogesterone in plasma[179] and blood spots[68] from such infants. Direct assays always gave higher values for A_4 than extraction assays; this was also the earlier experience in the determination of 17-hydroxyprogesterone in neonatal blood spots.[68] Raised A_4 concentrations were found in all neonatal blood spots tested from cases of CAH.

On the basis of the results presented, it would be feasible to screen for CAH by determining A_4 by direct assay and then reassess after solvent extraction those samples giving high values. This strategy was successfully employed in a pilot screening study in Scotland for CAH using 17-hydroxyprogesterone estimations.[68] The working range of the A_4 assay described, in addition to the suitability of the blood spot specimen, is also appropriate for home monitoring of glucocorticoid replacement therapy in patients with CAH. Several groups have recommended this[180-182] because when serum androgens were studied in children with CAH to evaluate adequacy of treatment, A_4 was shown to reflect best the degree of control.[103] Concentrations of A_4 in serum can be used to monitor control in patients of all ages[180,182] and are less affected by individual steroid doses than are 17-hydroxyprogesterone measurements.[183] Others have described the use of reagents produced as part of this study in home monitoring of glucocorticoid replacement therapy in patients with CAH.[178]

The selected reagents described, provide a sensitive specific, precise, and robust immunoassay. It has been confirmed that elevated concentrations of A_4 occur in untreated classical cases of 21-hydroxylase deficiency (and 11β-hydroxylase deficiency) and are modestly raised in late-onset cases. Some increase may also occur in polycystic ovarian disease but further tests, such as the response of A_4 and 17-hydroxyprogesterone to Synacthen[184] and/or measurement of urinary steroid metabolites[185] are required to assist in differential diagnosis. The sensitivity of this RIA allows A_4 to be measured in a very small volume of sample which facilitates neonatal screening for CAH and home monitoring of the efficacy of replacement therapy by measurement of A_4 in dried

blood spots. The major advantages of A_4 over 17-hydroxyprogesterone determinations are that CAH due to 11β-hydroxylase deficiency can be diagnosed in addition to 21-hydroxylase deficiency and that it may be a better indicator of efficacy of glucocorticod replacement therapy.

One result of this project was the replacement of a cumbersome radioimmunoassay for serum androstenedione employing a tritiated radioligand. Now the method described in this thesis is used for routine diagnostic purposes in the Institute of Biochemistry, Royal Infirmary, Glasgow.

Using the androstenedione blood-spot assay developed for this thesis, I have shown that androstenedione is elevated in samples from infants with CAH due to either 21-hydroxylase deficiency or 11β-hydroxylase deficiency. The application of this assay to mass neonatal screening for CAH would have an important advantage over existing programmes. Now it is possible to extend screening to include the detection on CAH due to 11β-hydroxylase deficiency in addition to CAH caused by 21-hydroxylase deficiency.

REFERENCES

1. Cameron E D H, Jones T, Jones D, Anderson A B M, Griffiths K. Further studies on the relationship between C19- and C21 - steroid synthesis in the human adrenal cortex. J Endocrinol 1969; **45**: 215-30.

2. Miller W L. Molecular biology of steroid hormone synthesis. Endocrine Reviews 1988; **9**(3): 295-318.

3. Guillemin R, Schally A V. Recent advances in the chemistry of neuroendocrine mediators originating in the central nervous system. In: Nalbandov A V, ed. Advances in Neuroendocrinology. Urbana: University of Illinois Press, 1963; 314-28.

4. Ganong W F, Biglieri E G, Mulrow P J. Mechanisms regulating adrenocortical secretion of aldosterone and glucorticoids. Recent Prog Horm Res 1966; **22**: 381-414.

5. Ganong W F, Alpert L C, Lee T C. ACTH and the regulation of adrenocortical secretion. N Engl J Med 1974; **290**: 1006-10.

6. Mason P A, Fraser R, Semple P F, Morton J J. The interaction of ACTH and angiotensin II in the control of corticosteroid plasma concentration in man. J Steroid Biochem 1979; **10**: 235-9.

7. Jirasek J E. In: Development of the Genital System and Male Pseudo-hermaphroditism. Baltimore, Maryland: John Hopkins Press, 1971; 10-23.

8. Seron-Ferre M, Lawrence C C, Siiteri P K, Jaffe R B. Steroid production by definitive and fetal zones of the human fetal adrenal gland. J Clin Endocrinol Metab 1978; **47**(3): 603-9.

9. Huhtaniemi I. Studies on steroidogenesis and its regulation in the human fetal adrenal and testis. J Steroid Biochem 1977; **8**: 491-7.

10. Gower D B, Fotherby K. Biosynthesis of androgens and oestrogens. In: Mackin H L J, ed. Biochemistry of Steroid Hormones. Oxford, London: Blackwell Scientific Publications, 1975; 77-104.

11. de Crecchio L. Sopra un di caso apparenze virile in una donna. Morgagni 1865; **7**: 151-83.

12. Bartter F C, Forbes A P, Leaf A. Congenital adrenal hyperplasia associated with the androgenital syndrome: an attempt to correct its disordered hormone pattern. J Clin Invest 1950; **29**: 797-802.

13. Wilkins L, Lewis R A, Klein R, Rosenberg E. Suppression of androgen secretion by cortisone in a case of congenital adrenal hyperplasia: preliminary report. Bull John Hopkins Hosp 1950; **86**: 249-52.

