ON THE SYNERGISM OF GROWTH HORMONE AND ANDROSTENEDIONE IN CHILDREN

BY

DR. SAMI NOOH HASSAN

M.B., Ch.B., D.C.H., (GLASGOW)

THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

FROM THE

DEPARTMENT OF CHILD HEALTH, FACULTY OF MEDICINE

MAY 1990

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

PREFACE AND ACKNOWLEDGEMENTS

The approval for the clinical aspect and the use of the patients in the treatment program of this work was obtained from the Ethics Committee of the Royal Hospital for Sick Children and Queen Mother's Hospitals. Patient clinical studies, follow up and samples collection were done by the author under the clinical supervision of Dr. W. Hamilton in the Day Bed Area or Ward 7B, Royal Hospital for Sick Children, Glasgow.

The laboratory studies were carried out by the author in the Steroid Laboratories of the University Department of Child Health, Royal Hospital for Sick Children, Glasgow. The investigation was supported financially by the Ministry of Higher Education and Scientific Research, of Iraq.

I am specially indebted to Dr. William Hamilton for his unfailing patience and constant encouragment and guidance. I would also like to express sincere thanks to Professor Forrester Cockburn for his constant encouragment and stimulating comments throughout this work.

Thanks are also due to Dr. Robert Anderson of the Department of Forensic Medicine and Science of Glasgow University for his technical help to solve one problem of this work by identifying an unknown peak in the g.l.c tracings by the use of mass-spectrometer-gas liquid chromatography system and to Professor Charles J. W. Brooks for his helpful discussion on the subject.

I would like also to express my thanks to the Department of Biochemistry, Royal Hospital for Sick Children, Glasgow specially to Dr. J. Farquharson, Dr.R.D. Paton and Miss C. Jamieson for

allowing me to use the Gamma Counter and for their technical help in that matter and in some other situations related to this work.

I am grateful to Mrs Catherine Clark and Miss Jean Hyslop of the Department of Medical Illustrations, Royal Hospital for Sick Children, Glasgow for adding an artistic touch to some of the Figures in this thesis. Other Figures e.g. 2, 5, 15, 24, and 25 and all Tables are completely of my own doing.

Thanks are also due to Mr. John Fleming of the Department of Computers, Queen Mother's Hospital, Glasgow for allowing me to use the Department computer and typewriter facilities for the typing of this volume.

The patience and unfailing confidence of my family has been a great source of strength to me throughout the preparation of this work.

CONTENTS

Subjects	Page
PREFACE AND ACKNOWLEDGMENTS	1
CONTENTS	3
LIST OF TABLES	5
LIST OF FIGURES	8
STEROID NOMENCLATURE	10
ABBREVIATIONS	11
SUMMARY	12
CHAPTER ONE INTRODUCTION	18
Jacob-Creutzfeldt disease.	22
Synthetic human growth hormone.	23
Somatomedins.	25
Testing for growth hormone deficiency.	28
Treatment of short stature with growth hormone.	31
Androgens, oestrogens and growth hormone.	31
Somatic effects of androgens and oestrogens.	35
Synthetic steroid analogues of testosterone.	39
Androstenedione.	42
CHAPTER TWO METHODOLOGY	47
Clinical materials and methods.	47
Androstenedione preparation for oral use.	52
Laboratory materials.	54
Radioactive labelled steroids.	54
Unlabelled steroids.	54
Other reagents.	55
Gases.	56
Equipment.	56
Laboratory Methods.	62

Determination of urinary androgen metabolites. 62
Practical procedures for determination of urinary androgens. 79
Determination of urinary creatinine. 80
Plasma androstenedione assay. 85
Plasma testosterone assay. 93
CHAPTER THREE RESULTS 102
Analyses of the plasma concentrations of androstenedione and testosterone. 102
Group 1 patients. 102
Group 2 patients. 112
Group 3 patients. 117
Intermediate summary. 122
Analyses of anthropometric data in Group 3 patients. 123
Analyses of urinary androgen metabolites. 143
Group 3 patients. 144
Group 2 patients. 149
Group 1 patients. 153
CHAPTER FOUR DISCUSSION 163
Introduction. 163
Analyses and discussion on data. 174
Group 1 patients. 174
Group 2 patients. 178
Group 3 patients. 182
PROTOCOLS FOR GROUP 3 PATIENTS 191
CHAPTER FIVE REFERENCES 197

LIST OF TABLES

Tables		Page
I	The recovery of radioactive androgens in the nuclei of rat prostate.	37
II	The common anabolic hormones used clinically.	41
III	Plasma concentrations of androstenedione and testosterone in cord blood and in peripheral blood in the first day of life.	44
IV	Anthropometric data of Group 1 patients.	48
V	Anthropometric data of Group 2 patients.	49
VI	Anthropometric data of Group 3 patients.	50
VII	Anthropometric data of Group 4 patients.	50
VIII	Recovery . . . of creatinine added to urine.	83
IX	The anthropometric data and plasma concentrations of androstenedione and testosterone in Group 1 patients.	104
X	Plasma concentrations of LH and FSH following GnH-RH test in Group 1 patients.	108
XI	The ratios of plasma androstenedione to testosterone in Group 1 patients.	110
XII	The plasma concentrations of androstenedione and tesosterone in Group 2 patients.	115
XIII	Plasma concentrations of androstenedione and testosterone in Group 3 patients.	120
XIV	Basal anthropometric data in Group 3 patients.	125
XV	The anthropometric data of Group 3 patients basally and one year after treatment with growth hormone and androstenedione.	126

XVI	Three monthly incremental height increases expressed as calculated annual growth velocities in Group 3 patients.	128
XVII	Some clinical anthropometric data of Group 3 patients one year after treatment with growth hormone and androstenedione.	129
XVIII	The ratios of weight to height in Group 3 patients before, after combined treatment with growth hormone and androstenedione and one year on growth hormone alone.	137
XIX	Percentages of the patients weight:height ratios compared to the 50^{th} centile weight:height ratios at the three periods of observation in Group 3 patients.	138
XX	The anthropometric data of Group 4 patients over the first year of treatment with growth hormone alone.	140
XXI	The ratios of weight:height in Group 4 patients basally and one year after treatment with growth hormone alone.	142
XXII	Plasma concentrations of testosterone and the corresponding urinary metabolites in Group 3 patients.	145
XXIII	Plasma concentrations of androstenedione and the corresponding urinary metabolites in Group 3 patients.	148
XXIV	Plasma concentrations of testosterone and the corresponding urinary metabolites in Group 2 patients.	150
XXV	Plasma concentrations of androstenedione and the corresponding urinary metabolites in Group 2 patients.	152
XXVI	Plasma concentrations of testosterone and the corresponding urinary metabolites in Group 1 patients.	155

XXVII Plasma concentrations of androstenedione and the corresponding urinary metabolites in Group 1 patients. 159

XXVIII The totals and means of urinary androgen metabolites in Group 1 patients. 176

XXIX The means of plasma concentrations of androstenedione and testosterone and the corresponding urinary metabolites in Groups 1 and 2 patients. 181

XXX The means of plasma concentrations of androstenedione and testosterone and the corresponding urinary metabolites in Group 3 patients. 184

XXXI The means of plasma concentrations of androstenedione and testosterone and the corresponding urinary metabolites in Groups 1, 2 and 3 patients. 186

XXXII The clinical anthropometric data and their means in Group 3 patients basally, after one year of treatment with growth hormone and androstenedione and after one further year of treatment with growth hormone alone. 188

LIST OF FIGURES

Figures		Page
1	Human growth hormone molecular structure.	21
2	Actions of human growth hormone.	27
3	Over-night profile of sleep stages and serum growth hormone concentrations.	30
4	Linearity of the flame ionization detector of the gas liquid chromatography system for urinary steroid detection.	72
5	Flow diagram of the steps in the hydrolysis, extraction and quantitation of urinary androgen metabolites.	73
6	Gas liquid chromatographic analysis with flame ionization detection of a standard steroid-hepto-fluorobutyrate mixture.	76
7	Gas liquid chromatographic analysis with flame ionization detection of an esterified urinary extract.	77
8	Gas liquid chromatographic analysis with flame ionization detection of a standard steroid-heptofluoro-butyrate mixture and urinary androgen metabolite-hepto-fluorobutyrates.	78
9	Optical density of creatinine as assayed by the Jaffe colour reaction. Obedience to Beer-Lambert law.	82
10	Summary of the radioimmunoassay technique for the plasma androstenedione assay.	88
11	Plasma androstenedione radioimmunoassay standard curve.	90
12	Plasma testosterone radioimmunoassay technique (summary).	96

13 Plasma testosterone radioimmunoassay standard curve. 98

14 The histogram of the plasma concentrations of androstenedione and testosterone in Group 1 patients. 106

15 The molecular structure of the three components of Sustanon. 113

16 The histogram of the plasma concentrations of androstenedione and testosterone in Group 2 patients. 116

17 The histogram of the plasma concentrations of androstenedione and testosterone in Group 3 patients. 121

18 The height-growth chart and follow-up pattern for Patient 1 in Group 3 patients. 130

19 The height-growth chart and follow-up pattern for Patient 2 in Group 3 patients. 131

20 The height-growth chart and follow-up pattern for Patient 3 in Group 3 patients. 132

21 The height-growth chart and follow-up pattern for Patient 4 in Group 3 patients. 133

22 The height-growth chart and follow-up pattern for Patient 5 in Group 3 patients. 134

23 The actual weights and trends of weights in Group 3 patients. 136

24 Androstenedione and testosterone interconversion relationship. 168

25 The mechanism of testosterone action at cell level and resultant effects. 171

STEROID NOMENCLATURE

To facilitate comprehension, trivial names of steroids have been used where possible in this thesis. The trivial names and abbreviations in brackets used are listed below together with the systematic nomenclature.

Trivial names	Systemic names
Aetiocholanolone (AE)	5β-androstane-3α-ol-17-one.
5α-Androstanediol (5α-ASD)	5α-androstane-3α,17β-diol.
5β-Androstanediol (5β-ASD)	5β-androstane-3α,17β-diol.
5α-3β,17β-Androstanediol	5α-androstane-3β,17β-diol.
Δ5-Androstenediol (Δ5-diol)	5-androstene-3β,17β-diol.
Androstenedione (AD)	4-androstene-3,17-dione.
Androsterone (A)	5α-androstane-3α-ol-17-one.
Dehydroepiandrosterone	5-androstene-3β-ol-17-one.
5α-Dihydrotestosterone (5α-DHA)	5α-androstane-17β-ol-3-one.
Epiandrosterone (EpiA)	5α-androstane-3β-ol-17-one.
17β-Oestradiol	1,3,5 (10)estratriene-3β,17β-diol.
Testosterone (T)	4-androstene-17β-ol-3-one.

ABBREVIATIONS

The main abbreviations used in this thesis are listed below.

ACTH	Adenocorticotrophic hormone.
AD	Androstenedione.
AGV	Annual growth velocity.
BA	Bone age.
CA	Chronological age.
cf.	Compare with.
c.p.m	Counts per minute.
d.p.m	Disintegrations per minute.
FID	Flame ionization detection.
FSH	Follicular stimulating hormone.
GH	Growth hormone.
GLC or g.l.c	Gas liquid chromatography.
GnH-RH	Gonadotrophin hormone releasing hormone.
HA	Height-age.
HCG	Human chorionic gonadotrophin.
Ht	Height.
LH	Luteinizing hormone.
GLC-MS	Gas liquid chromatography-mass spectrometer.
SA	Specific activity.
T	Testosterone.
T3	Triiodothyronine.
T4	Thyroxine.
TLC or t.l.c	Thin layer chromatography.
TRH	Thyrotrophin releasing hormone.
TSH	Thyroid stimulating hormone.
Wt	Weight.

SUMMARY

In Chapter one I have reviewed the origins of thinking concerning growth hormone and how clinicians like Pierre Marie, Minkowski and Cushing carefully related clinical conditions to pathology and came to hallowed conclusions regarding growth hormone. The catastrophe of the appearance of Jacob-Creutzfeldt disease in some patients who had received cadaveric growth hormone was subsequently avoided by the early synthesis of purified growth hormone by DNA recombinant techniques which has led to the current use of authentic growth hormone indistinguishable in structure and physiological action from the naturally occurring hormone. However since the naturally occurring hormone was really never extracted in a 100 per cent pure form who then can tell what are all its physiological actions?

Thereafter follows a consideration of other circulating substances such as the insulin-like growth factors IGF-I and IGF-II which are also necessary for the intimate actions of growth hormone at its sites of action. Included is brief reference to the synergistic actions of the sex hormones with growth hormone.

A review of the current variety of clinical tests used to determine whether or not a patient has complete or partial growth hormone deficiency follows with the highlighting of the insulin-hypoglycaemia test as being probably the best, since blood concentrations of glucose can be easily monitored and controlled so as to achieve a uniform nadir (concentration) for all patients.

The status of the synthetic anabolic hormones is reviewed but importantly androstenedione, a naturally occurring, easily assayable anabolic hormone is emphasized in its physiological,

interconvertible and degradation activities for it was used clinically as therapy with growth hormone in a Group of children with growth hormone deficiency.

Androstenedione is converted in the body to testosterone. Testosterone through intracellular conversion to 5α-dihydrotestosterone stimulates cell differentiation but for this, optimum concentrations must be within the range 0-120 nM at nuclear level. This can only be achieved with cytoplasmic concentrations within the range 0-10 nM. Low plasma testosterone concentrations cannot maintain these requirements and all our patients had subnormal plasma testosterone concentrations. It seemed justifiable, therefore, that to give growth hormone alone without ensuring adequate plasma concentrations of testosterone, less than optimum growth responses would be achieved. This then is the burden of the clinical exercise namely, first to study the utilization of androstenedione and then to use it intelligently and cautiously combined with growth hormone in the treatment of five boys with growth hormone deficiency.

In Chapter Two the materials and methods used are detailed according to accepted style. The patient cohort consisted of eleven boys age-range 9.25 to 15.83 years on whom the effects of oral androstenedione were observed with reference to plasma concentrations of androstenedione and testosterone and their urinary metabolites. A second Group of boys age range 10.67 to 15.3 years had been given testosterone (as 3 esters) 100 mg intramuscularly as part of an insulin hypoglycaemia test. Similar observations were made in a third Group of boys (growth hormone deficient) age-range 6.28 to 13.8 years who were given subcutaneous growth hormone (4 IU thrice

weekly) and oral androstenedione (100 mg) with the same measurements being made of the plasma concentrations of androstenedione and testosterone as well as their urinary metabolites. Determinations were undertaken basally and at three-monthly intervals during the first year of combined treatment. Anthropometric data for these five patients were recorded over a period of two years. These data are presented in Chapter 3.

In Chapter 3 the data presented shows that in children less than 12.0 chronological years 17β-reductase is not an enzyme as active as it is in children above that age. I adduce this on the finding that the ratios of plasma AD/T are greater in the younger age group following oral AD (100 mg). The administration of intramuscular testosterone (100 mg) raises plasma testosterone concentrations to supraphysiological concentrations but judged by the excretion in these patients of 5α-androstane-3α,17β-diol (5α-ASD), there was no increase in general utilization of these high plasma concentrations.

When growth hormone (4 IU subcutaneously) and AD (100 mg orally) were administered long term (both thrice weekly) to 5 boys with idiopathic growth hormone deficiency (IGHD), the plasma concentrations of both AD and testosterone although raised above basal concentrations did not rise above physiological concentrations for age. Happily the combined therapy resulted in a marked increase in AGV of approximately 2 years for one year of treatment which was not maintained during a second year of treatment with GH alone. Regrettably osseous maturation advanced at about the same rate increasing by approximately 2.0 years per year of treatment.

As the result of Chapter 3 were analysed it become clear that

in the first group of boys to whom an oral bolus of AD (100 mg) had been given and which had resulted in plasma concentrations of AD and testosterone well above their physiological normals, evidence of their ability to utilize these concentrations maximally was lacking in that the urinary excretion of 5α-ASD remained low, being not greater over 5 hours than 11.6 μg for those with an osseous maturation less than 12.0 years. In those boys who received testosterone (100 mg) intramuscularly as priming for an ITT, on the third day their excretion rate of 5α-ASD over a 5 hour period was 12.7 μg. These comparable excretion rates indicate that no matter what the plasma concentration of AD and testosterone were, utilization was restricted to an equivalent degree in both groups. In the group of 5 IGHD boys on chronic GH and AD treatment for one year, the equivalent 5α-ASD excretion over a comparable 5h period was 27.0 μg. This indicates that a mechanism for utilizing testosterone had developed permitting excretion of urinary 5α-ASD in these increased quantities.

The argument, therefore, is offered that in small for age children in whom there is a reduced plasma testosterone concentration a combination of immature or numerically deficient testosterone receptors could account for part of the short stature since it is known that a threshold concentration of plasma testosterone is required to create an intracellular concentration of 5 -DHT which in turn will activate nuclear replication. Indeed specific concentrations for these compartments are known. The cytoplasmic concentration of 5α-DHT must be within the range 0 - 10 nM to produce a nuclear concentration of 5α-DHT within the range of 0 - 120 nM with an upper limit of 250 nM. When plasma testosterone

concentrations are subphysiological the cytoplasmic concentration of 5α-DHT greater than 1-2 nM cannot be maintained and the nuclear concentration falls to a level at which full cell differentiation cannot be maintained. If this is the case then low excretion rates for urinary 5α-ASD reflect this failure to utilize these plasma concentrations of testosterone. This is exactly what I found basally in the 5 boys with IGHD. Their initial excretion rate of 5α-ASD was 0.46 μg per 5h but following the one year of GH and AD therapy their comparable mean urinary excretion rate of 5α-ASD was 27.0 μg per 5h and this compared with a mean of 11.6 μg per 5h in Group 1 patients (oral AD 100 mg) and a mean of 12.7 μg per 5h in Group 2 patients (IM testosterone 100 mg).

It could be argued that in Groups 1 and 2 patients the androgen receptors if adequately present were inactive and hence the circulating testosterone was not being transported within the cells. If this were so then Group 3 patients (IGHD) shared this defect initially. But following the prolonged use of oral AD evidence is offered to indicate that either the androgen receptors increased in number or the respective domains on the receptors matured so that physiological concentrations of plasma testosterone facilitated the transport of testosterone within the cell, to be converted to 5α-DHT which in turn produced nuclear concentration of 5α-DHT sufficient to stimulate mitogenesis in chondroblasts at the growth plate and thereafter osteoblastosis.

The good first year AGV was not maintained during a second year on growth hormone alone and from the bone maturation score at the end of the first and second years of treatment it was deduced that AD contributed only for 1.0 bone age year per chronological year and 1.7 bone age years was due to the growth hormone therapy.

It is suggested that smaller quantities of AD could be used in conjunction with GH so as to limit the amount of growth hormone given for the latter is currently a very expensive drug.

CHAPTER ONE

INTRODUCTION

The subject of this work is the contribution of anabolic steroids to the growth response resulting from growth hormone given to children of short stature who have been diagnosed as having complete growth hormone deficiency. In this introductory Chapter I shall review the historical aspects of growth hormone and its action in the growth process, factors which act together with that hormone such as the somatomedins and the present understanding of the nature and role of the anabolic hormones in relation to their growth-promoting effects.