14. Federman D D. In: Abnormal Sexual Development. Philadelphia: W B Saunders, 1968; 121-33.

15. Kuhnle U, Chow D, Rapaport R, Pang S, Levine L S, New M I. The 21-hydroxylase activity in the glomerulosa and fasciculata of the adrenal cortex in congenital adrenal hyperplasia. J Clin Endocrinol Metab 1981; **52**: 534-44.

16. Bouchard P, Kuttenn F, Mowszowicz I, Schaison G, Raux-Eurin M C, Mauvais-Jarvis P. Congenital adrenal hyperplasia due to partial 21-hydroxylase deficiency. A study of five cases. Acta Endocrinol (Copenh) 1981; **96**: 107-11.

17. Rosenwaks Z, Lee P A, Jones G S, Migeon C J, Wentz A C. An attenuated form of congenital virilising adrenal hyperplasia. J Clin Endocrinol Metab 1979; **49**: 335-9.

18. Kohn B, Levine L S, Pollack M S, *et al.* Late-onset steroid 21-hydroxylase deficiency. A variant of classical congenital adrenal hyperplasia. J Clin Endocrinol Metab 1982; **55**: 817-27.

19. Dupont B, Oberfield S E, Smithwick E M, Lee T D. Levine L S. Close genetic linkage between HLA and congenital adrenal hyperplasia (21-hydroxylase deficiency). Lancet 1977; **2**(1): 1309-11.

20. Levine L S, Sachmann M, New M I, *et al.* Genetic mapping of the 21-hydroxylase deficiency gene within the HLA linkage group. N Engl J Med 1978; **299**: 911-5.

21. Blankstein J, Faiman C, Reyes F I, Schroeder M L, Winter J S D. Adult-onset familial adrenal 21-hydroxylase deficiency. Am J Med 1980; **68**: 441-8.

22. Migeon C J, Rosenwaks Z, Lee P A, Urban M D, Bias W B. The attenuated form of congenital adrenal hyperplasia as an allelic form of 21-hydroxylase deficiency. J Clin Endocrinol Metab 1980; **51**: 647-9.

23. Pollack M S, Levine L S, O'Neill G T, *et al.* HLA linkage and B14, DRI, Bfs haplotype association with genes for late-onset and cryptic 21-hydroxylase deficiency. Am J hum Genet 1981; **33**: 540-50.

24. New M I, Levine L S. Congenital adrenal hyperplasia. In:Harris H, Hischhorn K, eds. Advances in Human Genetics. Vol.4, New York:Plenum Press, 1973; 251-326.

25. Bongiovanni A.M. Congenital adrenal hyperplasia and related conditions. In Stanbury, Wyngaarden, Fredrickson, eds. The Metabolic Basis of Inherited Disease. (4th Edn). New York: McGraw-Hill, 1978; 868-93.

26. Werder E A, Siebenmann R E, Knorr-Murset G, *et al.* The incidence of congenital adrenal hyperplasia in Switzerland. A survey of patients born in 1960 to 1974. Acta Paediatr Helv 1989; **35**: 5-11.

27. Shepard T H II, Clausen S W. Case of adrenogenital syndrome with hypertension treated with cortisone. Pediatrics 1951; **8**: 805-13.

28. Wilkins L, Crigler Jr J F, Silverman S H, Gardner L I, Migeon C J. Further studies on the treatment of congenital adrenal hyperplasia with cortisone. III. The control of hypertension with cortisone with a discussion of variations in the type of congenital adrenal hyperplasia and report of a case with probable defect of carbohydrate-regulating hormones. J Clin Endocrinol Metab 1952; **12**: 1015-30.

29. Eberlein W R, Bongiovanni A M. Congenital Adrenal hyperplasia with hypertension: unusual steroid pattern in blood and urine. J Clin Endocrinol Metab 1955; **15**: 1531-4.

30. Eberlein W R, Bongiovanni A M. Plasma and urinary corticosteroids in the hypertensive form of congenital adrenal hyperplasia. J Biol Chem 1956; **223**: 94-95.

31. Reynolds J W, Ulstrom R A. Studies of cortisol metabolism in a case of the hypertensive form of congenital adrenal hyperplasia: demonstration of the absence of 11β-dehydroxylation. J Clin Endocrinol Metab 1963; **23**: 191-6.

32. Bricaire H, Aubert P, Laudat P. Congenital adrenal hyperplasia due to disorders of 11β-hydroxylation. Prensa Med Argen 1966; **53**: 225-34.

33. Blunck W, Bierch J R. Congenital adrenal hyperplasia with 11β-hydroxylase deficiency. A case report and contribution to diagnosis. Acta Paediatr Scand 1968; **57**: 157-61.

34. Frasier S D, Horton R, Ulstrom R A. Androgen metabolism in congenital adrenal hyperplasia due to 11β-hydroxylase deficiency. Paediatrics 1969; **44**: 201-8.

35. Green O C, Migeon C J, Wilkins L. Urinary steroids in the hypertensive form of congenital adrenal hyperplasia. J Clin Endocrinol Metab 1960; **20**: 929-46.

36. New M I, Seaman M P. Secretion rates of cortisol and aldosterone precursors in various forms of congenital adrenal hyperplasia. J Clin Endocrinol Metab 1970; **30**: 36-71.