Pièrre Marie (1886)[1] described two cases of a condition which he termed "acromegaly" (acro. Gk. extremity; mega. Gk. large). Both patients exhibited broadening of the limb bones, the mandible and the skull bones. In addition there was thickening of the tongue. He suspected that the pituitary gland was in some way involved but in the absence of a postmortem examination his suspicion remained unverified. It was left to Minkowski (1887)[2] to make the association on postmortem examination of some of his patients whom he identified as having the same clinical condition as those patients described by Marie, where he observed enlargement of the pituitary gland. This led interested physicians and scientists to consider the relationship between clinical acromegaly and the pituitary gland. Laboratory animals were introduced as vehicles for this study which involved pituitary gland transplantation to promote linear growth and hypophysectomy to retard linear growth. These investigations confirmed the fact that the pituitary gland played a part in linear growth. A few

years later Cushing (1909)[3] postulated the existence of an hormonal substance in the pituitary gland responsible for linear growth. He thought that this substance was produced excessively in patients with acromegaly.

Attempts were then made to extract this hormonal substance from pituitary glands. Success is attributed to Isovesco (1913)[4] who injected young rabbits with a bovine pituitary extract and observed promotion of linear growth. Evans and Long (1921)[5] gave immature rats a bovine pituitary extract intraperitoneally and observed that gigantism resulted. However such procedures were not without risk. Johns et al., (1927)[6] reported on their experience with pituitary extracts in dogs where diabetes mellitus resulted. Houssay and Biasotti (1931)[7] followed up this observation by showing that the diabetes mellitus induced by pituitary extracts given to dogs and toads was reversible by hypophysectomy, only to be manifest again by the administration of pituitary extracts.

An interesting and important observation about this time was made by Smith (1930)[8] namely that hypophysectomy in rats resulted in a cessation of linear growth and an atrophy of the thyroid and adrenal glands. Both the growth and the glands were restored to near normal following the administration of pituitary gland extracts. A decade had to elapse before it was announced that the active substance which had been sought in the pituitary extracts was a "growth hormone" (Fraenkel-Conrat et al., 1940)[9]. By 1943 Evans et al.,[10] had been able to demonstrate by their personally devised test and assay method for "growth hormone", the growth response of hypophysectomized immature rat tibial epiphyses to administered rat pituitary extracts. This assay method

enabled Li and Evans (1944)[11] to isolate and to some extent purify bovine growth hormone.

From then until the present great strides in our knowledge of growth hormone have been made. For instance, Li and Papkoff (1956)[12] found that the human growth hormone extracted from cadaveric pituitaries does not result in linear growth in monkeys, while growth hormone extracted from monkey pituitary glands does give linear growth when administered to their own species. Knobil and Greep (1959)[13] found that growth in primates is stimulated only by primate growth hormone while in hypopituitary human patients there was no growth response to administered bovine growth hormone. Thus species specificity was established.

Researchers in this field have continued to report technical achievements in the extracting of growth hormone from human cadaveric pituitaries. The now historical work of Li and Papkoff (1956)[12], of Raben (1957)[14] and of Wilhelmi (1961)[15] will long be remembered for their names are linked to various human growth hormone preparations now consigned to obscurity following the appearance of several cases of Jacob-Creutzfeldt disease developing in American patients who had received human cadaveric growth hormone. Whether this disease will ever be linked specifically to a slow virus acquired either during the procedure of the hormone preparation or from "rogue" pituitaries in the collections remains to be seen. However the "scare" brought to an abrupt end the treatment of growth-retarded children with human growth hormone. Important in this historical review is the fine work of Li et al.,(1966 & 1969)[16 & 17] who characterized the molecular structure of human growth hormone, (Figure 1). The stage was thus set for the recently introduced technique of

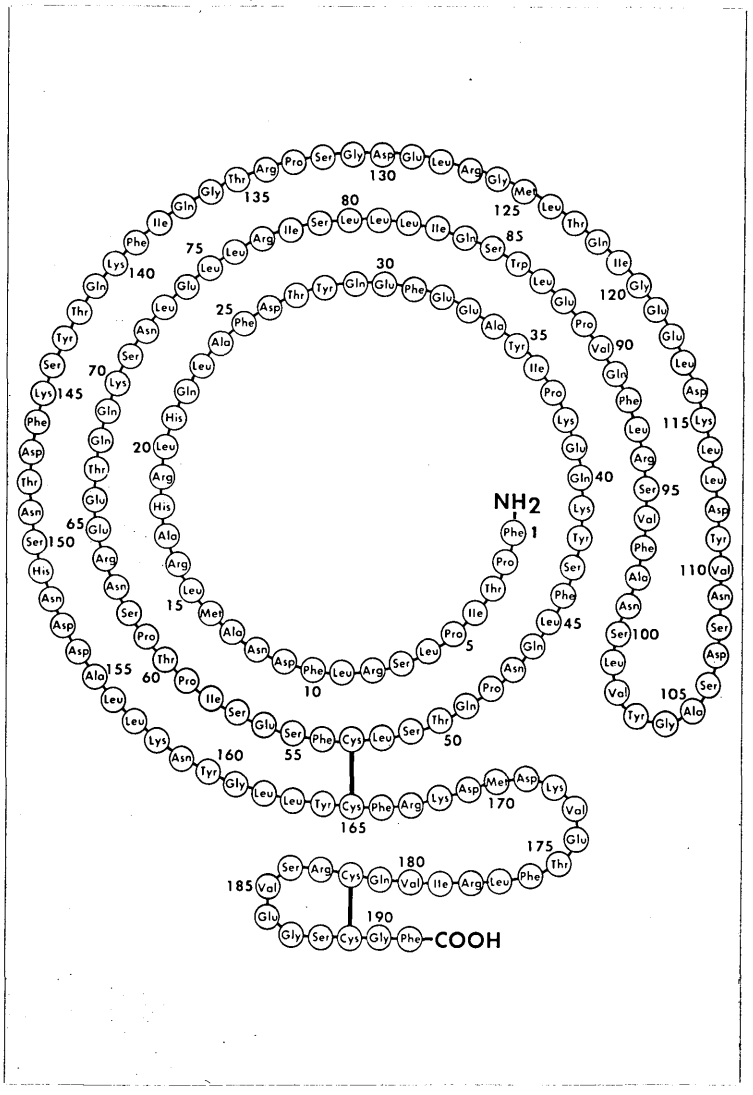


Figure 1: Human growth hormone molecular structure. It is a single polypeptide chain with 191 aminoacids, its molecular weight is 21,734 D. and it has two disulphide bridges between cysteine residues at loci number 53-165 and 182-189.

recombinant DNA synthesis of growth hormone to fill the gap created by the loss of the human glands. Bacteria and rodent mammary gland cells became the important tools of research rather than the human pituitary glands.

Jacob-Creutzfeldt disease.

This is a spongiform subacute fatal encephalopathy characterized by progressive presenile dementia with central nervous system disorders such as ataxia, gait disorders, myoclonus, convulsions, extrapyramidal and motor neurone signs (Simpson, 1984)[18]. It is a world-wide disease with an average seven months clinical course to a fatal termination through prodromal and dementing-myoclonic phases. The cerebral cortex, cerebellum, thalamus and hippocampal areas are involved. Death usually occurs after six to twelve months. The incidence of the disease is about one per million deaths in USA (Underwood et al., 1985)[19]. Duffy et al., (1974)[20] were the first to report the transmission of the disease from man to man following corneal transplantation. Bernoulli et al., (1977)[21] later reported two further cases of transmission due to the use of silver electrode implantation in stereostatic electroencephalographic exploration. The electrodes had been used previously in a patient proven to have the disease but they had been sterilized with 70 per cent alcohol and formaldehyde vapour. Now that the Jacob-Creutzfeldt syndrome in human growth hormone-treated patients has been recorded and the cause identified, it should not occur in future in such patients as a result of therapy.

Synthetic human growth hormone.

It would be untrue to say that the Jacob-Creutzfeldt scare which resulted in withdrawal of cadaveric growth hormone as treatment in the Spring of 1985, was the stimulus for the production of a synthetic growth hormone suitable for replacement therapy. But it certainly was a stimulus to the early marketing of a product which was nearing perfection in the commercial laboratories of companies such as Eli Lilly, Kabi Vitrum, Serona and Nordisk. There had however been a growing realization that hormone extracted from human pituitaries was in insufficient supply for the demand which existed even before 1985. Further, other protein hormones were in demand also and purity, monomerism and nonantigenicity were the main requirements. Monocomponent insulin had arrived and clinicians in many fields had made demands on the scientists to produce monocomponent human growth hormone, Factor VIII and other blood clotting factors, and the gonadotrophins which until then had all the defects inherent in cadaveric growth hormone preparations. Added to these demands came the risk of the AIDS virus. Being neurotrophic, there was a possibility that an HIV positive individual without the active disease could become the donor of a pituitary by sudden death and postmortem examination.

Characterization of human growth hormone structure and the determining of its aminoacid sequence by Li et al., (1966 & 1969)[16 & 17] stimulated a search for the active core within the 191 aminoacids. Much work in animal models followed and it was in 1979 that Goeddel et al.,[22] first described the DNA sequence coding for human growth hormone synthesis in Escherichia coli.

The end result of these researches resulted in production and clinical use of methionyl growth hormone in January 1986 at least in the Glasgow area.

Flodh (1986)[23] described a method of synthesizing authentic human growth hormone thus removing the methionine from the previous preparation. His method was to use a sequence known as a signal peptide which informed the cells used that the protein connected to their C-terminal was to be "exported". The DNA sequence corresponding to the 23 aminoacids of the signal peptide was inserted between the "promoter" and the gene for human growth hormone. Thus the bacteria elaborated under their particular culture conditions, the 191 aminoacid sequence plus the 23 aminoacids of the signal peptide. The 23 aminoacid sequence was then cleaved from the authentic human growth hormone by signal peptidase. This new product has been under clinical trial by many workers in this country, in Europe and in the Far East and has been proven to have all the attributes for growth promotion in children shown to be growth hormone deficient.

One of the great advantages of this new preparation is its minimal degree of antigenicity (Takano and Shizume, 1986)[24].

At the time of the binding of this thesis the new authentic growth hormone preparation is on the market and is being freely prescribed.

Somatomedins.

The ability of growth hormone to stimulate growth was believed to be a direct action of the hormone on the target tissues. Salmon and Daughaday,(1957)[25] however showed that normal serum stimulated the uptake of sulphate by the cartilage taken from immature hypophysectomized rats. They designated a factor in the serum as sulphation factor for it was not present in the serum of rats which had been hypophysectomized. But following administration of rat growth hormone to previously hypophysectomized rats, the factor returned to the serum. A similar situation obtained in children who were short statured because of growth hormone deficiency (Almqvist, 1960)[26]. Subsequent study of this factor revealed that its plasma level was age-dependent (Almqvist and Rune, 1961)[27], growth hormone dependent (Salmon and Daughaday, 1957)[25] and nutrition dependent (Grant et al., 1973)[28]. Daughaday et al., 1972[29] showed that the factor was active in the growth process of both skeletal and nonskeletal tissues and proposed the name somatomedin as a suitable designation.

Many and varied research projects have been devoted to somatomedin since these early days and it will suffice here to state the present thinking on this substance(s). The mitogenic factor(s) were extracted from normal serum, purified and characterized. They were found to be polypeptides[30 & 31] with the end chains of proinsulin hence the adopted terminology of insulin-like growth factors. Since two factors of differing molecular weight containing different numbers of aminoacids were characterized the terms IGF-I and IGF-II were adopted to replace

the earler terms Somatomedin A and C (IGF-II). The IGFs are elaborated in several tissues particularly in the liver and kidneys but also by many other tissues where their effects are mediated locally through growth hormone. The major portion of IGF´s circulating are of hepatic and less so renal origin. They circulate bound to high affinity binding proteins whose precise role is uncertain. There are specific receptors for the IGFs. The Type-I receptor is similar to the insulin receptor and binds preferentially IGF-I while not surprisingly the IGF-II receptor preferentially binds IGF-II. IGF-I is predominantely the factor which with growth hormone, promotes linear growth. It is probably still speculative but IGF-II may play a role in the other metabolic effects of growth hormone. The scheme of interplay of IGF-I and growth hormone may be represented as in Figure 2.

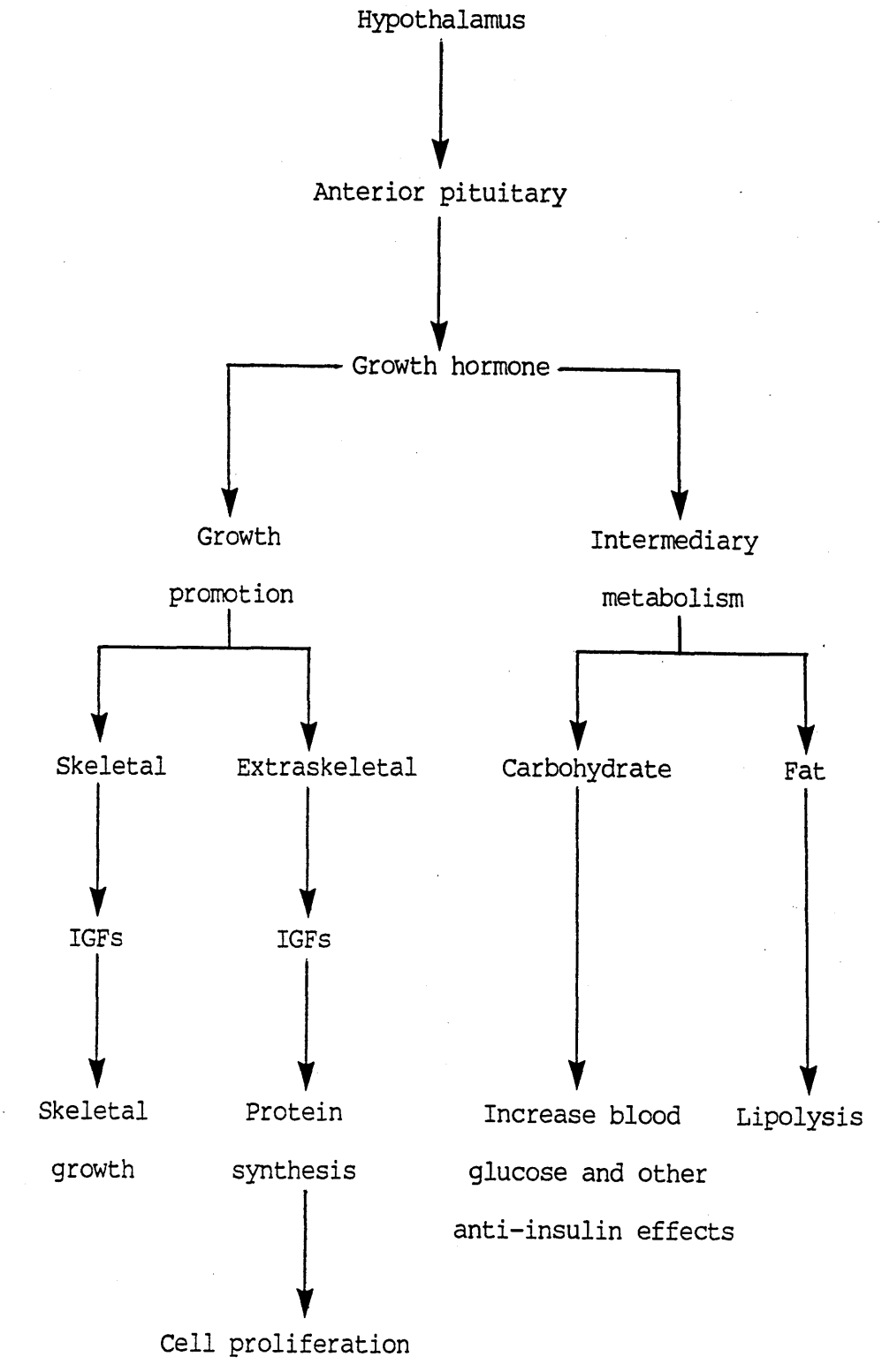


Figure 2: Actions of the human growth hormone.

Modified from: Buchanan C. Growth and growth disorders. KabiVitrum Ltd., Middlesex and Dublin, 1988; 17-17.

Testing for Growth Hormone Deficiency.

The early biological methods for determining the potency of extracts of the cadaveric pituitaries for growth hormone content are now of historical interest. The radioimmunoassay technique for measuring growth hormone was made possible by the advent both of purified hormone and the availability of isotope-labelling methodologies,[32].

Clinically the insulin unresponsiveness test was previously used as an indirect assay of growth hormone adequecy. In this test insulin was given to the patients (I.V.) and the level of blood sugar noted over a period of two and a half hours. Nonrecovery from a hypoglycaemia of half the basal level by two to two and a half hours was taken as an indication of growth hormone deficiency. This test has remained the best test of growth hormone status but with the adjunct of radioimmunoassay of plasma growth hormone as well as the blood sugar concentration; the latter values are now used to gauge the adequacy of the hypoglycaemic response. Additionally in the same plasma samples it is possible to assay cortisol since another effect of hypoglycaemia is to raise the plasma ACTH concentration. It is currently accepted that following adequate hypoglycaemia, plasma growth hormone concentrations of less than 7.0 mU/L represent complete growth hormone deficiency while levels between 7.0 and 15.0 mU/L indicate a partial deficiency state. Not every clinician accepts the partial deficiency state[33], while others accept higher concentrations of plasma growth hormone even up to 20 mU/L as indicative of a deficiency state in relation to puberty[34].

In the normal individual there is a diurnal rhythm of growth

hormone secretion. For example on wakening, plasma concentrations are around 6 mU/L and during the day higher concentrations are observed in response to food and exercise. Highest plasma concentrations have been found during sleep[35 & 36]. This simple observation has become a useful tool alongside the insulin hypoglycaemia test. Although the early work on growth hormone concentrations in response to REM sleep utilized 30 minutes sampling, in the light of the results and in general clinical practice such frequent sampling is neither necessary nor practical. It will be seen from Figure 3 that sampling at four-hourly intervals is a practical and useful assay of the neurosecretory discharge of growth hormone. Thus patients with an adequate response to insulin hypoglycaemia may well lack this neurosecretory sleep response which will undoubtedly prevent normal growth.

Several other stimulatory tests have been devised such as the response to exercise[37], to (I.M.) glucagon[38], to infused methyl DOPA[39], to (I.V.) arginine[40] and more recently to oral clonidine[41]. In the work here presented the insulin hypoglycaemia test was used.