37. Loras B, Dazord A, Roux H, Bertrand J. Study of the level of production of cortisol, corticosterone, aldosterone and deoxycorticosterone in 4 cases of congenital adrenal hyperplasia due to 11β-hydroxylase deficiency. Revue Etud Clin Biol 1971; **16**: 585-90.

38. Kowarski A, Russell A, Migeon C J. Aldosterone secretion rate in the hypertensive form of congenital adrenal hyperplasia. J Clin Endocrinol Metab 1968; **28**: 1445-9.

39. Imai M, Igarashi Y, Sokabe H. Plasma renin activity in congenital virilising adrenal hyperplasia. Paediatrics 1968; **41**: 97-104.

40. Sizonenko P C, Riondel A M, Kohlberg I J, Paunier L. 11β-Hydroxylase deficiency: steroid response to sodium restriction and ACTH stimulation. J Clin Endocrinol Metab 1972; **35**: 281-7.

41. Sizonenko P C, Schindler A M, Kohlberg I J, Paunier L. Gonadotrophins, testosterone and oestrogen levels in relation to ovarian morphology in 11β-hydroxylase deficiency. Acta Endocrinol (Copenh) 1972; **71**: 539-50.

42. Rosler A, Russell A. Normotensive congenital adrenogenital syndrome with 11β-hydroxylase deficiency of 17α-hydroxylated steroids and high plasma aldosterone and testosterone. Isr J Med Sci 1975; **11**: 523 (abstract).

43. Solomon I L, Schoen E J. Blood testosterone values in patients with congenital virilising adrenal hyperplasia. J Clin Endocrinol Metab 1975; **40**: 355-62.

44. Wilkins L. In: The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence (3rd Edn). Springfield, Illinois: C C Thomas, 1965; 401-27.

45. Bergman P, Sjorgren B, Hakansson B. Hypertensive form of congenital adrenocortical hyperplasia. Analysis of a case with co-existing polycystic ovaries. Acta Endocrinol (Copenh) 1952; **40**: 555-64.

46. Madsen P O. Familial female pseudohermaphroditism with hypertension and penile urethra. J Urol 1963; **90**: 466-9.

47. Riddick D H, Hammond C B. Long term steroid therapy in patients with androgenital syndrome. Obstet Gynec (N Y) 1975; **45**: 15-20.

48. Gregory T, Gardner L I. Hypertensive virilising adrenal hyperplasia with minimal impairment of synthetic route to cortisol. J Clin Endocrinol Metab 1976; **43**: 769-74.

49. Gandy H M, Keutmann E H, Izzo A J. Characterisation of urinary steroids in adrenal hyperplasia: isolation of metabolites of cortisol, compound S, and deoxycorticosterone from a normotensive patient with androgenital syndrome. J Clin Invest 1960; **39**: 364-77.

50. Zachmann M, Vollmin J A, New M I, Curtuis H C, Prader A. Congenital adrenal hyperplasia due to deficiency of 11β-hydroxylation of 17α-hydroxylated steroids. J Clin Endocrinol Metab 1971; **33**: 501-8.

51. Gabrilove J L, Sharma D C, Dorfman R I. Adrenocortical 11β-hyroxylase deficiency and virilism first manifest in the adult woman. N Engl J Med 1965; **272**: 1189-94.

52. Maschler I, Weidenfeld J, Muller A, *et al.* A case of adrenogenital syndrome with aberrant 11β-hydroxylation. Acta Endocrinol (Copenh) 1977; **85**: 832-9.

53. Cathelineau G, Brerault J L, Fiet J, Julien R, Dreux C, Canivet J. Adrenocorticol 11β-hydroxylation defect in an adult women with postmenarchal onset of symptoms. J Clin Endocrinol Metab 1980; **51**: 287-91.

54. Rosler A, Levine L S, Schneider B, Novogroder M, New M I. The interrelationship of sodium balance, plasma renin activity and ACTH in congenital adrenal hyperplasia. J Clin Endocrinol Metab 1977; **45**: 500-12.

55. Finkelstein M, Schaefer J M. Inborn errors of steroid biosynthesis. Physiol Rev 1979; **59**: 353-406.

56. Bartter F C. Albright F, Forbes A P, Leaf A, Dempsey E, Carroll E. The effects of adrenocorticotrophic hormone and cortisone in the adrenogenital syndrome associated with congenital adrenal hyperplasia. An attempt to explain and correct its disordered hormonal pattern. J Clin Invest 1951; **30**: 237-51.

57. Hughes I A. Congenital and acquired disorders of the adrenal cortex. In: Bailey J D, ed. Clinics in Endocrinology and Metabolism. W B Saunders, Paediatric Endocrinology 1982; **11**(1): 89-125.

58. Conte F A, Grumbach M M. Abnormalities of sexual differentiation. In: Greenspan F S, Forsham P H, eds. Basic and Clinical Endocrinology. Los Altos, California: Lange Medical Publications, 1983; 414-37.

59. Grant J K, Beastall G H. Congenital hyperplasia and the measurement of 17-hydroxyprogesterone In: Clinical Biochemistry of Steroid Hormones Methods and Applications. Kent: Croom Helm, 1983; 209-13.