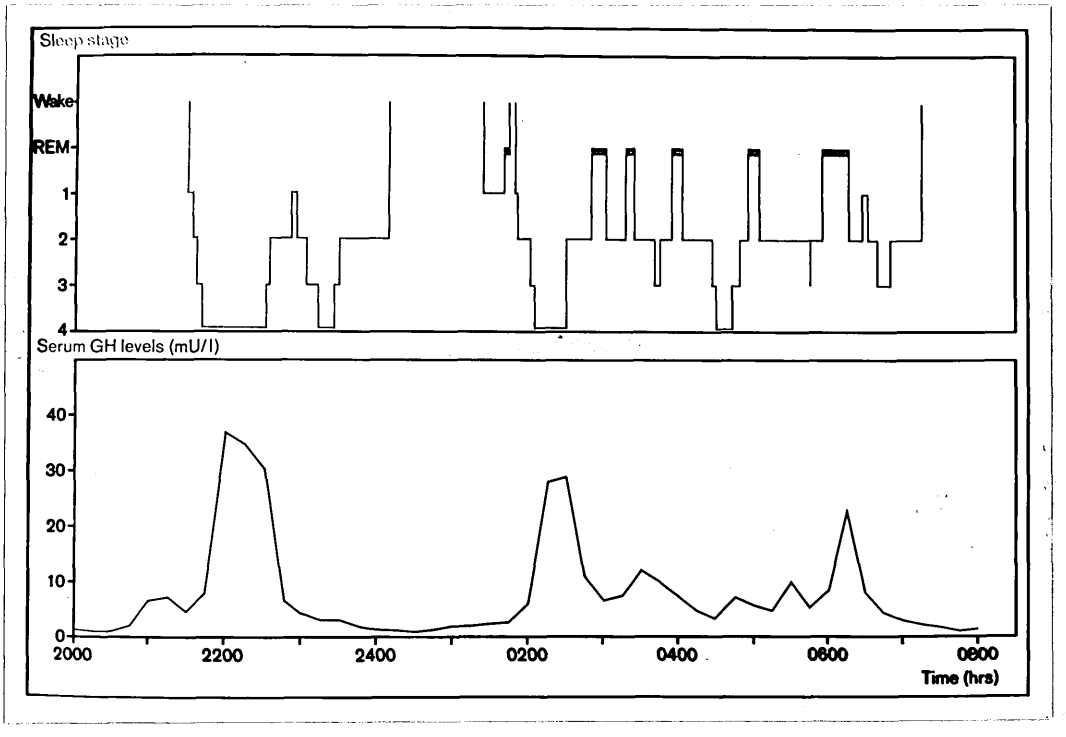


Figure 3: Over night profile of sleep stages and serum growth hormone concentrations.

Taken from: Buchanan C. Growth and growth disorders. KabiVitrum Ltd., Middlesex and Dublin, 1988; 11-11.

Treatment of Short Stature with growth hormone.

This subject will be picked up in its modern context. Administered growth hormone has an initial half-life of around 20 to 25 minutes. Thus it might be expected that large frequent doses of growth hormone would be required for adequate treatment of growth hormone deficient children. In fact it has been found that the administration of hormone in 4 I.U. doses on alternate days for three doses per week gives a good response and this has been the classical therapeutic regimen. Recent studies[42 & 43] have indicated that small daily doses of growth hormone given subcutaneously approximates better to the physiological state and improves the growth response.

Androgens, Oestrogens and Growth Hormone.

It has long been recognized that there is an increased output of growth hormone at least in boys[44] at the age of puberty. This observation has led to the practice of priming significantly short statured patients who are of pubertal age but who have an osseous maturation score less than ten years, with testosterone. Girls in the same category have been given ethinyloestradiol. Those papers reviewed do not indicate the mechanisms of action of these drugs and authors who report on this practice do not give the plasma concentrations of either the testosterone or ethinyloestradiol achieved. Certainly superphysiological concentrations must be achieved following either a stat dose of testosterone (I.M. 100 mg) or ethinyloestradiol (oral 100 µg daily for 3 days). Clinicians may not be concerned with

the plasma concentrations achieved but there are few laboratories which could raise antibodies to ethinyloestradiol to effect an assay system. In an informative paper Hamilton and Khattab, (1986)[33] suggested that the 17β- hydroxyl grouping of both testosterone and ethinyloestradiol may be the effective moiety. This led them to test the effectiveness of 17β-oestradiol as a priming agent for boys as well as girls. The estimation of plasma 17β-oestradiol a naturally occurring steroid, is much easier. Their findings were that 17β-oestradiol for both sexes is a satisfactory drug and offers the advantages that the drug is given orally, plasma concentrations are easily assayed and being a naturally occurring substance normal plasma concentrations are established within 5 to 7 days. Induced high concentrations of testosterone following an injection of a triple ester of testosterone (100 mg) up to 100 nmol/L may persist for more than 30 days. Ethinyloestradiol inactivation by the liver or other tissues is very slow and its high intrinsic potency and persistence are thereby explained. The same authors also observed that following adequate hypoglycaemia (< 1.0 mmol/L) plasma concentrations of growth hormone (< 7.0 mU/L) were not improved significantly by priming either with testosterone or 17β-oestradiol.

The question of the mechanism of action of androgens and oestrogens in the priming procedure remains unanswered. Undoubtedly androgens and oestrogens account for that intangible attribute called masculinity and femininity in the sexes. Nature has performed for us an experiment in the complete expression of the testicular feminizing syndrome where in the chromosomal male there is complete feminization in psyche and soma despite high

concentrations of plasma testosterone. In the partial form masculinization of both psyche and soma occurs in the untreated patient at puberty. By what mechanism then is the psyche and for that matter other parts of the brain within the hypothalamo-pituitary axis affected and by what prosthetic grouping of the steroid nucleus?. Interestingly adult patients with the complete form of testicular feminization syndrome have a considerable sexual libido.

Some extremely interesting knowlege has accrued from animal experiment and it is highly probable that humans share like functions. For instance it has been shown[45 & 46] that there are androgen and oestrogen neurones in brain tissue. Androgen neurones are capable of converting naturally occurring androgens, (androstenedione) to oestrogens[47]. Oestrogen neurones while accepting oestrogens do not alter the oestrogen nuclear structure[48].

Other workers[49] have studied the metabolism of testosterone by neurones from various areas of the brain. Testosterone is converted to 5α-dihydrotestosterone, 5α-androstane,3α,17β-diol and androstenedione, and the greatest conversion is by the anterior hypothalamo-thalamic and midbrain neurones followed by the pituitary gland itself. If we now combine these findings it is clear that both these androgens are important in the "endocrine" areas of the brain. Despite the fact that testosterone is seven times more potent than androstenedione in its peripheral action[50], in their endocrine neural activity both display molar equivalence[51]. It is unlikely that the 5 -reduced metabolites of testosterone have a physiological activity at the endocrine brain level[52], but circulating 17β-oestradiol, the 17β-oestradiol derived from circulating androstenedione by the

action of the endocrine neurones and the 17β-oestradiol derived from the androstenedione, a neural metabolite of testosterone, is the principal stimulus to hypothalamo-pituitary activity. Thus it is most probable that it is the unreduced A-ring of the 17β-oestradiol nucleus and not the 17β-hydroxyl group, which is the active moiety activating endocrine events in the endocrine neural pathways. The relationship with the work which will be presented in this thesis is the now well recognized action of orally administered oestrogen on the biphasic release of gonadotrophins from the pituitary namely their initial inhibitory and later stimulatory actions. Thus it is likely that there are at least two populations of oestrogen-sensitive neurones, one group sensitive to brief intense oestrogen stimulation and the other to long term low level stimulation[53]. There is a strong possibility that both circulating and locally elaborated oestrogens within the endocrine hypothalamus activate also growth hormone releasing-hormone as they do gonadotrophin-releasing hormone. The logic of utilizing 17β-oestradiol as a priming agent is enhanced by the low circulating androgens and oestrogens in that group of patients whom we are pleased to term "growth hormone deficient" and is more understandable. Whether the low target organ synthesis of androgens and oestrogens is the cause or result of the low growth hormone production, the correlation in paediatric practice is striking and probably significant. In this thesis I will present some work which will strongly suggest a positive influence of androstenedione as a stimulant to growth hormone action or at least as a synergistic agent.

Somatic effects of androgens and oestrogens.

The principle and the most physiologically active circulating androgen is testosterone. Its mode of action is to facilitate DNA synthesis in the cell nucleus. However it is not testosterone itself but a metabolite, dihydrotestosterone which is the ultimate initiator of the synthesis. Like other steroids, testosterone diffuses passively into the cell to become attached to cytoplasmic receptors. It is here that the conversion of testosterone to dihydrotestosterone takes place and thus the concentration of the receptors in the cell cytoplasm determines the conversion rate, the passage of both testosterone and its metabolites through the cytoplasm and the nuclear membrane and the ratio of testosterone and dihydrotestosterone transferred into the nucleus. An important point here is that at moderately low cytoplasmic levels of dihydrotestosterone there is a greater change in the nuclear concentration of this substance in the absence of testosterone. But there is a required concentration of circulating testosterone to maintain optimum intranuclear concentrations of dihydrotestosterone for DNA synthesis. Thus in patients such as are the subject of this work, where there is a failure of testosterone synthesis, adequate levels of nuclear dihydrotestosterone to maintain the cell in a state of full differentiation are not maintained by virtue of a deficiency of cytoplasmic testosterone and dihydrotestosterone.

Optimum concentrations of nuclear dihydrotestosterone are within the range 0 to 120 nM which requires a cytoplasmic concentration within the range 0 to 10 nM. With low plasma concentrations of testosterone resulting in cytoplasmic

concentrations of dihydrotestosterone in the range 1 to 2 nM, the concentration of nuclear dihydrotestosterone falls as low as 25 nM, a level at which DNA synthesis can not be maintained[54].

In this work I shall be presenting studies on the action of androstenedione. In the light of the above observation it is important to note that 3α- and 3β-androstanediol, androsterone, androstanedione, androstenedione and dehydroepiandrosterone do not as a rule enter the nucleus but are converted to dihydrotestosterone in the cytoplasm. Only those androgens which are capable of forming androgen-receptor complexes (notably testosterone and dihydrotestosterone) contribute to the nuclear concentration of dihydrotestosterone[55]. Table I shows the concentration of the nuclear dihydrotestosterone obtained from rat prostate after injection of various tritiated androgens. The rats had been castrated, hepatectomized and eviscerated thus avoiding endogenous production of the androgens either by synthesis or metabolism. In my patients, of course, no such operations were feasible and hence interpretation of my data may not be quite so simple. But several of the androgens mentioned were detected in the urine in increased quantity following androstenedione administration.

The anabolic effects of the androgens are thus mediated through the cell cytoplasmic androgen-receptor complex formation and the production of dihydrotestosterone for the nuclear activity of cell replication. Clearly different body cells will contain varying amounts of cytoplasmic receptors and the recognized androgen—dependent tissues are specifically endowed. But there are other anabolic actions of the androgens not mediated by the above considerations and these seem to be due to the androgens

Table I: The recovery of radioactive androgens in the nuclei of rat prostate. Modified from Bruchovsky and Lesser (1976)[54].

	^{3}H-Steroids recovered (10^3 x nmoles/g) wet weight of prostate	
	Dihydrotestosterone	
^{3}H-Steroid	Cytoplasm	Nuclei
Testosterone	2.9	2.6
Dihydrotestosterone	1.6	2.0
3α-androstanediol	2.0	2.8
Androstenedione	0.5	0.8
Androsterone	1.0	0.7
Androstanedione	0.6	0.5
3β-androstanediol	0.8	0.2
Dehydroepiandrosterone	0.7	0.1

themselves or metabolites other than dihydrotestosterone, eg. on skeletal muscle[56], on sexual behaviour[52], and on bone marrow [57].

Interestingly it has been thought that there are no specific androgen or oestrogen receptors in bone, progesterone being the only steroid and even then it competes with glucocorticosteroids for a common receptor. The action of androgens and perhaps to a lesser extent oestrogens on bone is through their action on protein synthesis so that as protein is formed osteogenesis takes place with subsequent mineralization. It is currently thought that only a small subpopulation of bone cells respond to physiological concentrations of sex steroids, the majority of the other bone cells being inactive. However the known activity of the cells at the epiphyseal plate where growth occurs is undoubtedly responsive to normal circulating concentrations of sex hormones as well as to growth hormone and the synthetic anabolic hormones.

The endocrine effects of oestrogens are well known but it is important to remember that oestrogens also increase plasma lipids, lipoproteins and calcium. At cell level oestrogens increase protein and DNA synthesis. The actions therefore of oestrogens on the liver, kidneys, muscle and blood are quite different from their actions on the uterus and the vagina. The former organs seem to "mop up" circulating oestrogens whereas the sex organs display a slow uptake and a long retention of the hormones over a prolonged period. In the uterus and vagina the steroid is retained as 17β-oestradiol whereas in other tissues there is a mixture of conjugated and unconjugated metabolites. The oestrogens at cell level, like the androgens, form in the cytoplasm

oestrogen-receptor complexes. But there is no conversion of the oestrogen to another compound as with testosterone to dihydrotestosterone, for the oestrogen-receptor complex moves into the nucleus, there to have its specific action.

The ovary is not the only source of oestrogen for the aromatase enzyme is widely distributed in the body tissues so that testosterone is converted to 17β-oestradiol locally where specific need exists. This thesis is less concerned with oestrogens except at the hypothalamic level where the conversion of testosterone and androstenedione to 17β-oestradiol is of prime importance.

Synthetic steroid analogues of testosterone.

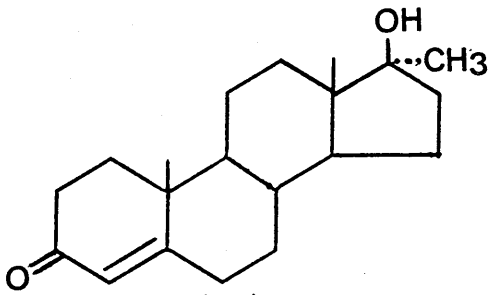
This group of compounds have been termed anabolic steroids and are part of a wider group of chemically modified testosterone and oestrogens, modified in such a way as to reduce their androgenicity and oestrogenicity respectively, but with retention of their anabolic activity. It must be remembered that testosterone acts both by its conversion to 5α-dihydro-testosterone through the steroid-receptor cytoplasmic complex system in androgenic dependent tissues and by virtue of the Δ4-3-oxo function of the A-ring. Specific receptors in muscles and in other sites of testosterone anabolic activity, have not been characterized or found.

There is no immediate action from the anabolic activity of testosterone or its analogues, and the molecular basis for the use of these compounds is wanting, but clinically anabolic effects are observed e.g. in production of muscle bulk and increase in

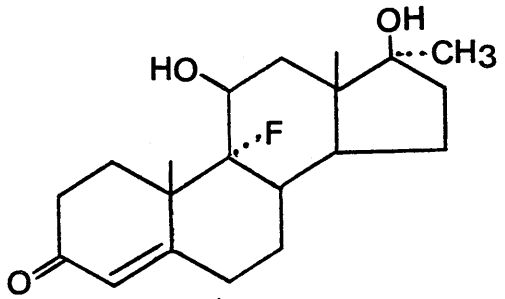
stamina of athletes. The common anabolic hormones are as in Table II.

There is a voluminous literature on the uses of these compounds as stimulants of linear growth either alone or in conjunction with growth hormone. Simpson et al., 1944[58] must have been among the first to observe that linear growth in rats was greater when growth hormone and testosterone propionate were used together than when either was used singly. Several groups of clinicians have used fluoxymesterone[59, 60, 61, 62, 63 & 64], others have used oxandrolone[65, 66, 67, 68, 69, 70, 71, 62, 72, 73,74 & 75], while others have experience with testosterone or one of its esters[76, 77, 78, 79, 80, 81, 82 & 83]. Considerable work has been done using various anabolic hormones in cases of Turner's Syndrome[84, 85, 86, 87, 88, 89, 90, 91, 92 & 93]. Undoubtedly from the clinical point of view there is an improved growth velocity when these drugs are used alone, or in combination with growth hormone. Although there is an immediate growth response which to some extent is maintained over the periods of steroid adiminstration, the unwanted action of the increasing osseous maturation accompanying their use tends to limit their ultimate value for chronic administration. The various authors have used dosage schedules for these anabolic hormones on a per kilogram body weight basis, and regrettably none gives the plasma concentrations achieved during their period of use. Thus there is an unfortunate non-scientific but rather clinical basis for assessment. Were it possible to be able readily to measure plasma concentrations of the anabolic substance used and to know its intimate metabolism and whether or not its metabolites had metabolic activity, a considerable step forward would be made into

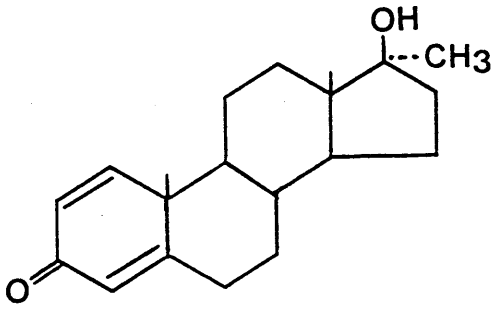
Table II : The common anabolic hormones used clinically.



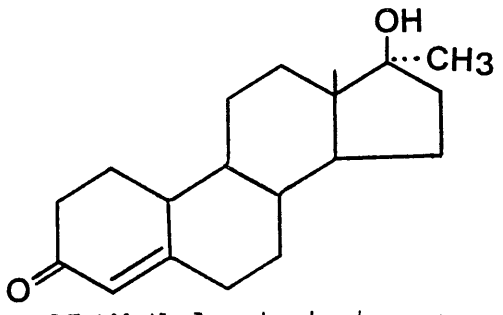
Methyltestosterone



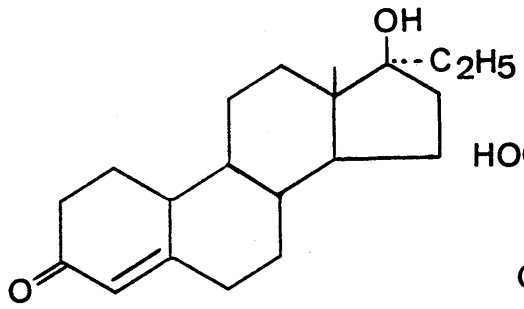
Fluoxymesterone



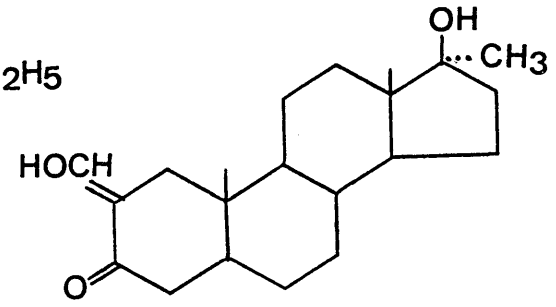
Methandrostenolone



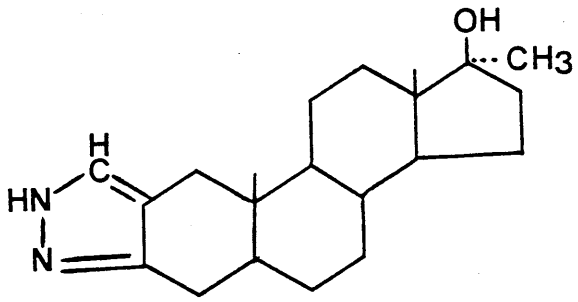
17α-Methylnortestosterone



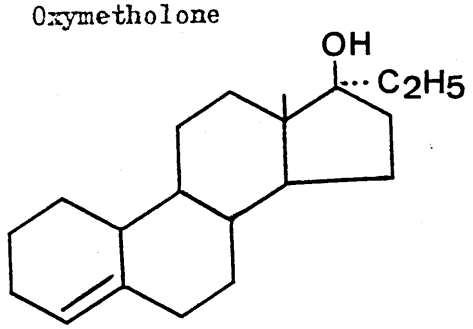
Norethandrolone



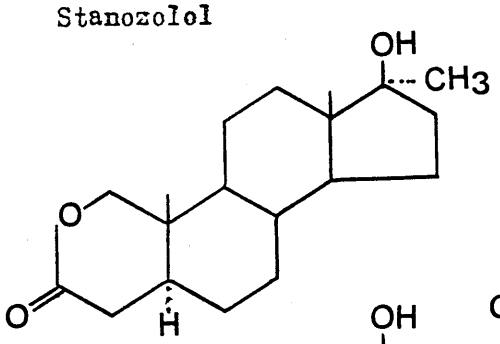
Oxymetholone



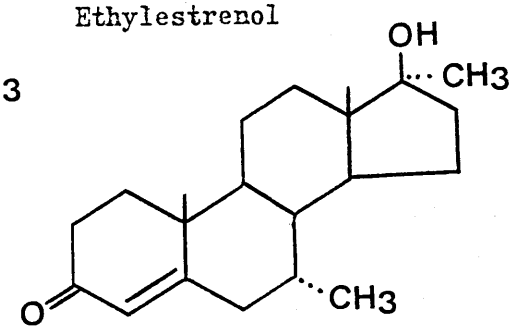
Stanozolol



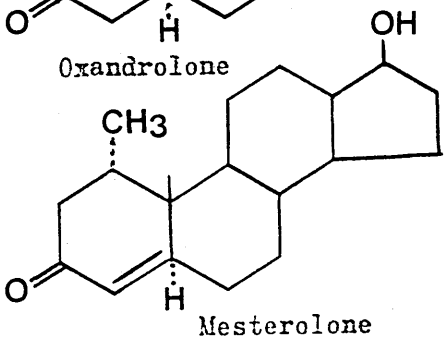
Ethylestrenol



Oxandrolone



Bolasterone



Mesterolone

the assessment of the part played by the anabolic hormone itself and its contribution to the growth stimulus in association with growth hormone. Fortunately there is such an anabolic hormone, naturally occurring, of adrenal origin, a precursor of testosterone, freely available and synthetically manufactured. It is androstenedione and its plasma concentration is easily estimated. This compound was used in this study as an anabolic hormone given in conjunction with growth hormone.