60. Korth-Schultz S, Virdis R, Saenger P, Chow D, Levine L S, New M I. Serum androgens as a continuing index of adequacy of treatment of congenital adrenal hyperplasia. J Clin Endocrinol Metab 1978; **46**(2): 425-58.

61. Hirschfeld A J, Fleshman J K. An unusually high incidence of salt-losing congenital adrenal hyperplasia in the Alaskan Eskimo. J Paediatr 1969; **75**: 492-4.

62. Childs B, Grumbach M M, Van Wyk J J. Virilising adrenal hyperplasia. A genetic and hormonal study. J Clin Invest 1956; **35**: 213-22.

63. Natoli G, Moschini L, Acconcia P, Albino G, Costa P, Pansa G. Newborn screening by microassay of 17-hydroxyprogesterone. In: Chiumello G, Sperling M, eds. Recent progress in Paediatric Endocrinology. New York: Raven Press, 1983; 285-90.

64. Prader A. Die Haufigkeit des kongenitalen androgenitalensyndrome. Acta Paediatr Helv 1958; **13**: 426-31.

65. Mauthe I, Laspe H, Knorr D. Zur haufigkeit des kongenitalen androgenitalen syndroms: Munchen 1963-1972. Klin Paediatr 1977; **189**: 172.

66. Muller W, Prader M, Kofler J, Glatzl J, Geir W. Zur haufigkeit des kongenitalen androgenitalen syndroms. Paediatr Paedol 1979; **14**: 151-5.

67. Hubble D. Congenital adrenal hyperplasia. In: Holt K.S, Raine D.H, eds. Basic concepts of Inborn Errors and Defects of Steroid Biosynthesis. Proceedings of the 3rd Symposium of the Society for the Study of Inborn Errors of Metabolism. Edinburgh: Livingston Press, 1966; 68-74.

68. Wallace A M, Beastall G H, Cook B, *et al.* Neonatal screening for congenital adrenal hyperplasia: A programme based on a novel radioimmunoassay for 17-OH-progesterone in blood spots. J Endocrinol 1986; **108**: 299-308.

69. Dorche C, Dhondt J L, Bozon D, *et al.* Systematic neonatal screening for congenital adrenal hyperplasia. Report of a pilot study in two centres in France. In: Proceedings of the Sixth International Congress series. Amsterdam: Elsevier, 1987; 289-92.

70. Pang S, Wallace A M W, Hofman L, *et al.* Worldwide experience in newborn screening for classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Paediatrics 1988; **81**(6): 866-74.

71. Pang S, Murphey W, Levine L S, *et al.* A pilot newborn screening for congenital adrenal hyperplasia in Alaska. J Clin Endocrinol Metab 1982; **55**(3): 413-20.

72. Therell B L, Brown L O, Aldis B. Addition of congenital adrenal hyperplasia screening to the Texas department of health newborn screening programme. In: Program and Absracts of the Seventh National Neonatal Screening Symposium, New Orleans 1989; 39 (abstract).

73. Cacciari E, Balsamo A, Cassio A, *et al.* Neonatal screening for congenital adrenal hyperplasia. Lancet 1982; **1**: 1069.

74. Cacciari E, Balsamo A, Cassio A, *et al.* Neonatal screening for congenital adrenal hyperplasia in a homogeneous Caucasian population. Ann N Y Acad Sci 1985; **458**: 85-9.

75. Lyon I C T, Webster D. Newborn screening for congenital adrenal hyperplasia in New Zealand. In: Therell B L, ed. Proceedings of the Sixth International Neonatal Screening Symposium. Excerpta Medica International Congress series. Amsterdam: Elsevier, 1987; 297-300.

76. Suwa S Y, Igarishi I, Kato K, *et al.* A case survey study for congenital adrenal hyperplasia in Japan: I Study for incidence. Acta Paediatr Jpn 1981; **85**: 204-10.

77. Suwa S Y, Shimozawa K, Kitagawa T, *et al.* Collaberative study on regional neonatal screening for congenital adrenal hyperplasia in Japan. In: Therell B L, ed. Proceedings of the Sixth International Neonatal Screening Symposium. Excerpta Medica International Congress series. Amsterdam: Elsevier, 1987; 279-80.

78. Fujieda K, Matsuura N, Takisuga N, Fukushi M, Arai O, Mizushima Y. Five years of newborn screening for congenital adrenal hyperplasia. In: Program and Abstracts of the Sixth International Neonatal Screening Symposium, Austin 1986; 21 (abstract).

79. Glass M W, Thuline H C, Neier S. Results of screening tests to detect congenital adrenal hyperplasia in Washington state newborns. In: Proceedings of the Sixth National Neonatal Screening Symposium, Portland 1988; 28-30.

80. Qazi Q H, Thompson M W. Incidence of salt-wasting form of congenital virilising adrenal hyperplasia. Arch Dis Child 1972; **47**: 302-4.

81. Pang S, Kling S, Craft W, Dobbins R H, Clark A. Illinois experience in newborn screening for congenital adrenal hyperplasia (CAH): A new guideline for follow-up approach. Absracts of the Seventh National Neonatal Screening Symposium, New Orleans 1989; 31 (abstract).

82. Dobbins R H, Kling S. Infant screening for congenital adrenal hyperplasia in Illinois. Proceedings of the Sixth National Neonatal Screening Symposium, Portland 1988; 158-9 (abstract).

83. Hoffman L F, Ostack R. Screening for congenital adrenal hyperplasia (CAH) in North Carolina. Abstracts of the Seventh National Neonatal Screening Symposium, New Orleans 1989; 31 (abstract).

84. Rosenbloom A L, Smith D W. Congenital adrenal hyperplasia. Lancet 1966; **1**: 660.

85. Porter B, Finzi M, Leiberman E, Moses S. The syndrome of congenital adrenal hyperplasia in Israel. Paediatrician 1977; **6**: 100-5.

86. Wilkins L. Adrenal disorders. II. Congenital virilising adrenal hyperplasia. Arch Dis Child 1962; **37**: 231-41.

87. Baulieu E E, Peillon F, Migeon C J. Adrenogenital syndrome. In: Eisenstein, ed. The Adrenal Cortex. Boston: Little, Brown, 1967; 553-638.

88. Donaldson M D C, Thomas P H, Haytread A, Price K J, McNinch A W, Savage D C L. Survey of congenital adrenal hyperplasia in the South West of England 1968-1988. In: Abstracts of the 62nd Annual Meeting of the British Paediatric Association, Warwick 1990; (abstract).

89. Cohen J M. Salt-losing congenital adrenal hyperplasia. Pediatrics 1969; **44**: 621-2.

90. Riordan F A I, Wood P J, Wakelin K, Betts P, Clayton B E. Bloodspot 17α-hydroxyprogesterone radioimmunoassay for diagnosis of congenital adrenal hyperplasia and home monitoring of corticosteroid replacement therapy. Lancet 1984; **1**(1): 708-10.

91. Frankenberg W K. Selection of diseases and tests in paediatric screening. Paediatrics 1974; **54**: 616-6.

92. Pang S, Hotchkiss J, Drash A L, Levine L S, New M.I. Microfilter paper method for 17α-hydroxyprogesterone radioimmunoassay. Its application for rapid screening for congenital adrenal hyperplasia. J Clin Endocrinol Metab 1977; **45**: 1003-8.

93. Lorenzen F, Pang S, New M I, *et al.* Hormonal Phenotype and HLA-genotype in families of patients with congenital adrenal hyperplasia (21-hydroxylase deficiency). Paediat Res 1979; **13**: 1356-60.

94. Levine L S, Dupont B, Lorenzen F, Pang S, Pollack M, Oberfield S. Cryptic 21-hydroxylase deficiency in families of patients with classical congenital adrenal hyperplasia. J Clin Endocrinol Metab 1980; **51**: 1316-24.

95. Shackleton C H L, Honour J W. Simultaneous estimation of urinary steroids by semi-automated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis. Clin Chim Acta 1976; **69**: 267-83.

96. Steen G, Tas A C, Tennoeven de Braun M C, Drayen M M, Wolthers B C. The early recognition of the 21-hydroxylase deficiency variety of congenital adrenal hyperplasia. Clin Chim Acta 1980; **105**: 213-24.

97. Edwards P W H, Makins H L J, Barratt T M. The steroid 11-oxygenation index: a rapid method for use in the diagnosis of congenital adrenal hyperplasia. J Endocrinol 1964; **30**: 181-94.

98. Strott C A, Lipsett M B. Measurement of 17-hydroxyprogesterone in human plasma. J Clin Endocrinol Metab 1968; **28**: 1426-30.

99. Barnes N D, Atherden S M. Diagnosis of congenital adrenal hyperplasia by measurement of 17-hydroxyprogesterone. Arch Dis Child 1972; **47**: 62-5.

100. Youssefnejadian E, David R. Early diagnosis of congenital adrenal hyperplasia by measurement of 17-hydroxyprogesterone. Clin Endocrinol 1975; **4**: 451-4.

101. Hughes I A, Winter J S D. The application of a serum 17-hydroxyprogesterone radioimmunoassay to the diagnosis and management of adrenal hyperplasia. J Paediatr 1976; **88**: 766-73.

102. Ratcliffe W A, McClure J P, Auld W H R, Honour J W, Fraser R, Ratcliffe J G. Precocious pseudopuberty due to a rare form of congenital adrenal hyperplasia. Ann Clin Biochem 1982; **19**: 145-50.

103. Korth-Schutz S, Levine L S, New M I. Serum androgens in normal prepubertal and pubertal children and in children with precocious adenarche. J Clin Endocrinol Metab 1976; **42**: 117-24.

104. New M I, Dupont B, Pang S, Pollack M, Levine M I. An update of congenital adrenal hyperplasia. Recent Prog Horm Res 1981; **55**: 751-3.

105. Dupont B, Pollack M S, Levine L S, O'Neill G J, Hawkins B R, New M I. Congenital adrenal hyperplasia. In: Teraski, ed. Histocompatibility Testing 1980: 693-706. (UCLA Tissue Typing Laboratory, Los Angeles, 1980).

106. Lorenzen F, Pang S, New M I, *et al.* Studies of the C-21 and C-19 steroids and HLA genotyping in siblings and parents of patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Clin Endocrinol Metab 1980; **50**: 572-9.

107. Pollack M S, Levine L S, Zachmann M, *et al.* Possible genetic linkage disequilibrium between HLA and the 21-hydroxylase deficiency gene (congenital adrenal hyperplasia). Transplant Proc 1979; **XI**: 1315-6.