Androstenedione.

This naturally occurring androgen will be much spoken of in this thesis for it has been used in the clinical part of the study of the linear growth response to administered growth hormone. Androstenedione is its trivial name, androst-4-ene-3,17-dione its chemical name and because there will be frequent reference to it I shall use the abbreviation AD.

It was chemically synthesized in 1935 and shown to have an androgenicity in the capon's comb test of 12 per cent that of testosterone[94]. It is produced by the adrenal cortex[95], the Leydig cells of the testis[96], the ovary[97] and the placenta during pregnancy[98]. Formerly it was thought that its rate of production was relatively constant but more recently a diurnal rhythm has been noted in man[99] and a seasonal rhythmicity in gerbils[100]. Others have not found such a seasonal variation in rams[101]. ACTH increases its adrenal production[102] and LH its testicular and ovarian outputs[103]. AD plasma concentrations vary throughout infancy and childhood[104] being less than 4 nmol/L in pre-puberty and less than 1.0 nmol/L from the first

month to 4 years of age in both sexes. Interestingly immediately after birth peripheral and cord blood levels approach prepubertal values (Table III), and this is in keeping with many other hormones[105].

AD and testosterone are interconvertible[107] and this has been recognized as taking place in the liver. Interestingly testosterone is not synthesised by the adrenal gland. Recently it has been shown that lymphocytes[108], red blood cells[109], platelets[110], breast tissue[111], lung tissue[112] and skin[113] are capable of converting AD to testosterone. Indeed it has been demonstrated that AD may be a more important precursor of dihydrotestosterone in female skin than testosterone itself[114]. Degradation of AD to androsterone and aetiocholanolone[115] and to smaller amounts of epiandrosterone and the 5α- and 5β-androstanediols is the common pathway in the liver and other sites of action[50 & 113]. In the ovary AD is converted to 17β-oestradiol[116].

The plasma concentrations in health and disease have not been extensively used in clinical practice. Plasma concentrations are high in the Stein-Leventhal syndrome and some other causes of female hirsutism, in AD secreting adrenal tumours, in Cushing's syndrome, in congenital adrenal hyperplasia and in Leydig cell tumour[117]. Plasma concentrations of AD are normal in adult cases of Klinefelter's syndrome[118] and low in congenital adrenal hyperplasia due to lack of 17α-hydroxylase[119], following castration[120], in post-menopausal women and significantlly low in ovariectomized women[121].

AD circulates in the plasma in the free state and bound to sex binding globulin. Like other steroid hormones it is the free

Table III : Plasma concentrations of androstenedione and testosterone in cord blood and in peripheral blood on the first day of life. From Forest and Cathiard (1975)[106].

Site	Compound	nmol per litre	
		Male	Female
Cord blood	Androstenedione	3.03 ± 1.05	3.27 ± 1.32
Peripheral blood	Androstenedione	6.88 ± 3.32	6.10 ± 2.6
Cord blood	Testosterone	1.33 ± 0.36	1.01 ± 0.26
Peripheral blood	Testosterone	7.89 ± 4.46	1.61 ± 0.48

fraction which is physiologically active through the Δ4-3-oxo grouping of the A-ring. An equilibrium between bound and free AD exists so that as the free fraction is further bound to cellular receptors or converted to testosterone, release of the plasma bound fraction maintains normal plasma concentrations. A specific high-affinity binding mechanism for AD is present in the liver. Generally it is thought that AD itself has minimal androgenic activity save in its precursor capacity for testosterone. However it is being recognized that it may have an important action in fetal life in that it may take part in neonatal imprinting of hepatic enzymes[122].

The measurements of plasma AD in this work will be effected using an RIA Kit method. This method estimates in unextracted plasma both plasma bound and free AD. It is my intention to study the adjuvantal effects of orally administered AD to growth hormone-deficient children who are receiving growth hormone as treatment. Where others have failed when using synthetic anabolic hormones I hope to be able to estimate plasma concentrations of AD because its determination is possible, whereas with the lack of suitable antibodies for all the other synthetic anabolic steroid hormones the task of following these other substances biologically would require constant use of GLC-MS instrumentation not available in the Department of Child Health. Moreover, the metabolic pathway of AD is well documented so that it will be possible for me to correlate plasma concentrations of AD with the urinary excretions of its metabolites, samples of both blood and urine having been taken at the same time. It is my intention, however, to study first of all the immediate action of a bolus dose of AD given to non-growth hormone-deficient children in terms of

immediate conversion to testosterone and reduction to its urinary metabolites, and then to apply this information to the clinical cases of growth hormone deficiency. However since AD is converted to testosterone I shall also measure plasma testosterone concentrations and since testosterone is converted to AD I shall present an analysis of plasma and urinary steroids in eleven male patients who had received testosterone priming as part of their initial investigation (vide supra) for their short stature.

In Chapter two I shall outline the methodology used in fair detail.

CHAPTER TWO

METHODOLOGY

Statistical Methods

The statistics which have been used in this work were applied to only a limited number of data. The reason for this was the small number of patients included in the treatment programme (Group 3 patients) whom were followed for two years. The number of newly diagnosed, isolated growth hormone deficiency children attending the Paediatric Endocrine Clinic is approximately 3 - 5 patients per year. It is self evident, therefore, that to include a large number of patients would necessitate a study embracing many years or else involve a multi-centre study. Neither solution was practical. Another consideration is that ethical approval is essential were a new drug is being employed experimentally although scientifically.

The statistical methods which have been used in this thesis include the following. A common statistic used for all Groups (1 - 4) of patients (32) was the standard deviation score (SDS) for height. The mean, standard deviation and standard error of the mean were calculated and graphs were constructed showing the plasma concentrations of testosterone and AD in Group 1 patients. Also employed were means, the Wilcoxon test, the t-test, coefficient of variation and percentages where appropriate.

Clinical Materials and Methods

In this work I will present androstenedione (AD) as an anabolic hormone used clinically for the first time. I have been unable to find references to such a usage in a fairly extensive search (computer) of the world literature. It was, therefore, necessary first to study its fate in the body, assessing its absorption from the GI tract during fasting and in relation to food, blood concentrations achieved by a standard orally administered bolus, its possible biological half-life, its urinary metabolites and its reciprocal relationship to plasma testosterone. Approval for this research had been obtained from the Hospital Ethics Committee.

Twenty-seven patients forming three groups have been studied. Group 1 consisted of eleven boys to whom was given AD 100 mg orally. In Table IV are the anthropometric data for these boys. The decimal ages and standard deviation scores (SDS) based on height measurements were all performed according to Tanner (1984)[123]. The osseous maturation scores were estimated by the TW20 method[124]. Table V lists similar data for eleven boys who received testosterone (Sustanon) 100 mg I.M. as a priming exercise prior to their having an insulin hypoglycaemia test. Table VI contains data of 5 boys diagnosed as having complete growth hormone deficiency and who received as treatment synthetic growth hormone 4 IU S.C. thrice weekly and who received concurrently 100 mg AD orally on the same day as the growth hormone injection. These latter 5 patients were paired with 5 other children (Table

Table IV : Anthropometric data of Group 1 patients.

Patient	CA.(yrs)	HA.(yrs)	Ht.SDS	BA.(yrs)	Ht(cm)	Wt(kg)
1 SS	9.25	6.83	-2.23	4.4	119.5	20.4
2 AG	10.73	8.06	-2.14	9.6	126.5	24.3
3 SC	11.40	8.50	-2.21	10.1	128.9	26.8
4 SW	12.18	8.76	-2.47	8.6	130.3	28.3
5 SD[1]	12.98	10.64	-1.70	9.7	140.0	30.6
6 TW	13.00	9.41	-2.51	10.3	133.8	32.5
7 JK	13.14	10.34	-2.01	10.5	138.5	29.5
8 PM	13.96	9.45	-3.18	8.5	134.0	29.8
9 SC[2]	15.30	10.79	-3.65	11.9	140.8	30.1
10 DC	15.66	11.58	-3.48	13.0	145.1	35.5
11 GC[3]	15.83	13.59	-1.96	14.5	157.5	42.8

CA = Chronological Age. HA = Height Age.

SDS = Standard Deviation Score for height. BA = Bone Age.

Ht = Height. Wt = Weight.

yrs = years.

(1) Patient 5 in Table VI.

(2) Patient 11 in Table V.

(3) This patient was treated by 100 mg AD orally on alternating days for 6 months and the treatment was stopped 1 month before the test.

Table V : Anthropometric data of Group 2 patients.

Patient	CA.(yrs)	HA.(yrs)	Ht.SDS	BA.(yrs)	Ht(cm)	Wt(kg)
1 SK	10.67	6.74	-3.25	7.2	119.0	20.0
2 DO	11.65	8.32	-2.50	9.5	127.9	28.3
3 AM	11.66	9.02	-1.96	7.7	131.7	30.0
4 MA	12.31	9.50	-2.01	10.7	134.3	28.9
5 NM	12.59	8.82	-2.69	10.7	130.6	25.7
6 SM	12.64	9.66	-2.12	8.3	135.1	29.4
7 JP	12.74	8.89	-2.72	10.8	131.0	25.0
8 DK	13.53	11.04	-1.84	10.0	142.1	47.9
9 SS	14.16	9.86	-3.09	14.2	136.1	30.1
10 AD	14.77	10.91	-3.00	11.3	141.4	26.3
11 SC*	15.30	10.79	-3.65	11.9	140.8	30.1

CA = Chronological Age. HA = Height Age.

SDS = Standard Deviation Score for height. BA = Bone Age.

Ht = Height. Wt = Weight.

yrs = years.

* Patient 9 in Table IV.

Table VI : Anthropometric data of Group 3 patients.

Patient	CA.(yrs)	HA.(yrs)	Ht.SDS	BA.(yrs)	Ht(cm)	Wt(kg)
1 AS	6.28	3.68	-3.24	2.9	99.3	15.2
2 MR	7.03	4.29	-3.12	6.3	103.6	19.6
3 GY	9.50	5.21	-4.05	5.8	109.6	18.7
4 GM	12.80	6.74	-4.32	8.8	119.0	25.6
5 SD*	13.80	11.19	-1.96	10.6	143.0	34.6

* Patient 5 in Table IV.

Table VII : Anthropometric data of Group 4 patients.

Patient	CA.(yrs)	HA.(yrs)	Ht.SDS	BA.(yrs)	Ht(cm)	Wt(kg)
1 RB	6.06	2.06	-4.65	2.9	91.0	13.9
2 JC	7.38	4.34	-3.37	6.7	103.9	19.7
3 JA	9.62	6.30	-3.01	8.7	116.4	20.0
4 NM	12.16	6.88	-3.89	10.6	119.8	30.7
5 BD	13.95	10.64	-2.45	10.3	140.0	36.6

CA = Chronological Age. HA = Height Age.

SDS = Standard Deviation Score for height. BA = Bone Age.

Ht = Height. Wt = Weight.

yrs = years.

VII) who had formerly received only growth hormone therapy and were matched as far as possible in chronological age plus one or more of the other appropriate anthropometric data.

The 11 patients of Group 1 had been under investigation for growth hormone deficiency as the cause of their short stature. All of them were shown to have adequate growth hormone reserve on insulin hypoglycaemia testing except Patient 5 (SD) who was later retested and shown to have complete growth hormone deficiency. He was then included in Group 3 patients (combined treatment with growth hormone and AD). These patients were used to study the kinetics of orally administered AD. Permission to do so was received from fully informed parents. Blood (5ml heparinized) and urine were taken hourly over a 5-hour period. After the basal sample AD (100 mg) was given orally. The separated plasma was stored at $-20^{o}C$ and the urine preserved with chloroform (2 ml), was stored at $-5^{o}C$ until required for assay.

The 11 boys (Group 2) whose clinical history and anthropometric data indicated the need for a primed insulin hypoglycaemia test received testosterone (Sustanon) 100 mg I.M. after basal blood (5 ml heparinized) and urine had been obtained. The plasma and urine were similarly prepared and stored. Three days later on the morning of the insulin hypoglycaemia test, further single samples of blood and urine were taken as previously and stored.

The 5 boys (Group 3) who had been shown to be examples of complete growth hormone deficiency on an insulin hypoglycaemia test were given synthetic growth hormone (methionyl growth hormone) 4 IU S.C. thrice weekly at 22.00h and AD (orally) 100 mg thrice weekly at 22.00h on the same day as growth hormone administration. Acceptance of this regimen was given by fully

informed parents. These patients were reviewed at three monthly intervals for height, weight, growth velocity and pubertal status plus at 6 monthly intervals for a bone maturation score. Also single samples of blood and urine were taken at each visit to study the changes if any, in plasma concentrations of the appropriate steroids (AD and testosterone) and alterations if any in the metabolic pathways of excretion when compared with their pre-treatment status in these respects. The AD was stopped after 1 year and the patient's height, weight, growth velocity, pubertal status and bone maturation were recorded for one further year of treatment with growth hormone alone.

Androstenedione preparation for oral use.

Androstenedione was obtained from Sigma Chemical Company Ltd, Fancy Road, Poole, Dorset, BH17 7NH, England. Chemical purity of the material was confirmed by the obtaining of a single UV absorbing band at 242 nm in three t.l.c. systems. These were in order;

I. Benzene : Methanol::85:15 v/v. (Rf value of testosterone is 0.58 and Rs value of AD is 1.31) (s = testosterone).

2. Benzene : Ethyl acetate::60:40 v/v. (Rf value of testosterone is 0.30 and Rs value of AD is 1.67) (s = testosterone).

3. Cyclohexane : Ethyl acetate::50:50 v/v. (Rf value of testosterone is 0.41 and Rs value of AD is 1.46) (s = testosterone).

(Rf value is the ratio between the migration distance of the testosterone and the distance covered by the solvent front from the base line). (Rs value is the ratio between the migration distance of AD and that of testosterone as a standard).

AD (50 µl) was spotted on a 20x20 cm silica thin layer using chloroform as a vehicle. The chromatogram was developed in the solvent system I for two, 45-min., periods, allowing the plate to dry in air before the second development. The silica over the UV-absorbing area of AD was removed and the steroid eluted with dichloromethane (3 x 2 ml) and then methanol (3 x 2 ml). The total eluate was blown to dryness under nitrogen and the residue taken up again in chloroform and spotted for development in the second system. The procedure was again repeated using the third solvent system. In all the systems the AD was found to give one UV absorbing spot.

All glassware and porcelainware used for the preparation of AD for oral use were sterilized in dry heat ($100^{o}C$) for 1 hour. Rubber stoppers were sterilized in absolute alcohol for 1 hour at room temperature. AD (10 g) was placed in a conical flask containing absolute alcohol (100 ml). At $60^{o}C$ the solute was in complete solution. This solution was then filtered through a scintered glass funnel and the filtrate collected in a second conical flask which was stoppered using an appropriate ground glass stopper. The flask was then placed in a refrigerator at $-20^{o}C$ for 12 hours. The AD by then had crystallized as an macroscopic amorphous deposit. The supernatant alcohol was removed by careful tilting of the flask and after the addition of further absolute alcohol (100 ml) the procedure was twice repeated. The thrice crystallized AD was transferred to a porcelain basin (dia. 12 cm) and the residual alcohol evaporated at $40^{o}C$ in a hot air oven. The AD was then weighed out using an Electronic Reading Balance, in 100 mg quantities onto pharmaceutical powder-papers, which were then folded professionally

and stored in 12's in plastic (sealable) envelopes. Each envelope contain a sachet of dessicant.

The glassware was obtained from the hospital Pharmacy and the dispensing was undertaken in a clean place using a sterile platinum spatula. Packs of powders (12 x 100 mg) were dispensed at monthly intervals to the patients of Group 3.

Laboratory Materials

Radioactive Labelled Steroids

The radioactively-labelled steroids used in this work were (1,2,6,7-^{3}H)testosterone (SA 87 Ci per mM) and (9,11-^{3}H)androsterone (SA 40 Ci per mM). The former was used for the purpose of the recovery experiments of urinary androgen extraction and esterification, and was purchased from Radiochemical Centre, Amersham, Buckinghamshire, England. The latter was used for estimating the percentage recovery of the esterification of the standard steroid mixtures, and was purchased from New England Nuclear, Albany Street, Boston, Mass, USA.

Unlabelled Steroids

The following androgens were purchased from Sigma Chemical Co. Ltd., Norbitan Station Yard, Kingston-upon-Thames, Surrey, England.

Systemic names	Trivial names
5α-androstane-3α,17β-diol.	5α-androstanediol.

5β-androstane-3α,17β-diol.	5β-androstanediol.
5α-androstane-3β,17β-diol.	5α-3β,17β-androstanediol.
5α-androstane-3α-ol,17-one.	androsterone.
5β-androstane-3α-ol,17-one.	aetiocholanolone.
5α-androstane-3β-ol,17-one.	epiandrosterone.
5α-androstane-17β-ol,3-one.	5α-dihydrotestosterone.
Δ5-androstene-3β,17β-diol.	Δ5-androstenediol.
Δ5-androstene-3β-ol,17-one.	dehydroepiandrosterone.

Other Reagents

Beta-glucuronidase was obtained from Sigma Chemical CO. Ltd., as above. The potency was $1,47x10^6$ Fishman Units per gram of crystalline material. It was used in the hydrolysis of urinary steroid glucuronides at a dilution of 750 F.U. per ml urine.

Methanol (Analar Grade) was purchased from James Burrough Ltd. Montford Place, London, SE11, England.

Peroxide free diethyl ether (Analar Grade) and Heptafluoro-butyrate anhydride were purchased from May and Baker Ltd., Dagenham, Essex.

Toluene based scintillation fluid containing PPO(2,5-diphenyl-oxazol) (5 g/L), POPOP(1,4-di(2-(5-phenyl-oxozalyl-benzene))) (0.05 g/L) from Hopkin and Williams Ltd., Asschem, Redding Industrial, Estate, Redding, Falkirk, Scotland.

Other chemicals such as dichloromethane, hexane, chloroform, benzene, ethyl alcohol (all Analar Grade), sodium hydroxide, sodium acetate trihydride and glacial acetic acid were purchased from BDH Chemical Ltd., Poole, Dorset, England.

Gases

Cylinders of oxygen-free nitrogen, of compressed air and of hydrogen were purchased from British Oxygen Company Ltd., Possil Park, Glasgow. Cylinders of propane(2 per cent) in argon(98 per cent) were purchased from British Oxygen Company Ltd., Deer Park Road, London, England. Gas purification bottles (supplied by British Oxygen Company) were placed in-line on every cylinder of gas.

Equipment

Glassware

All glassware of silica quality was purchased locally . This included test tubes (stoppered and unstoppered), conical flasks (glass stoppered), separating funnels, measuring cylinders, pipettes, micropipettes, trident vials and elution thimbles.

The Gas Liquid Chromatograph

This was model 304 from Pye Unicam Ltd., Cambridge, England. It was fitted with both flame ionization and electron capture detectors reading to a single pen recorder (Philips) on standard chart paper (PM.9920/00).

Bubble Flow Meter

This was supplied by Pye Unicam Ltd., accompanying the purchase of the G.L.C. Equipment. It was used to meter the flow rate of gases both carrier (nitrogen) and flame (hydrogen and air).

The Liquid Scintillation Spectrophotometer

This was a Philips instrument Model PW 4540 Liquid Scintillation Analyser with a "print out" Model PM 2467 digital printer. It was purchased from Pye Unicam Ltd. as above. The instrument was calibrated using a three channel system for simultaneous counting of both ^{3}H and ^{14}C. Quenched standards (Philips) were used to establish quench correction curves employing external standard channels ratio techniques and the external standard of 10 micro Curies of Radium 226 incorporated into the scintillation counter.

Radiochromatogram Scanner

This was a PANAX Instrument Model RTLS 1A supplied by Panax Equipment Ltd., Redhill, Surrey, England in conjunction with a Smith's Flatbed Recorder (Smith's Industries Ltd., Industrial Division, Wembley Park, Middlesex, England). The detector gas was a mixture of argon (98 per cent) and propane (2 per cent) regulated to a gas flow rate of 50 ml per minute. All plates were scanned at the 15 x 2 mm detector aperture adjusted to approximately 1 mm above the plate (nearer if possible for maximum geometric localization). The detector voltage was set at 1,040 V, detector dead time 200 µS and discriminator bias at 10 mV. A time constant of 100 seconds and a scanning speed of 30 mm per hour were employed.

Gamma Counter

This was a PANAX AUTOBIOSCINT supplied by Panax ESI, Rotherone and Mitchell Ltd., Ruislip, Middlesex, England. This was for counting ^{125}Iodine in the estimation of plasma

testosterone and androstenedione.

Unicam SP600 Spectrophotometer

This instrument was purchased from Pye Unicam Ltd., York Street, Cambridge. Its use in this work was in the estimation of urinary creatinine in the urine samples obtained from boys of Groups 2 and 3.

Griffin Incubator

This instrument was bought from GallenKamp Co. Ltd., Technico Home, Christopher Street, London, EC2 2ER, England. The urinary steroid conjugates were hydrolysed with β-glucuronidase in this incubator, the temperature being set at 37°C.

pH-Meter

This was an Horiba M and L Type instrument, supplied by Scotlab Instrument Sales Ltd., Unit 15, Earn Avenue, Righead, Industrial Estate, Bellshill, MlA 3JQ. It was used to adjust the pH of urine to 4.6 using either sulphuric acid (10 per cent w/v) or sodium hydroxide (1M solution).

Elgastat Water Deioniser

This was a Cartridge type purchased from Elga Ltd., Lane End, High Wycombe, Buckinghamshire, HP14 3IH. The deionized water was used for rinsing all detergent-washed glassware. Other samples of the deionized water were degassed by boiling for 10 minutes and used with biological samples destined for g.l.c. separation.

Techni-Samples Concentrator

This apparatus which is really a manifold for delivering nitrogen through fine metal tubes which are directed into test tubes containing organic solvent. The stream of nitrogen blows to dryness the sample contained in suitable test tubes. The instrument was purchased from R and J Wood, 39 Balk Sheddon Street, Paisley, PA3 2DE.

Meter Mechanical Analytical Balance

Bought from GallenKamp (address above) it has a range of 1.0 mg to 99.999 g. It was used to weight milligram amounts of the various androgenic steroids used as standards in the g.l.c. estimation of the urinary androgen metabolites.

Drying Oven with Thermostat

Purchased from Baird and Tatlock London Ltd., Chadwell Heath, Essex. It was used to dry all glassware which had undergone soaking in detergent (Decon 90), followed by washing with ordinary liquid soap in water and thorough rinsing with deionised water. It was also used for hot air sterilization of the glassware used in the purification of AD.

Vacuum Motors

These were Type B0-1508-B obtained from AE Motor and Control, Gear Division, Newcastel-upon-Weir, Staffordshire. One was used for suction to remove loosened silica from t.l.c. plates to eluting thimble flasks and the other to create a vacuum in the desiccator.

Pyrex Desiccator with Stopcock

This was purchased from Camlab Ltd., Nuffield Road, Cambridge, CB4 1TH. Its use was to desiccate completely steroid residues prior to esterification. The slightest moisture content in these urinary steroid residues inhibits quite significantly the percentage esterification.

Ultraviolet Lamp

This instrument had separate tubes emitting UV light at 242 nm and 356 nm respectively. It was used to locate steroids with the Δ4-3-oxo configuration (242 nm). It was bought from Baird and Tatlock London Ltd..

Shaker Waterbath Incubator

Type BKS-300 with rack, cooling coil and thermostatic regulator supplied by GallenKamp Ltd. (address as above). It was used to incubate plasma samples in the assays of plasma testosterone and androstenedione.

Thin layer Silica Plates

These were bought from Anderman and Co. Ltd., Central Avenue, East Molesy, Surrey. They were Type 60 F 254 precoated with silica gel (0.25 mm thickness). These plates are ready for use without further processing. Although the laboratory of the Department of Child Health has a plate spreader for hand use, the preparation of the starch/silica mixture, the uneveness of the layer, the washing, the drying and storage stages reduced the usefulness of that equipment.

Rotamixer

This is a mechanical agitator to facilitate solution of solute in solvent. It is a very useful instrument for use with small volumes. It was purchased from Hook and Tucker Instrument Ltd., Vulcan Way, New Addington, Croydon, CR0 9UG.

Centaur-2 MSE Centrifuge

The instrument was purchased from Fison plc., Scientific Equipment Division, 41 Gatwick Road, Crowley, Sussex, RH10 2UL. It is a bench top centrifuge and was used for separating plasma from blood cells and at times to separate organic and aqueous phases in extraction techniques.

Glass Vacuum Thimbles

These were obtained from McQuilkin and Co., Polmadie Avenue, Glasgow. They consist of a scintered glass filter disc incorporated into a glass tube expanded above and tapered beneath the scintered glass level. They were used in the elution of steroids from the silica which had been removed from the t.l.c. plates corresponding to the UV positive loci of the steroids.

Trident Vials

These were purchased from Scientific Suppliers, Scientific House, Vine Hill, London. They were used to contain the tritiated steroid residues plus scintillant fluid for counting (dpm's) radioactivity in the Scintillation Spectrophotometer.

Microsyringes (SGE)

These of 1 microlitre and 10 microlitre capacity were

purchased from Scientific Glass Engineering (UK) Ltd., Potters Lane, Kilnfarm, Milton Keynes, England. They were used for injection of samples to the g.l.c. column.

Silanized Glass Wool

This was bought from Phase Separation Ltd., Deeside Industrial Estate, Queen's Ferry, Flintshire. Small wedges of this material were used as loose plugs at both ends of the column (support material) in the g.l.c. oven. The purpose of these small plugs is to contain the support material .

Laboratory Methods

Several methodologies were used in this work each one utilizing different physicochemical procedures. I shall describe them in order of their having been used in relation to the three groups of boys studied. Basic to all three groups was the fractionation of the urinary androgens (C19-compounds) which involved the addition to the urine of ^{3}H-testosterone for recovery experiments, the hydrolysis of urinary steroid conjugates, their extraction, partial purification, esterification and quantitation by g.l.c..

Determination of Urinary Androgen Metabolites

Radioactive-Labelled Steroids

Radioactively labelled testosterone and androsterone were used in this work. Both these compounds were tritiated, testoster-

one 1,2,6,7-^{3}H (SA. 87.0 Ci per mM) and androsterone 9,11-^{3}H (SA. 40.0 Ci per mM). One millicurie of ^{3}H-testosterone in benzene (1 ml) was purchased from Radiochemical Centre, Amersham. The ampoule was opened and the testosterone transferred quantitatively to a glass stoppered test-tube using benzene (3 x 1 ml). The testosterone was blown to dryness under nitrogen and reconstituted with benzene / chloroform 6:1 (2 ml). This was stored at 5^{o}C as a stock standard. One hundred microlitres of this stock standard were spotted on a t.l.c. plate evenly streaked along the origin (20 x 20 cm) and developed in the system cyclohexane:ethyl acetate (60:40 v/v) for 45 minutes. Silica over the locus of the testosterone (outlined by the Panax Radiochromatogram) was removed and the testosterone eluted by ethyl acetate (3 x 5 ml), blown to dryness under nitrogen and reconstituted in benzene / chloroform 6:1 (100 ml). On counting, 1 ml contained 1,098,900 dpm's giving a 99 per cent recovery from this purification step. The solution was stored at 5^{o}C as the working standard. One half of a millilitre of this working standard testosterone was added for assessment of recovery in each volume of urine (pH 4.6) to be analyzed.

Androsterone was similarly handled having been purchased in an amount of 250 uCi from New England Nuclear, Mass, USA. An amount of 0.25 ml (68680 dpm's) was added to the standard steroid mixture for estimation of the percentage of esterification.

Solutions for pH adjustment of urine and buffer:

Sodium hydroxide (1M) solution was prepared by dissolving sodium hydroxide pellets (40 g) in freshly boiled, cooled, deionized water (1 L).

Sulphuric acid solution (10 per cent w/v) was prepared by

adding the fuming acid volume (11 ml) (SG. 1.84) to water (200 ml).

Acetate buffer (2M) was prepared by dissolving sodium acetate trihydrate (272 g) in glacial acetic acid (120 ml) to deionized water (1 L).

The urine was adjusted to pH 4.6 by the addition of small amounts of sulphuric acid (10 per cent) and 1M sodium hydroxide and to stabilize the pH during incubation, acetate buffer (20 ml) was added to maintain the pH.

Hydrolysis of urinary steroid conjugates

To the hourly samples of urine of Group 1 patients, to a measured volume of urine (25 - 100 ml) from Group 2 and Group 3 boys, β-glucuronidase (750 F.U. per ml) was added and thoroughly mixed prior to incubation at 37°C for 72 hours. The efficiency of the β-glucuronidase preparation as purchased was not verified but was taken according to Sigma reference value.

Extraction of free urinary steroids

The incubated urine was transferred quantitatively into a separating flask (500 ml) by filtration using Whatman GF/A glass fibre paper and the urine extracted with diethyl ether (1/2 vol. x 4). On each occasion the contents of the flask were allowed to settle for 10 minutes before collecting the ether layer in a conical flask (200 ml) the organic extractant being collected in a similar flask thereafter. The total volume of diethyl ether was placed quantitatively into a separating flask and washed with sodium hydroxide solution (5M; 10 ml) and thereafter with deionized water (20 ml) until the water washings were neutral to

"full range" Whatman test strip (pH 1-14). The diethyl ether was then transferred to a boiling tube (40 ml) and blown to dryness under a stream of nitrogen. The residue was taken up in methanol: water::70:30 (10 ml) and to this was added hexane (10 ml). After mixing, separation of the phases was allowed and the supernatant (hexane) layer (containing non steroidal material) was removed for disposal[125]. The aqueous methanol layer was dried under nitrogen in a water bath at 40°C and the residue transferred quantitatively to a small-test tube (12 ml) (conical glass stoppered) using benzene:chloroform::6:1 (3 x 10 ml) and again blown to dryness. The dried residue was placed in a vacuum desiccator for one hour and the residue then taken up in dichloromethane (2 ml). An aliquot (0.2 ml) was removed for radioactive counting which showed recovery rates uniformly between 86 and 89 per cent (average 87.5 per cent). To the remainder heptafluorobutyrate anhydride (100 µl) was added for esterification at room temperature for 30 minutes. This procedure favours esterification at hydroxyl groups and prevents enol formation.

Assessment of Esterification

The esters in solution were blown dry at room temperature under a stream of nitrogen and then dissolved in hexane (2 ml) partitioned against 70 per cent (v/v) methanol in water (2 ml) to remove unesterified steroids. A mean recovery of 92 per cent radioactivity from the hexane fraction at this stage when compared to the radioactivity in the stage before esterification indicated near total esterification. The results of the recovery are as follows:

Results of Recovery Experiments following Esterification:

Samples	dpm's added	dpm's before esterification	% of recovery	dpm's after esterification	% of * recovery
1	549450	478002	87.0	449320	94.0
2	549450	475270	86.5	424900	89.4
3	549450	472530	86.0	453625	96.0
4	549450	485715	88.4	449285	92.5
5	549450	486260	88.5	437632	90.0
6	549450	475825	86.6	425860	89.5
7	549450	489012	89.0	440115	90.0
8	549450	480770	87.5	464410	96.6
9	549450	483518	88.0	435170	90.0
10	549450	486260	88.5	447365	92.0
11	549450	474720	86.4	443370	93.4
12	549450	475275	86.5	449140	94.5
13	549450	478020	87.0	439782	92.0
14	549450	480220	87.4	456215	95.0
15	549450	474170	86.3	422021	89.0
16	549450	486800	88.6	457592	94.0
17	549450	488000	88.7	436265	89.4
18	549450	483518	88.0	435170	90.0
19	549450	472530	86.0	419135	88.7
20	549450	489010	89.0	459640	94.0
Mean	549450	480771	87.5	442301	92.0

*(% esterified).

In the urinary androgen quantitation, 87.5 per cent was used as a recovery rate of urinary androgen extraction for the

calculations. The loss during esterification steps was similar in both standard steroid and urinary samples preparation (see below).

Preparation of Standard Androgen Heptafluorobutyrates

A standard solution containing 1 mg of each of the following steroids 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 5α-androstane-3α-ol,17-one, 5β-androstane-3α-ol,17-one, 5α-androstane-3β-ol,17-one, 5α-androstane-17β-ol,3-one, Δ 5-androstene-3β,17β-diol and Δ5-androstene-3β-ol,17-one was prepared in dichloromethane (2 ml). Radioactively labelled (9,11-^{3}H)androsterone (SA. 40 Ci per mM) 0.25 ml (68680 dpm's) was added to the prepared mixture. The steroids in this solution were esterified with heptafluorobutyrate anhydride (100 ul) at room temperature for 30 minutes. Excess reagent was removed by evaporation to dryness at room temperature under a stream of nitrogen. The dried esterified steroids were dissolved in hexane (2 ml) and partitioned against methanol:water::70:30 (2 ml) to remove unesterified androsterone. The hexane layer containing the esterified steroids was blown to dryness under a stream of nitorgen at room temperature and the residue taken up in hexane (1.0 ml). Of this an aliquot (0.1 ml) was taken into a counting vial, blown to dryness, scintillation fluid (10 ml) added and the contained radioactivity estimated in the counter. The mean percentage esterification was 92.0 per cent. The lower methanol:water layer was similarly blown to dryness in a counting vial to which was then added scintillation fluid (10 ml) and the contained radioactivity counted. Over the number of

esterification assays performed (20) the esterified fraction was consistently greater than 90.0 per cent of the total dpm's subjected to esterification. By back calculating, at no time was that figure greater than 100.0 per cent as shown below:

Samples	dpm's added	dpm's in esterified part	dpm's in unesterified part	% esterification
1	68680	61812	6866	90.0
2	68680	62155	6523	90.5
3	68680	62842	5835	91.5
4	68680	62499	6180	91.0
5	68680	62845	5834	91.5
6	68680	62705	5970	91.3
7	68680	63186	5495	92.0
8	68680	63184	5450	92.0
9	68680	63598	5075	92.6
10	68680	63665	5015	92.7
11	68680	63460	5221	92.4
12	68680	65933	2747	96.0
13	68680	63185	5496	92.0
14	68680	64560	4118	94.0
15	68680	63871	4810	93.0
16	68680	62706	5974	91.3
17	68680	63190	5490	92.0
18	68680	62900	5780	91.6
19	68680	62230	6448	90.6
20	68680	63190	5494	92.0
Mean	68680	63186	5491	92.0

Gas Liquid Chromatography

Liquid phase and operating conditions for the g.l.c. method were required by which separation of the steroid esters present in the samples (standard and urine extracts) could be effected. After several liquid phases had been tested, the highly polar OV-225 was chosen as this gave good base-line separation of the steroid heptafluorobutyrates studied.

An already available five-foot column (ID. 4 mm) was prepared with 3 per cent coating of temperature stabilized OV-225 on Gas Chrom-Q, (mesh 100 to 120).

Silanization: The empty glass column was rinsed separately with three times the column volume of methanol, acetone and chloroform and washed with deionized water. The column was then dried in an oven at 100°C. Silanization of the dry column was effected using dimethyl dichlorosilane (5 per cent) in toluene for 15 minutes to deactivate the glass column (Horning et al.,1968)[126]. The solution was removed by suction and the column washed with three column volumes of methanol. The column was then thoroughly dried at 100°C in the oven for one hour. The two ends were then closed until required for packing using rubber plugs.

Preparation of packing material: Gas Chrom-Q (100-120 mesh) was used as a support without further purification. A solution of OV-225 (3 per cent), (w/v) was prepared by dissolving OV-225 (3 g) in chloroform (100 ml). Gas Chrom-Q (100 g) was then added slowly to the chloroform solution, to give a 3 per cent coating of Gas Chrom-Q. The chloroform was then slowly evaporated and swirled in a rotatory evaporator under reduced pressure to dislodge the trapped air and to facilitate coating of the support.

The vacuum was then released and the remaining chloroform evaporated to dryness with air at 37°C. The flask containing the coated Gas Chrom-Q was left in the oven at 100°C overnight to permit complete drying. In the morning the coated support was transferred to a watch glass (dia. 20 cm) ready for column packing.

Packing and conditioning of the column: The coated support was added to the column through a small glass filter funnel attached to the injection end of the column by a small rubber tube. The filling was facilitated by suction from a water pump applied to the detector end of the column and by gentle vibrations applied from a hand vibrator to the wall of the column. The injection end of the column was not completely filled so as to accommodate the flash heater. A small wedge of silanized glass wool was trapped into position at the injection end of the column and a smaller one was trapped at the detector end from inside before filling. The packed column was placed in the oven of the g.l.c. system, by connecting its injection end to the injection port of the instrument, leaving the detector end free so as to avoid contamination of the detector during the conditioning procedure.