108. Coullin P, Nicolas H, Boue J, Boue, A. HLA typing of amniotic-fluid cells applied to prenatal diagnosis of congenital adrenal hyperplasia. Lancet 1979; **1**: 1076 (letter).

109. Brautbar C, Rosler A, Landau H, *et al.* No linkage between HLA and congenital adrenal hyperplasia due to 11β-hydroxylase deficiency. N Engl J Med 1980; **300**: 205-6.

110. Hughes I A, Laurence K M L. Antenatal diagnosis of congenital adrenal hyperplasia. Lancet 1979; **2**: 7-9.

111. Pang S, Levine L S, Cederquist L L, *et al.* Amniotic fluid concentrations of Δ-5 and Δ-4 steroids in fetuses with congenital adrenal hyperplasia due to 21-hydroxylase deficiency and in anencephalic fetuses. J Clin Endocrinol Metab 1980; **51**: 223-9.

112. Warsos S L, Larsen J W, Kent S G, *et al.* Prenatal diagnosis of congenital adrenal hyperplasia. Obstet Gynecol 1980; **55**: 751-3.

113. Ekins R P. The estimation of thyroxine in human plasma by an electrophoretic technique. Clin Chim Acta 1960; **5**: 453-9.

114. Yalow R S, Berson S A. Immunoassay of endogenous plasma insulin in man. J Clin Invest 1960; **39**(2): 1157-75.

115. Landsteiner K, van der Scheer J. On cross reactions of immune sera to azoproteins. J Exptl Med 1936; **63**: 325-39.

116. Sela M. Antigenicity: some molecular aspects. Science 1969; **166**: 1365-74.

117. Erlanger B F, Borek F, Beiser S M, Lieberman S. Steroid-protein conjugates. I. Preparation and characterisation of conjugates of bovine serum albumin with testosterone and cortisone. J Biol Chem 1957; **228**: 713-27.

118. Lieberman S, Erlanger B F, Beiser S M, Agate F J. Steroid-protein conjugates: Their chemical immunochemical and endocrinological properties. Recent Prog Horm Res 1959; **15**: 165-200.

119. Abraham G E. Solid phase radioimmunoassay for oestradiol-17β. J Clin Endocrinol Metab 1969; **29**: 866-70.

120. Freund J. The effect of paraffin oil and mycobacteria on antibody formation and sensitization. Am J Clin Path 1951; **21**: 645-9.

121. Neischlag E, Kley H K, Usadel K H. Production of steroid antisera in rabbits. In: Cameron E D H, Hillier S G, Griffiths K, eds. Steroid Immunoassay. Cardiff: Alpha Omega Publ.Ltd, 1975; 87-132.

122. Niswender G D, Migley A R. Hapten radioimmunoassay for steroid hormones. In: Peron F G, Caldwell B V, eds. Immunological Methods in Steroid Determination. New York: Appelton, Century, Crofts, 1970; 149-66.

123. Lindner H R, Perel E, Friedlander A, Zeitlin A. Specificity of antibodies to ovarian hormones in relation to the site of attachment on the steroid hapten to the peptide carrier. Steroids 1972; **19**: 357-75.

124. Midgley A R, Niswender G D, Gay V L, Reichert L E. Use of antibodies for characterisation of gonadotrophins and steroids. Recent Prog Horm Res 1971; **27**: 236-86.

125. Gilby E D, Jeffcoate S L, Edwards R. ^{125}Iodine tracers for steroid radioimmunoassay. J Endocrinol 1973; **58**: xx (abstract).

126. Oliver G C, Parker B M, Brasfield D L, Parker C L. The measurement of digoxin in human serum by radioimmunoassay. J Clin Invest 1968; **47**(1): 1035-42.

127. Lindberg P, Edquist L E. The use of 17β-oestradiol-6-(o-carboxymethyl)oxime-^{125}[I] tyramine as tracer for the radioimmunoassay of 17β-oestradiol. Clin Chim Acta 1974; **53**: 169-74.

128. Hunter W M, Greenwood F C. Preparation of iodine-^{131}labelled human growth hormone of high specific activity. Nature 1962; **194**: 495-6.

129. Hunter W M, Nars P W, Rutherford F J. Preparation and behaviour of ^{125}I-labelled radioligands for phenolic and neutral steroids. In: Cameron E D H, Hillier S G, Griffiths K, eds. Steroid Immunoassay. Fifth Tenovus Workshop. Cardiff: Alpha Omega Publ. Ltd., 1975; 141-52.

130. Herbert V, Lau K S, Gottlieb C W, Bleicher S J. Coated Charcoal Immunoassay of Insulin. J. Clin Endocrinol Metab 1965; **25**: 1375-84.

131. Dawes C C, Gardner J. Radioimmunoassay of digoxin employing charcoal entrapped in magnetic polyacrylamide particles. Clin Chim Acta 1978; **86**: 353-6.

132. Utiger R D, Parker M L, Daughaday W H. Studies on human growth hormone. I. A radioimmunoassay for human growth hormone. J. Clin Invest 1962; **41**: 254-61.

133. Midgley A R, Rebar R W, Niswender G D. Radioimmunoassay employing double antibody techniques. Acta Endocrinol Suppl. 1969; **142**; 247-54.