The column was then conditioned using a column temperature of 230°C and a carrier gas flow rate (oxygen free nitrogen) of 40 ml per minute, for 24 hours to "bleed-off" the excess liquid phase. The column was allowed to cool slowly, and when the column temperature dropped to 40°C, the oven door was opened and the detector end of the column connected to the detector.

The optimal conditions for the g.l.c. isothermal temperature separation of the steroids from the urine samples were determined.

The column temperature was 195°C, the detector temperature was 250°C and the injector temperature was 230°C. The flow rates of the carrier gas and the hydrogen were both set at 30 ml per minute. The air flow rate was set at 300 ml per minute. The g.l.c. with the newly packed and conditioned column was allowed to stabilize for 30 minutes before becoming operational.

Linearity of the detector: The detector for this work was flame ionization (FID). The linearity of the detector was determined using peak areas, calculated from the product of the height of each peak to the outside of the pen line and the width to the inside of the pen line at half the peak height[127]. Various concentrations of 5α-androstane-3α,17β -diol-HFB, 5β-androstane-3α,-17β-diol-HFB, androsterone-HFB and aetiocholanolone-HFB were studied and peak areas for each calculated. The linearity of the detector is shown in Figure 4. See Figure 5 for flow diagram of these procedures.

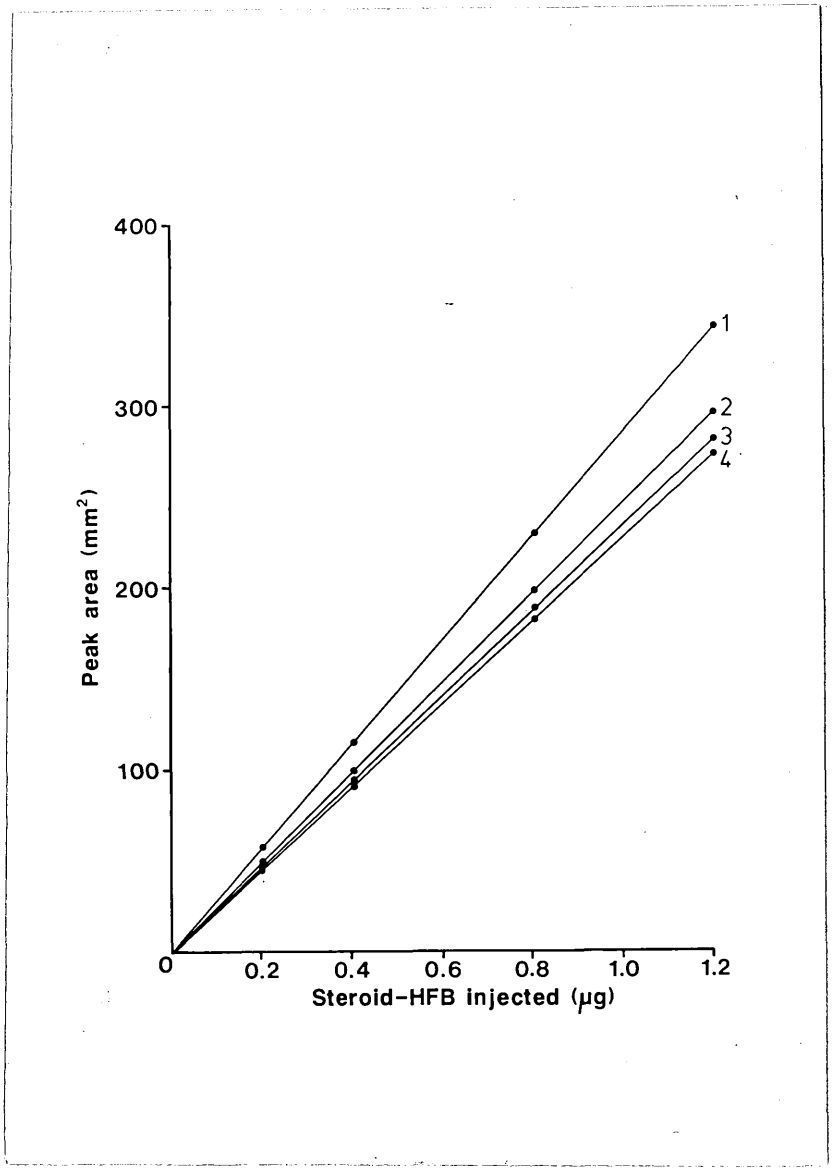


Figure 4: Linearity of the flame ionization detector determined from peak area measurements using varying concentrations of aetiocholanolone-HFB (1), 5β-androstane-3α,17β-diol-HFB (2), 5α-androstane-3α,17β-diol-HFB (3) and androsterone-HFB (4).

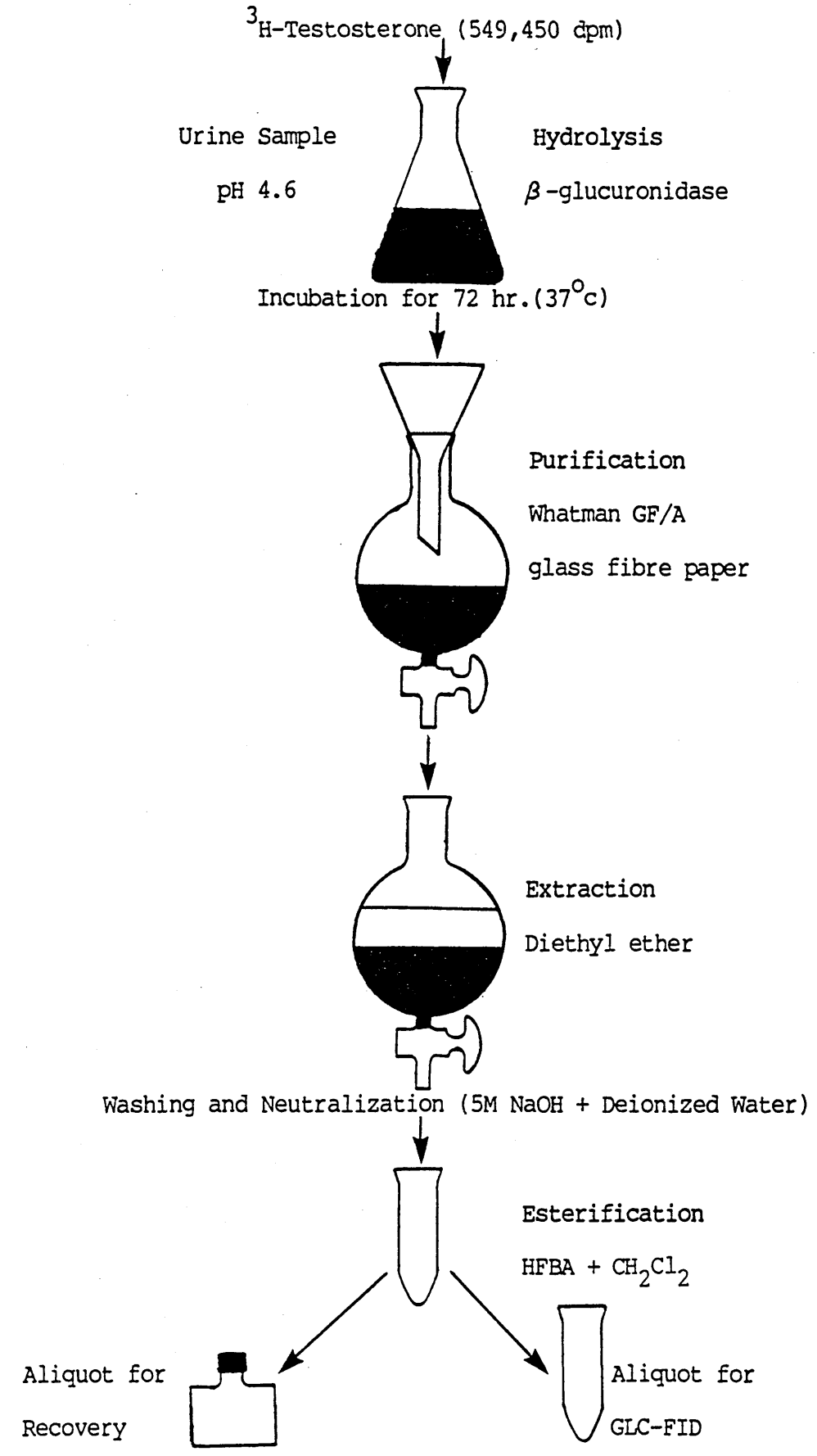


Figure 5: Flow diagram of the steps in the hydrolysis, extraction and quantitation of urinary steroid metabolites.

Quantitation of Urinary Androgen Metabolites

A standard steroid mixure was injected into the g.l.c. prior to the injection of each batch of samples (Figure 6). The identification of the peaks of the steroids in the urine samples, (Figure 7) was made by comparison of the retention times of the peaks with those of the standard steroids (Figure 8). Quantitation was made by comparison of the peak areas of each sample with the peak area of the respective steroid standards-HFB. Correction was made for the mass of individual steroids injected and the percentage recovery of ^{3}H-testosterone added initially to each urine sample. The results were back calculated to the total urine volume in each sample. For Groups 2 and 3 patients the results then were calculated to 100 mg excretion of urinary creatinine. The principle of the calculations was as follows:

If ;

Peak area of the individual urinary steroid injected = A.S

Peak area of the individual standard steroid injected = A.STD

The mass of standard steroid = M.STD

Volume of sample injected = V.I

Volume of urine used in extraction = V.E

The total volume of urine sample = V.T

Then ;

$$\frac{A.S \times M.STD}{A.STD} = \text{ng of the steroid-HFB in the sample injected.}$$

And ;

$$\frac{A.S \times M.STD \times 100}{A.STD \times V.I} = \text{ng of the steroid-HFB recovered in the extracted volume of the urine sample.}$$

But 1/10 of the residue was taken to calculate the pre-esterification recovery rate. The recovery up to pre-esterification was 87.5 per cent. Therefore if the recovery of the native androgen metabolites is equivalent to the recovery of ^{3}H-testosterone added intially to the urine, then there were;

$$\frac{A.S \times M.STD \times 100 \times 10 \times 100}{A.STD \times V.I \times 9 \times 87.5} = \text{ng of steroid in the urine extracted}$$

Then the mass of the urinary steroids was calculated to the total volume of the urine sample;

$$\frac{A.S \times M.STD \times V.T \times 100 \times 10 \times 100}{A.STD \times V.I \times V.E \times 9 \times 87.5} = \text{ng}$$

For Group 1 patients this mass represent the amount of urinary androgen metabolites excreted hourly. For Group 2 and 3 patients the results were further calculated to a constant excretion of urinary creatinine (100 mg). If the creatinine excreted in the urine sample is CR, then the mass of steroids in urine for a 100 mg excreted urinary creatinine was;

$$\frac{A.S \times M.STD \times V.T \times 100 \times 10 \times 100 \times 100}{A.STD \times V.I \times V.E \times CR \times 9 \times 87.5} = \text{ng}$$

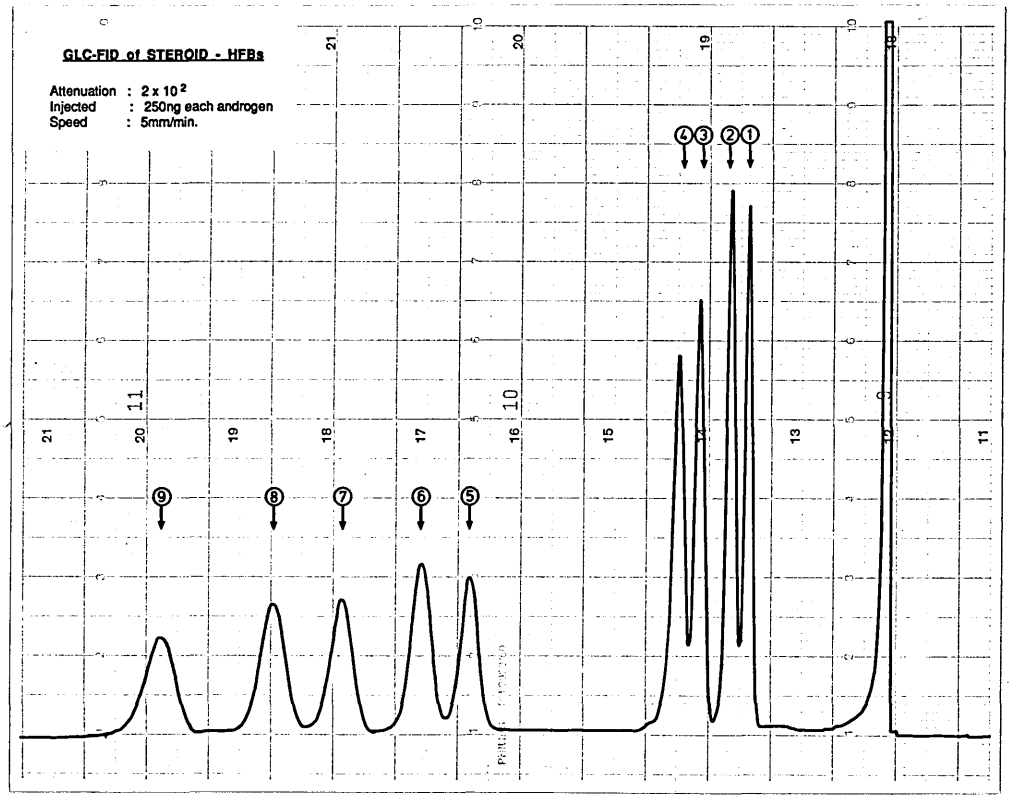


Figure 6: Gas liquid chromatographic analysis with flame ionization detection of a standard steroid-heptafluorobutyrate mixture.

1. 5α-androstane-3α,17β-diol-HFB (5α-androstanediol-HFB).
2. 5β-androstane-3α,17β-diol-HFB (5β-androstanediol-HFB).
3. Δ5-androstene-3β,17β-diol-HFB (Δ5-androstenediol-HFB).
4. 5α-androstane-3β,17β-diol-HFB (5α-3β,17β-androstanediol-HFB).
5. 5α-androstane-3α-ol-17-one-HFB (androsterone-HFB).
6. 5β-androstane-3α-ol-17-one-HFB (aetiocholanolone-HFB).
7. Δ5-androstene-3β-ol-17-one-HFB (dehydroepiandrosterone-HFB).
8. 5α-androstane-3β-ol-17-one-HFB (epiandrosterone-HFB).
9. 5α-androstane-17β-ol-3-one-HFB (5α-dihydrotestosterone-HFB).

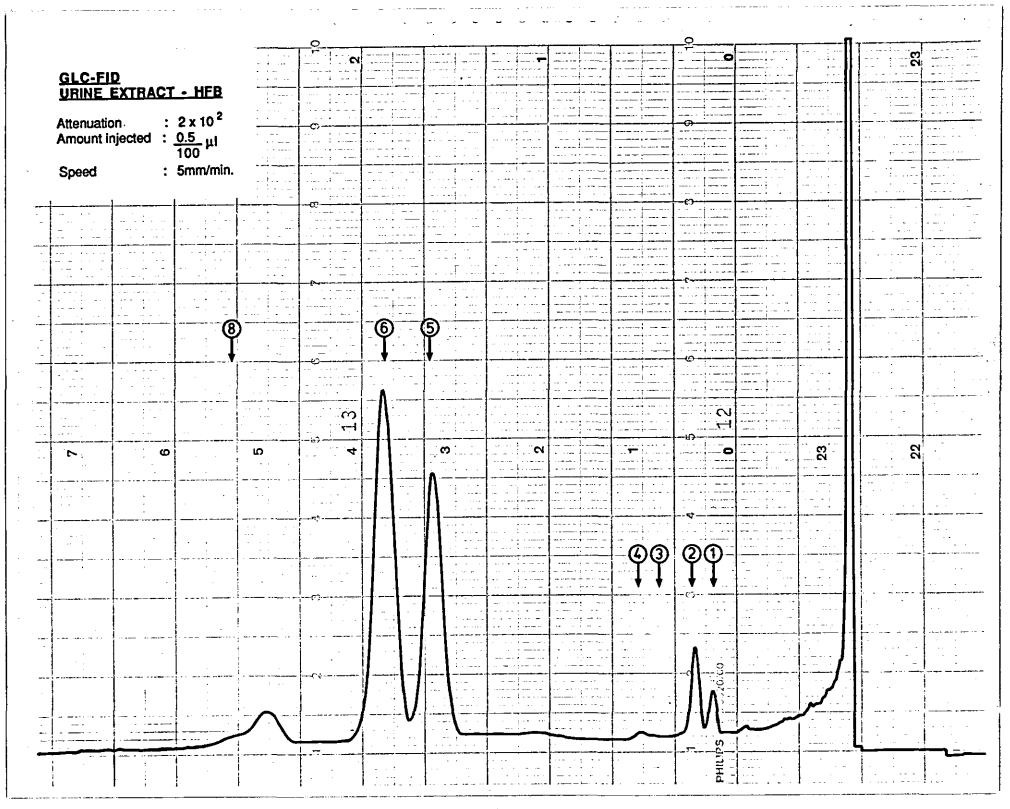


Figure 7: Gas liquid chromatographic analysis with flame ionization detection of an esterified urinary extract. The key to peaks is as in Figure 6. For the peak which is seen before peak 8 see Figure (8).

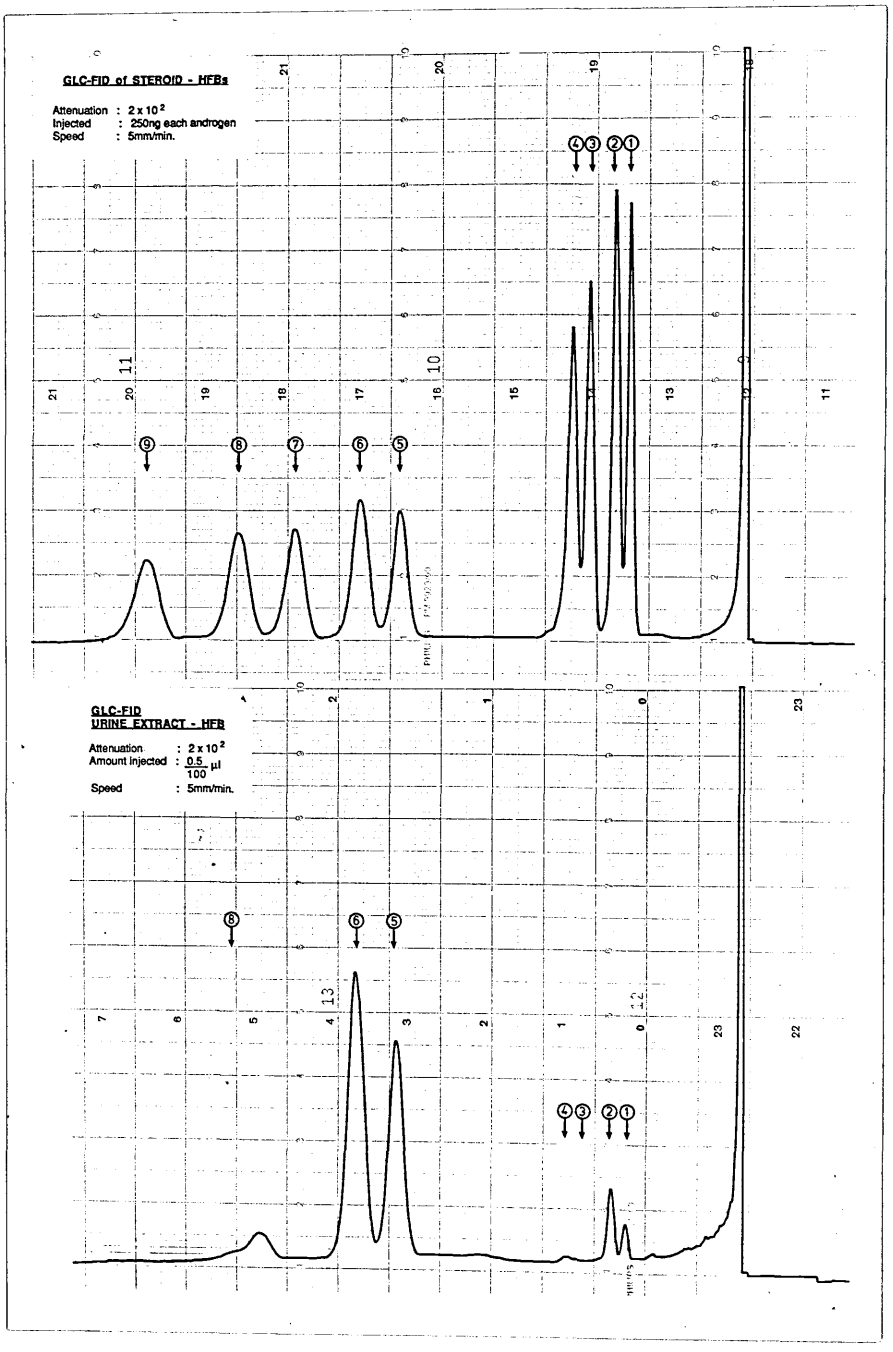


Figure 8: Gas liquid chromatographic analysis with flame ionization detection of a standard androgen-HFB's mixture (above) and urinary androgen metabolite-HFB's (below). The key to peaks is as in Figure 6. The peak immediately before peak 8 but not identical to peak 7 is non steroidal contamination by dioctyl phthalate as had been identified by Mass Spectrophotometer-GLC System. This will be discussed in detail in Chapter 4.

Practical Procedure for Determination of Urinary Androgens

1. Urine (a selected vol.) was adjusted to pH 4.6.
2. The following were added:
   a. β-glucuronidase (750 FU per ml).
   b. ^{3}H-testosterone (approx. 550,000 dpm).
   c. 2M acetate buffer (20 ml).
3. The contents of the flask were mixed thoroughly and the flask placed in an incubator at 37^{o}C for 72 h.
4. The contents of the flask were quantitatively transferred to a separating funnel (500 ml) by filtration using Whatman GF/A glass fibre paper and the urine extracted with diethyl ether.
5. The total volume of diethyl ether was washed with sodium hydroxide (5M, 10 ml) and then with deionized water (20ml) until the washings were neutral to full range Whatman test paper.
6. The diethyl ether was transferred quantitatively to a conical flask and blown to a small volume at room temperature using air.
7. This small volume was finally quantitatively transferred to a suitable sized boiling tube and blown to dryness using air at room temperature.
8. Methanol:water::70:30 (10 ml) and hexane (10 ml) were added to the boiling tube; stoppered (glass) and the contents thoroughly mixed. After settling, the hexane top layer was discarded and the methanol/water layer containing the steroids, transferred quantitatively to a conical test tube (12 ml) using benzene:chloroform::6:1 (3 x 10 ml) and the

solvent blown to dryness. The tube was then placed in a vacuum desiccator for 1h.

9. To the dried residue dichloromethane (2 ml) was added and the contents thoroughly mixed. An aliquot (0.2 ml) was taken to assess recovery up to this stage.
10. Heptafluorobutyric anhydride (100 µl) was added to the steroids in dichloromethane and esterification allowed to take place over 30 minutes at room temperature.
11. The esters were blown to dryness at room temperature under nitrogen and hexane (2 ml) and Methanol/water (70 per cent, v/v) (2 ml) were then added to remove unesterified steroids which were in the methanol/water phase.
12. The lower methanol/water layer was removed by pipette and discarded.
13. The hexane layer was blown to dryness and the residue taken up in hexane (100 µl) for both esterification assessment (10 µl) and g.l.c. quantitation (0.5 - 1.0 µl).

Determination of Urinary Creatinine

A modified Folin (1905)[128] adaptation of the colour reaction of creatinine with alkaline sodium picrate first described by Jaffe (1886)[129] was used for the determination of creatinine in the urine samples. Several of the modifications suggested by Bonsnes and Taussky (1945)[130] were applied in the method.

After measuring the total urine volume, duplicate aliquots (0.5 - 2.0 ml) containing 0.7 to 1.2 mg creatinine were pipetted into volumetric flasks (100 ml). A reagent blank containing

deionized water (1 ml) and a standard containing creatinine stock standard (creatinine 1 g/L in 0.1 N HCl) (1 ml = 1 mg) were prepared in similar flasks. Saturated picric acid (15 g picric acid per litre deionized water) (2 ml) was added to each flask followed by the addition at timed intervals, of 1M sodium hydroxide (1 ml). The flask contents were thoroughly mixed and allowed to stand exactly 10 minutes at room temperature before dilution to volume with deionized water. After dilution the contents were thoroughly mixed again and an aliquot transferred by Pasteur pipette to a cuvette of 10 mm light path. Optical densities were read at 490 nm on a Unicam SP 600 Spectrophotometer with zero set against the reagent blank.

The milligram equivalents of the test samples relative to the standard creatinine were calculated, and after adjustment for the aliquot of urine taken, and for the total urine volume, the final results were expressed as mg creatinine per total urine volume.

Standard creatinine was found to obey the Beer-Lambert Law (Figure 9) at the concentrations used. This permitted direct quantitation of creatinine in test samples. The recovery of creatinine added to urine was consistently greater than 95 per cent. Results of typical recovery experiments are shown in Table VIII. Duplicates were consistently within 3 per cent and inter batch differences were less than 5 per cent.

As long ago as 1905 Folin[131] observed that although considerable individual variation was noticed, the daily quantity of urinary creatinine was remarkably constant for the individual and that this constancy of creatinine excretion could be used as a gauge of the completeness of a 24-hour urine specimen. Smith 1942[132] found the creatinine test of great use in assessing the

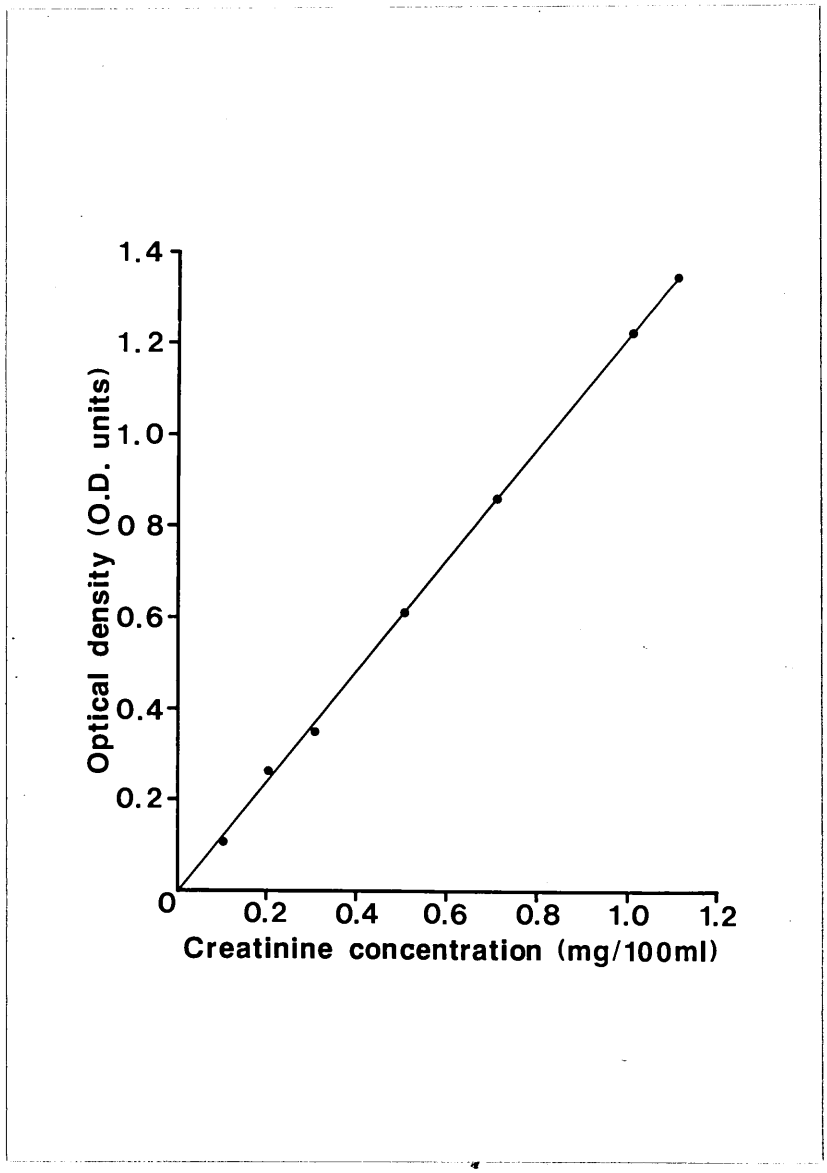


Figure 9 : Optical density of creatinine as assayed by the Jaffe colour reaction. The results were corrected for reagent blank. Note the obedience of the results to the Beer-Lambert Law at creatinine concentrations between 0.5 and 1.1 mg/100 ml.

Table VIII : Recovery rates of creatinine added to urine.

Test	O.D.* 490 nm	% Recovery of Creatinine **
Standard creatinine (1 mg)	1.20	-
Urine A	0.25	-
Urine A + creatinine (1 mg)	1.40	95.8
Urine A + creatinine (0.75 mg)	1.13	97.8
Urine A + creatinine (0.5 mg)	0.865	102.5
Urine A + creatinine (0.2 mg)	0.47	91.7
Urine A + creatinine (0.1 mg)	0.365	95.8
Urine A + creatinine (0.05 mg)	0.30	83.3
Urine A + creatinine (0.02 mg)	0.268	75.0

* O.D. = Optical Density.

** Calculated on mg recovered creatinine.

Recovery is acceptable (98.7 per cent) if the aliquot analysed contains between 0.7 and 1.2 mg creatinine.

accuracy of collection of 24-hour specimens. Wary and Scott Russell (1960)[133] and Paterson (1967)[134] confirmed that the 24-hour excretion of creatinine was significantly more constant than the 24-hour urine volume.

In this work the urinary excretion of the steroid metabolites were related to 100 mg of urinary creatinine in Groups 2 and 3 patients.

Practical Procedure

1. Urine (1 ml) was pipetted into a volumetric flask (100 ml).
2. Stock standard creatinine (1 g/L) (1 ml) was also pipetted into a volumetric flask as above.
3. To each flask was added sodium hydroxide (1M; 1 ml), and saturated picric acid (2 ml) and the flask allowed to stand for 10 minutes.
4. A blank flask containing the reagents only was prepared.
5. Each flask was then filled to volume with deionized water and the contents thoroughly mixed.
6. The SP 600 spectrophotometer was "zeroed" against the blank (1 cm light path cuvette) and the standard and test urines read at 490 nm.
7. The creatinine (mg) per total volume of urine was calculated thus:

$$\frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \text{total volume of urine (ml)}$$

(O.D. = Optical Density)

Plasma Androstenedione Assay

Androstenedione is a compound which is not frequently measured in the blood. However before the development of the radioimmunoassay methods, procedures used to estimate the plasma concentration utilized chromatography or colorimetry. These methods were unreliable for analytical work because of their very low recovery rates. Most of these methods required large amounts of plasma, and other procedures required the conversion of AD to testosterone before derivatization. Analytical inaccuracies arose because of the interference of the endogenous plasma testosterone. The very low recovery rate and the existence of the Δ4-3-oxo group which may have resulted in two isomeric derivatives rendered the g.l.c. system undesirable for AD estimation.

One of the important factors which contributed to the rapid advancement in the understanding of the endocrine diseases was the development of a precise, sensitive and accurate estimation technique. This involved radioimmunoassay. More recently the development of direct methods for determinating plasma steroids such as AD and testosterone have contributed further to this advancement. These new methods utilizing unextracted plasma or serum, have abolished difficulties associated with extraction, recovery rates, time involved in the assay and other factors contributing to imprecision.

In this work a direct RIA technique for estimating plasma AD was used. The tubes, antibodies and ^{125}I-androstenedione were purchased as a kit from Immunodiagnostics Ltd., Usworth Hall, Washington, Tyne and Wear, NE37 3HS. The principles of the test are a radioimmunoassay wherein there is a competition between

radioactive and non-radioactive antigens for a fixed number of antibody binding sites. The amount of ^{125}I-androstenedione bound to the antibody is inversely proportional to the concentration of AD present in the plasma. The separation of free from bound antigen on the wall of the tube is achieved by decanting or aspirating the free antigen from the tube.

Reagent and Test tubes Used:

Each kit consist of:

1. Androstenedione standards: Five vials containing separately, 0.1; 0.3; 1.0; 3.0 and 10.0 ng per ml in 0.5 ml of AD standard and one vial of 1.0 ml (0.0 ng per ml) AD standard in human serum (androstenedione free) containing 0.1 per cent sodium azide as a preservative were set up. These standards in solution can be stored at 2 to 8^{o}C for up to three weeks, and if a longer period is necessary stored at -15^{o}C or lower. In this work the assay was completed within 24 to 48 hours from the time of kit arrival.

2. ^{125}I-Androstenedione: The vial (50 ml) contained ^{125}I-AD (< 5 uCi) in a protein based buffer containing themerosal (0.05 per cent w/v) as a preservative. The vial was stored at 2-8^{o}C till used.

3. Anti-Androstenedione Ig G-Coated Tubes: These were polystyrene tubes (100) each coated internally with rabbit anti-androstenedione immunoglobulin. These were stored at 2-8^{o}C until used.

4. Control Serum: These were two vials each containing 0.5 ml, one low (0.66 or 0.79 ng per ml) and one high (4.57 or 5.919 ng per ml) concentrations of AD in human serum with sodium

azide (0.1 per cent w/v) as a preservative. These also were stored at 2-8°C until used.

All the above reagents and the plasma samples were equilibrated at room temperature and individually mixed thoroughly by gentle inversion before use.

Plasma Samples Used

Blood (5 ml) was taken from a suitable antecubital vein and placed in a lithium-heparin-containing tube. The plasma was separated by centrifugation and transferred to a plain tube immediately. It is appreciated that the concentration of plasma AD is slightly lower than that of serum AD concentration, but the blood taken was being used for other than the AD assay hence the use of plasma . Only unhaemolysed plasma samples were used. Likewise lipidaemic samples were discarded (i.e. those taken immediately after a meal).

The Assay Procedure

The method of assay was as follows:

1. All reagents and samples used were allowed to reach room temperature. The liquid reagents were mixed thoroughly but gently by inversion. The standards, controls and plasma samples were all assayed in duplicate.

2. The Ig G coated tubes (100) were set out in duplicate according to the protocol shown in Figure 10.

3. Standards, controls and unknown plasma samples (all 50 µl) were placed at the bottom of the individual tubes.

4. ^{125}I-AD (500 ml) was added immediately to each tube including two tubes for total counts. These latter were

Tube	Sample ——— Amount (µl)		^{125}I-AD(µl)
1,2	Total count	-	500*
	Standard (ng/ml)		
3,4	0.0	50	500
5,6	0.1	50	500
7,8	0.3	50	500
9,10	1.0	50	500
11,12	3.0	50	500
13,14	10.0	50	500
	Controls		
15,16	I	50	500
17,18	II	50	500
	Unknowns		
19,20		50	500

* leave aside until counting.

↓

Mixed by shaking the rack

↓

Incubation at 37°C (40 minutes)

↓

Decanting

↓

Radioactivity counting in Gamma Counter

Figure 10 : Summary of the RIA technique for plasma AD assay.

stoppered and set aside until the final counting.

5. The contents of the tubes were mixed thoroughly by agitating the tube rack vigorously by hand.

6. The tubes were then incubated in a water bath at 37°C for 40 minutes.

7. All tubes except those for total count, were transferred to a sponge rack and the liquid decanted by inversion of the rack. The radioactive waste was placed in the waste receptacle. The inverted tubes while in the rack were pressed sharply onto an absorbent pad for three minutes to insure that all the liquid phase was removed.

8. The radioactivity in each tube was then assayed in a Gamma Counter over a period of one minute.

Calculation of the Results

The mean counts per minute (cpm) for each pair of tubes, for the standards, controls and samples were determined. These means (cpm) were expressed as a percentage of the mean for the zero standard (i.e. B/B0).

$$B/B0 = \frac{\text{Mean(cpm) of each Standard, Control or Sample}}{\text{Mean (cpm) of zero Standard}} \times 100$$

A standard curve was prepared on semilogarithmic paper by plotting (B/B0) against the concentration of AD on the abscissa, Figure 11. The concentration of plasma AD in each unknown sample was determined from the standard curve (ng per ml). The results were then converted to (nmol per litre) as follows:

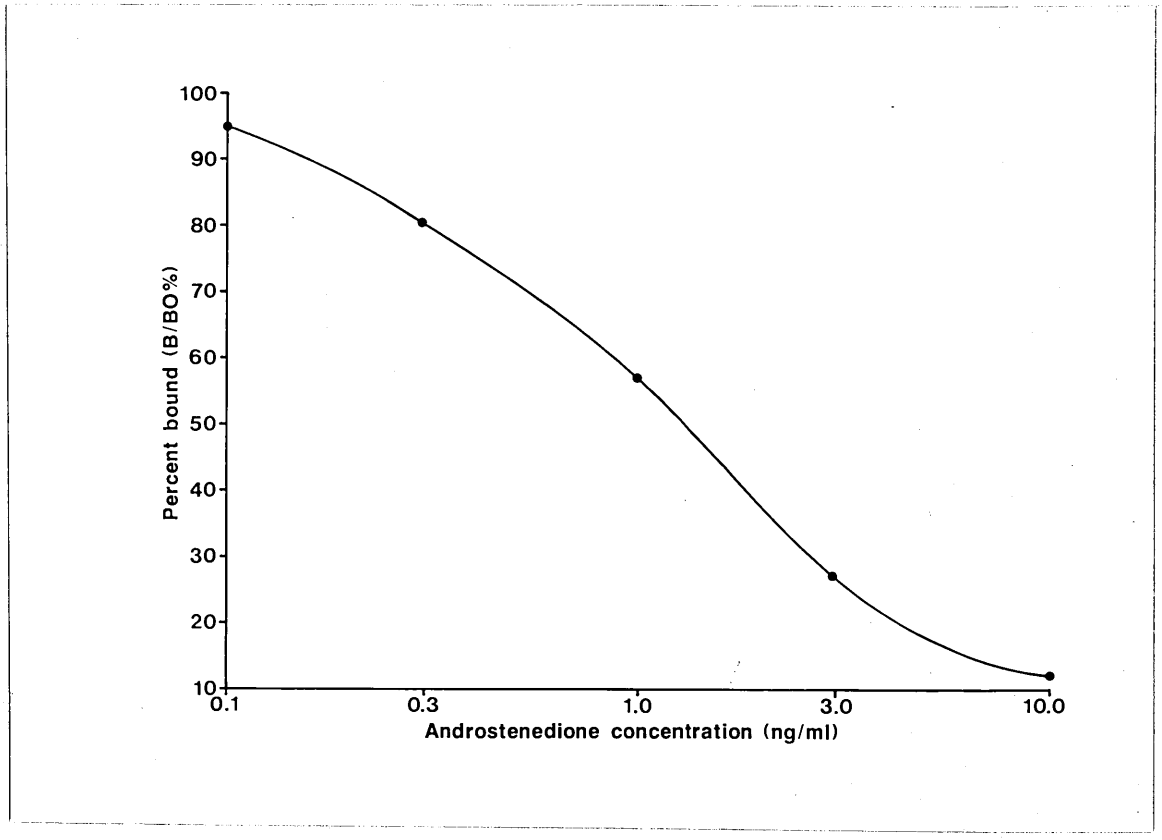


Figure 11 : Plasma androstenedione RIA standard curve.

$$\frac{\text{Results (ng/ml) x 1000}}{\text{286.42 (Mol. Wt. AD)}} = \text{nmol/L}$$

In this assay system the lower limits of detection were 0.035 ng per ml (0.12 nmol/L) and 0.042 ng per ml (0.15 nmol/L) and the upper limit was 10.0 ng per ml (34.9 nmol/L). (Three kits used).