134. Edwards R. The development and use of P E G assisted second antibody as a separation technique in radioimmunoassay. In: Hunter W M, Corrie J E T, eds. Immunoassays for Clinical Chemistry. Edinburgh: Churchill, Livingstone, 1982; 139-47.

135. Den Hollander F C, Schuurs A H W M. Double antibody solid phase (D A S P). In: Kirkham K E, Hunter W M, eds. Radioimmunoassay Methods. Edinburgh: Churchill, Livingstone, 1971; 419-22.

136. Chapman R S, Ratcliffe J G. Covalent linkage of antisera to microparticulate cellulose using 1.1^{1}Carbonyldiimadazole: a rapid, practical method with potential use in solid-phase immunoassay. Clin Chim Acta 1982; **118**: 129-34.

137. Nye L, Forest G C. Greenwood H, *et al.* Solid phase magnetic particles radioimmunoassay. Clin Chim Acta 1969; **69**: 387-96.

138. Chang T M S. Semi-permeable microcapsules. Science 1964; **146**: 524-5.

139. Chang T M S. Artificial cells. Springfield, Illinois: C C Thomas, 1972.

140. Chang T M S. Encapsulation of enzymes, cell contents, cells vaccines, antigens antiserum, cofactors, hormones and proteins. In: Chang T M S, ed. Biomedical Applications of Immobilised Enzymes. Vol 1. New York: Plenumn Press, 1977; 69-90.

141. Halpern E P, Bordens R W. Microencapsulated antibodies in radioimmunoassay. I. Determination of digoxin. Clin Chem 1979; **24**: 860-2.

142. Ashkar F S, Buehler J, Chan T, Hourani M. Radioimmunoassay of free thyroxine with pre-bound anti T4 microcapsules. J Nucl Med 1979; **20**: 956-60.

143. Halpern E P, Bordens R W. Microencapsulated antibodies in radioimmunoassay. II. Determination of free thyroxine. Clin. Chem 1979; **25**: 1561-3.

144. Wallace A M W, Aitken S, Duffy F A, Fraser W D, Beastall GH. Measuring free thyroxine by using magnetic antibody-containing microcapsules. Clin Chem 1990; **36**/4: 614-619.

145. Bordens R W, Halpern E P. Microencapsulated antibodies in radioimmunoassay. III. Determination of cortisol. Clin Chem 1980; **26**: 633-4.

146. Wallace A M, Wood D A. Development of a simple procedure for the preparation of semi-permeable antibody-containing microcapsules and their analytical performance in a radioimmunoassay for 17-hydroxyprogesterone. Clin Chim Acta 1984; **140**: 203-12.

147. Rambourg P, Levy J, Levy MC. Microencapsulation III. Preparation of invertase microcapsules. J Pharm Sci 1982; **71**: 753-8.

148. Westphal U. Assay properties of cortisol binding globulin and other steroid binding proteins. Meth Enzym 1969; **15**: 761-96.

149. Dunn J F, Nisula B C, Rodbard D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 1981; **53**: 58-68.

150. Grant J K, Beastall G H, eds. Steroid saturation analysis. In: Clinical Biochemistry of Steroid Hormones. Methods and Applications. Kent: Croom Helm, 1983; 45-90.

151. Ratcliffe W A. Evaluation of four methods for the direct assay of progesterone in unextracted serum. Ann Clin Biochem 1982; **19**: 362-7.

152. Ratcliffe W A, Corrie J E T, Dalziel A H, Macpherson J S. Direct ^{125}I-radioligand assays for serum progesterone compared with assays involving extraction of serum. Clin Chem 1982; **28/6**: 1314-8.

153. McConway M G, Chapman R S, Beastall GH, *et al.* How sensitive are immunometric assays for thyrotropin? Clin Chem 1989; **35**: 289-91.

154. Jeffcoate S L. Hapten-specificity of antisera to steroid protein conjugates. In: Kirkham K E, Junter W M, eds. Radioimmunoassay Methods. Edinburgh: Churchill, Livingstone, 1971; 151-5.

155. Pratt J J. Steroid immunoassay in clinical chemistry. Clin Chem 1978; **24**(11): 1869-90.

156. Corrie J E T. ^{125}Iodinated tracers for steroid radioimmunoassay: The problem of bridge recognition. In: Hunter W M, Corrie J E T, eds. Immunoassays for Clinical Chemistry. Edinburgh: Churchill, Livingstone, 1983; 353-57.

157. Webb R, Baxter G M, McBride D, Nordblom G D, Shaw M P K. The measurement of testoserone and oestradiol-17β using iodinated tracers and incorporating an affinity chromatography extraction procedure. J Steroid Biochem 1985; **23**: 1043-51.

158. Jeffcoate S L Use of ^{125}Iodine tracers in steroid radioimmunoassays. In: Gupta D, ed. Radioimmunoassay for Steroid Hormones (2nd Edn). Weinheim: Verlag Chemie, 1980; 209-19.

159. Perry L A, Al-Dujaili E A S, Edwards C R W. A direct radioimmunoassay for 11-deoxycortisol. Steroids 1982; **39**: 115-28.

160. Al-Duijali E A S, Edwards C R W. Optimisation of a direct radioimmunoassay for plasma aldosterone. J Steroid Biochem 1981; **14**: 481-7.

161. Dyas J, Read G F, Riad-Fahmy D. A single robust assay for testosterone in male plasma using an ^{125}I-radioligand and a solid phase separation technique. Ann Clin Biochem 1979; **16**: 325-31.

162. Besch N F, Buoy M E, Haller W S, Johnson N J, Besch P K. RIA for 4-androsten-3,17-dione, including synthesis, production and characterization of antiserum. Clin Chem 1986; **32**: 1357-67.

163. Vihko R, Ruokonen L. Steroid sulphates in human adult testicular steroid synthesis. J Steroid Biochem 1975; **6**: 353-6.

164. Goodall A B, James V H T. Observations on the nature of conjugated androstenedione in human plasma. J Steroid Biochem 1981; **14**: 465-71.

165. Rousell R H, Hewet A J, McEwan J, Allen R M, Adams K, Redshaw M R. Determination of serum progesterone levels using a direct ^{125}I-radio-assay. La ricerca in Clinica e in Laboratorio 1979; **9**: 185-95

166. Jurgens H, Pratt J J, Woldring M G. Radioimmunoassay of plasma oestradiol without extraction and chromatography. J Clin End Metab 1975; **40**: 19-25.

167. Pratt J J, Wiegman T, Lappohn R E, Woldring M G. Estimation of plasma testosterone without extraction and chromatography. Clin Chim Acta 1975; **59**(3): 337-46.

168. McGinley R, Casey J H. Analysis of progesterone in unextracted serum: a method using danazol (17 - pregn-4-en-20-yno(2,3-d)isoxazol-17-ol) a blocker of steroid binding to proteins. Steroids 1979; **23**: 127-38.

169. Haynes S P, Corcoran J M, Eastman C J, Doy F A. Radioimmunoassay of progesterone in unextracted serum. Clin Chem 1980; **26**: 1607-9.

170. Foster L B, Dunn R T. Single-antibody technique for radioimmunoassay of cortisol in unextracted serum or plasma. Clin Chem 1974; **20**: 365-8.

171. Seth J,Brown L M. A simple radioimmunoassay for plasma control. Clin Chim Acta 1978; **86**: 109-20.

172. Brock P, Eldred E W, Woiszwillo J E, Doran M, Shoemaker H J. Direct solid-phase ^{125}I-radioimmunoassay of serum cortisol. Clin Chem 1978; **24**: 1595-8

173. Kane J W. Use of sodium salicylate as a blocking agent for cortisol-binding-globulin in a radioimmunoassay for cortisol on unextracted plasma. Ann Clin Chem 1979; **16**: 109-212.

174. Hummer L, Nielsen MD, Christiansen C. An easy and reliable RIA of serum androstenedione: age-related normal values in 252 females aged 2 to 70 years. Scand J Clin Lab Invest 1983; **43**: 301-6.

175. Smith MR, Rudd BT, Shirley A, *et al.* A RIA for the estimation of serum dehydroepiandrosterone sulphate in normal and pathological serum. Clinica Chimica Acta 1975; **65**: 5-13.

176. Vermeulen A. The androgens. In: Gray CH, James VHT, eds. Hormones in Blood (3rd Edn). London: Academic Press, 1979; 356-416.

177. Vermeulen A, Verdonck L. RIA of 17β-hydroxy-5α-androstane-3-one, 4-androstene-3,17-dione, dehydroepiandrosterone, 17-hydroxyprogesterone and progesterone and its application to human male plasma. J Steroid Biochem 1976; **7**: 1-13.

178. Egan S M, Betts P, Thomson S, *et al.* A blood spot androstenedione assay suitable for home monitoring of steroid replacement therapy in congenital adrenal hyperplasia. Ann Clin Biochem 1989; **26**: 262-7.

179. Murphy J F, Joyce B G, Dyas J, Hughes I A. Plasma 17-hydroxyprogesterone concentrations in ill newborn infants. Archives of Disease in Childhood 1983; **58**: 532-4.

180. Korth-Schutz S, Virdis R, Saenger P, *et al.* Serum androgens as a continuing index of adequacy of treatment of congenital adrenal hyperplasia. J Clin Endocrinol Metab 1978; **46**: 452-8.

181. Cavallo A, Corn C, Bryan GT, *et al.* The use of plasma androstenedione in monitoring therapy of patients with congenital adrenal hyperplasia. J Pediatr 1979; **95**: 33-7.

182. Lee PA, Urban MD, Gutai JP, *et al.* Plasma progesterone, 17-hydroxyprogesterone, androstenedione and testosterone in prepubertal, pubertal and adult subjects with congenital virilizing adrenal hyperplasia as indicators of adrenal suppression. Horm Res 1980; **13**: 347-57.

183. Meyer WJ, Gutai JP, Keenan BS, *et al.* A chrono-biological approach to the treatment of congenital adrenal hyperplasia. In: Lee PA, Plotnick LP, Kowarski AA, Migeon CJ, eds. Congenital adrenal hyperplasia. Baltimore: University Park Press, 1977; 203-15.

184. White PC, New MI, Dupont B. Congenital adrenal hyperplasia. N Eng J Med 1987; **316**: 1519-24.

185. Honour J. Biochemical aspects of congenital adrenal hyperplasia. J Inher Metab Dis 1986; **9**: 124-34.