Performance of the assay procedure

Two quality control serum samples were assayed with each kit. The low level quality control serum samples were 0.66 and 0.796 ng per ml and the high level quality control serum samples were 4.57 and 5.919 ng per ml. The confidence limit of the assay for the low level control serum samples was 98.3 percent and for the high level control serum samples was 99.68 per cent. This indicates a high confidence limit of the assay procedure.

Non-specific binding (NSB) was determined by adding 50 ul of the 0.0 ng/ml AD standard to an uncoated polystyrene tube (duplicates). NSB was within the range 4-6 per cent indicating active agents and precision of the technique. The maximum binding i.e the percentage bound in the absence of unlabelled antigen was near 40 - 50 per cent in all kits used.

The precision of the work was determined by assaying three samples, four times each. The coefficients of variation shown below were highly satisfactory.

Sample	Mean level (ng/ml)± SD	% Coeff. of variation
1	7.57 ± 0.31	4.10
2	3.25 ± 0.20	6.15
3	0.57 ± 0.03	5.26

SD = Standard Deviation

Recovery Experiments

Two samples of plasma containing known concentrations of AD were "spiked" with AD of known concentration and the tube contents assayed for AD content. The recovery rate was calculated from the percentage of the observed concentration divided by the expected concentration and this was as follows:

Endogenous AD(ng/ml)	Added AD(ng/ml)	Expected AD(ng/ml)	Measured AD(ng/ml)	Percentage Recovery
3.41	3.0	6.41	6.29	98.13
3.41	0.3	3.71	3.77	101.62
0.43	3.0	3.43	3.45	100.58
0.43	0.3	0.73	0.69	94.50
Average recovery				98.71

In this work cross reactivity with other constituents (steroidal) of the plasma was not assayed. The cross reactivities with various steroids published by the manufacturer are shown overleaf.

Specificity

The cross reactivities for AD-RIA according to the manufacturer of the kits were as follows:

Steroid	% cross reactivity
Androstenedione	100.00
Androsterone	0.03
Cholesterol	0.002
Corticosterone	0.013
Cortisol	0.03
Cortisone	0.05
Cortexalone	0.06
Dehydroepiandrosterone	0.044
Dehydroepiandrosterone Sulfate	0.003
5α-Dihydrotestosterone	0.05
Deoxycorticosterone	0.03
11-Deoxycortisol	1.19
Oestradiol	0.009
Oestriol	0.03
Oestrone	0.08
Aetiocholanolone	0.014
17α-Hydroxypregnenolone	0.042
17β-Hydroxyprogesterone	0.002
Isoandrosterone	0.26
Pregnenolone	0.03
Progesterone	0.07

Plasma Testosterone Assay

Testosterone is the principal androgenic hormone synthesized by the testes. It is also produced by the ovary, from peripheral conversion of AD [135] and to a lesser extent from the liver[136]. The plasma concentration of testosterone is variable during the day with a nadir at 02.00h and a peak at 20.00 to 22.00h. Although some investigators have considered this variation as a nyctohemeral rhythm and reported the existence of an annualcadian rhythm[137], others have refuted its occurrence[138]. Other authors have attributed the penile erection and the erotic dreams during the rapid eye movement sleep (REM) to a high peak of plasma testosterone[139].

The normal plasma testosterone concentration in the adult male is 15.7 to 20.1 nmol per litre[140] and in the adult female 4.16 to 5.89 nmol per litre[141]. In pre-pubertal boys the plasma concentration of testosterone is very low. It is 0.28 to 1.18 nmol per litre up to the age of 10 years and 2.6 to 8.42 nmol per litre at the age between 10-14 years. From the age of 14-15 years the plasma concentration reaches that of the adult[142].

The catabolism of testosterone occurs mainly in the liver, primarily by a reduction process of the double bond at C4-5 then at the 3-oxo-group. Androsterone and aetiocholanolone are known to be the main metabolites. The 5α- and 5β-androstanediols are other end products of testosterone metabolism[143]. Interestingly the urinary excretion of 5α-androstanediol is considered to be an important index of testosterone activity in the target tissues. Its urinary excretion rate has been considered to represent the activity of testosterone on the target tissues via conversion to

dihydrotestosterone when the latter is below the limit of detection by the GLC-MS system[144]. In the androgen-dependent tissues the catabolism of testosterone occurs via the more active androgenic hormone dihydrotestosterone[145].

Since the concentration of systemic plasma testosterone is very low in young children and during the pre-pubertal period detection required very sensitive methods. Many methods have been described to fulfil this purpose. In 1961 Finklestein et al.,[146] described a method involving conversion of testosterone to 17β-oestradiol, the latter being measured by fluorimetry. This method required a large amount of blood. Direct methods were sought such as the double isotope derivitive method, fluorimetery and gas phase chromatography. With these methods many problems were encountered, the most important of which were problems of separation of testosterone from dihydrotestosterone and the requirement of a large quantity of blood (50 ml for some of these methods).

Recently success has been achieved in the detection of plasma testosterone in small volume (serum or plasma) by using the radioimmunoassay techniques and more recently the use of double antibody RIA technique to achieve a relatively good separation of antibody-bound from free testosterone. In this study the double antibody RIA was used.

Reagents and test tubes used in the assay

In this procedure the following reagents were obtained from Immunodiagnostic Ltd., Usworth Hall, Washington, Tyne and Wear, England.

1. Testosterone standards (A to H) (0.5 ml in each vial) which consisted of 0.0 ; 0.4; 1.0; 2.0; 4.0; 10.0; 20.0 and 40.0 nmol per litre.

2. Testosterone antiserum (52 ml) in one vial.

3. ^{125}I-testosterone (< 3.0 µCi) (52 ml vial).

4. Precipitating antibody (52 ml) in one vial.

5. Buffer (11 ml) in one vial.

These reagents were stored in -20°C and used within 48 h. of receipt of the kit.

Before the assay procedure commenced, all reagents and plasma samples were allowed to come to room temperature and each was gently mixed. Borosilicate glass tubes (I.D. 12 x 75 mm) obtained from Camlab Ltd., Nuffield Road, Cambridge, CB4 1TH were used.

The assay procedure

The assay procedure was performed as follows:

1. The test tubes were set up in duplicate as shown in Figure 12. Samples (each 50 µl) of unknowns, standards and controls were placed at the bottom of appropriate test-tubes.

2. To the tubes for non-specific binding, zero standard (50 µl) and buffer (500 µl) were added.

3. ^{125}I-testosterone (500 µl) was then added to all tubes including two for total counts which were set aside to be counted with the others after processing.

4. Testosterone antiserum (500 µl) was added to all tubes except the non-specific binding tubes.

5. The contents of all tubes were then gently mixed, foaming being prevented and thereafter all were incubated for 2 h at 37°C in a water bath.

Tube	Sample	Amount (µl)	^{125}-T. (µl)	T. antiserum (µl)
1,2	Total count	-	500*	-
	Standards (nmol/L)			
3,4	A 0.0	50	500	500
5,6	B 0.4	50	500	500
7,8	C 1.0	50	500	500
9,10	D 2.0	50	500	500
11,12	E 4.0	50	500	500
13,14	F 10.0	50	500	500
15,16	G 20.0	50	500	500
17,18	H 40.0	50	500	500
19,20	NSB	**	500	500
21,22	Controls & Unknowns	50	500	500

* Leave aside until final counting.

** Non Specific Binding (0.0 Std. 50 µl + Buffer 500 µl).

T = Testosterone.

Mix without foaming and incubate at 37°C for 2h in a water bath.

↓

Add 500 µl of second antibody to all tubes.

↓

Mix without foaming and incubate at room temperature for 15 minute.

↓

Centrifuge for 15 minute at 1000g in 5°C refrigerated centrifuge.

↓

Decant and Count in Gamma Counter for 1 minute.

Figure 12: Plasma testosterone-RIA technique (summary).

6. The second antibody (500 µl) was then added to all tubes and the contents gently mixed (again preventing foaming) by using the rotamixer for only a few seconds. The tubes were then incubated for 15 minutes at room temperature.

7. The tubes were thereafter centrifuged for 15 minutes at 1000 g in a refrigerated centrifuge (5°C). The supernatant fluid in each tube was removed by decanting and the tubes were drained onto a pad of absorbent tissue.

8. The radioactivity in each tube was then counted in a Gamma Counter for 1 minute.

Calculation of Results

1. B/B0 were calculated as follows:

$$\frac{\text{Counts for each standard - NSB counts}}{\text{Counts for zero standard - NSB counts}} \times 100$$

(NSB = Non Specific Binding Counts)

2. A standard curve was constructed (Figure 13) by plotting B/B0 against the concentration of the testosterone standards on three phase-semilogarithmic paper.

3. The B/B0 results of the test samples were then calculated as follows:

$$\frac{\text{Count for each sample - NSB counts}}{\text{Counts for zero standard - NSB counts}} \times 100$$

The B/B0 results were plotted against the standard curve to obtain the mass of testosterone (nmol/L) in each test sample. In this assay system for the kits used the lower limit of detection was 0.35 nmol/L and the upper limit of detection 34.7 nmol/L.

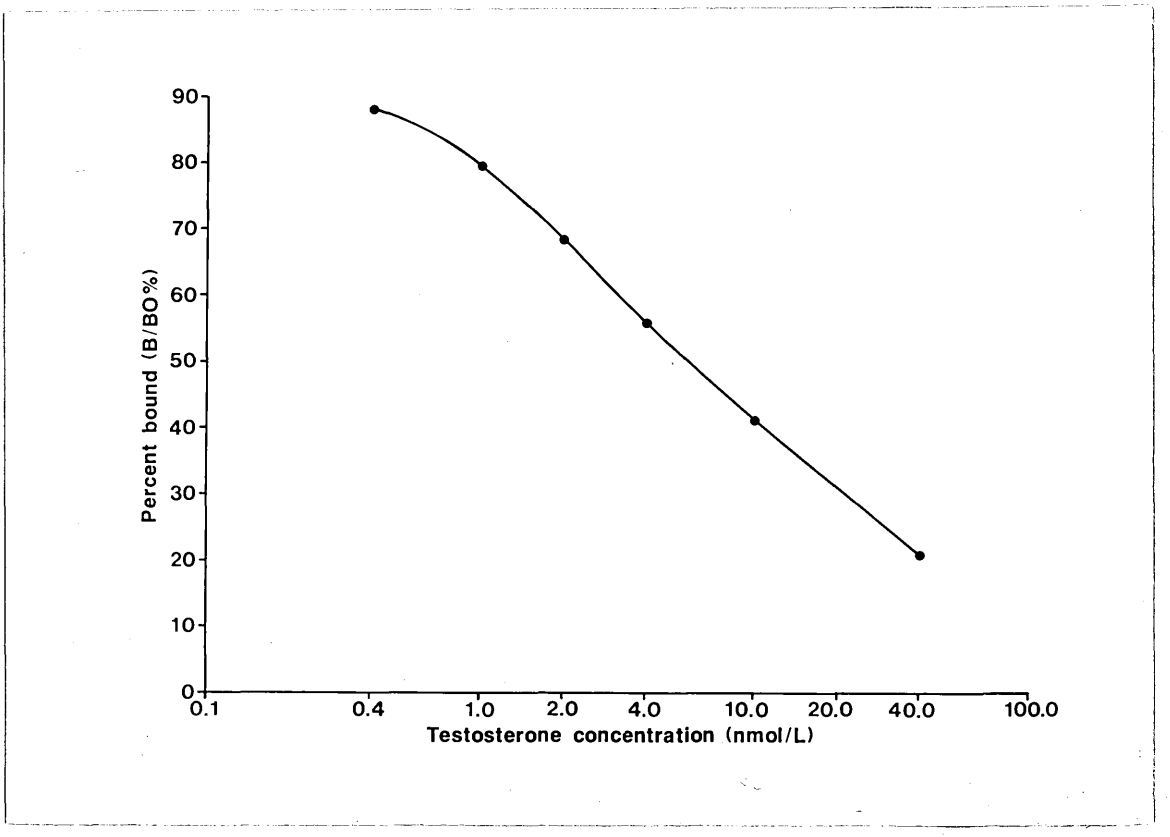


Figure 13 : Plasma testosterone RIA standard curve.

Performance of the assay procedure

Two quality control serum samples were assayed with each kit. The low level quality control serum sample was 1.6 nmol per litre and the high level quality control serum sample was 5.1 nmol per litre. The confidence limit of the assay for the low level quality control serum sample was 99.3 per cent and for the high level quality control serum sample was 99.7 per cent. This indicates high confidence limits for the assay procedure.

Non-specific binding (NSB) was within range 3.0-4.5 which indicated active reagents and precision of the technique. The maximum binding was within the range 60-80 per cent in all kits used (three kits).

Three samples were assayed four times each to determine the precision of the work. The data summarized below shows the results and the coefficients of variation, which are highly satisfactory.

Sample	Mean level (nmol/L) $\pm$ SD	% Coeff. of variation
1	3.40 $\pm$ 0.15	4.4
2	10.06 $\pm$ 0.133	1.32
3	0.57 $\pm$ 0.013	2.28

SD = Standard Deviation

The RIA technique for plasma testosterone assay was also tested for the accuracy of the dilution, which was required for some of the plasma samples from Group 2 patients. Only two-fold and three-fold dilutions were used. Two samples were used and diluted with 0.0 standard. The results indicated a highly satisfactory linearity and good recovery rates as shown below.

Sample	Dilution	Expected nmol/L	Measured nmol/L	Percentage recovery
1	-	-	4.90	-
	1:2	2.45	2.44	99.6
	1:3	1.63	1.59	97.5
2	-	-	20.50	-
	1:2	10.25	10.37	101.2
	1:3	6.83	6.59	96.5

Recovery Experiments

The recovery rate was assayed by adding a known concentration of testosterone to plasma samples previously assayed for testosterone (nmol/L) as follows:

Endogenous T. nmol/L	Added T. nmol/L	Expected nmol/L	Measured nmol/L	Percentage recovery
7.20	3.50	10.70	10.54	98.50
	8.70	15.90	15.70	98.74
0.73	3.50	4.23	4.03	95.27
	8.70	9.43	9.45	100.21
Average recovery				98.18

T = Testosterone

In this work the cross reactivities with other steroids of the plasma were not assayed. The cross reactivities with various steroids published by the manufacturer are shown overleaf.

The methods described in this Chapter have been used in the investigations of the children in Groups 1, 2 and 3 and the results obtained will be presented in the next Chapter.

Specificity

The cross reactivities for testosterone-RIA according to the manufacturer of the kits are as follows:

Steroid	% cross reactivity
Testosterone	100.00
5α-Dihydrotestosterone	3.40
11β-Hydroxytestosterone	<0.01
11-Oxotestosterone	2.00
11β-Hydroxyandrostenedione	<0.01
Androstenedione	0.50
5α-Androstanedione	<0.01
5β-Androstanedione	<0.01
5β-Dihydrotestosterone	0.63
Androsterone	<0.01
Epiandrosterone	0.20
5α-Androstane-3α,17β-diol	0.01
5α-Androstane-3β,17β-diol	2.20
Dehydroepiandrosterone	<0.01
6β-Hydroxytestosterone	0.95
Oestrone	<0.01
17β-Oestradiol	<0.01
Oestriol	<0.01
Progesterone	<0.01
Corticosterone	<0.01
Deoxycorticosterone	<0.01

CHAPTER THREE

RESULTS

In this chapter I will present the data derived from "the endocrine" analyses of plasma and urine from eleven boys who were given a bolus of androstenedione (100 mg) orally; from another eleven boys who received testosterone (100 mg once)intramuscularly (Sustanon: 20 mg propionate and 40 mg each of the phenyl-propionate and the isocaproate of testosterone) as a "priming" exercise preceding an insulin hypoglycaemia test and from five boys who received orally androstenedione (100 mg) thrice weekly on the same days as they received subcutaneous growth hormone (4 IU). In the latter, sampling was made basally and at three-monthly intervals over a one year period. Also will be presented the clinical effects of the combined treatment with androstenedione and growth hormone in these five boys with special reference to acceleration of growth velocity, 1 year post-treatment versus 1 year pre-treatment and then after one year of treatment with growth hormone alone. These latter five boys were matched with five other boys who had received only growth hormone therapy for their short stature (growth hormone deficiency) and were matched as far as possible in chronological age plus one or more of the other appropriate anthropometric parameters.

Analyses of Plasma Concentrations of AD and Testosterone.

Group 1 Patients Plasma concentrations of AD and testosterone.

In Table IX are shown the plasma concentrations of AD and testosterone basally and during the five consecutive hours following ingestion of AD (100 mg) by the eleven boys. The data

are set against chronological age, height age, bone age and standard deviation score (SDS)for height. Figure 14 presents the plasma concentrations over the 5-h. period in a histogram form.

The patients fall into two groups, namely those whose plasma androstenedione concentrations are greater than the plasma testosterone concentrations (Patients 1, 2 and 3) and those whose plasma testosterone concentrations exceed those of plasma androstenedione by 0.11 to 18.03 nmol/L (Patients 4, 5, 6, 7, 8, 10 and 11). This phenomenon is readily visualized in Figure 14. In Patient 9, sampling commenced four hours after the ingestion of androstenedione so as to observe the effects of time on the conversion of androstenedione to testosterone. It will be seen here that the 4th hour plasma testosterone concentrations exceeded those of plasma androstenedione but from then until the 7th hour the respective plasma concentrations are reversed, namely AD concentrations were greater than those of testosterone.

It is of considerable interest that high plasma concentrations of testosterone were achieved by the youngest patients (Patients 1, 2 and 3) comparable to the concentrations achieved by the two oldest patients (Patients 10 and 11). Whether or not these younger patients were able to utilize these high concentrations of testosterone will be considered later.

Two conclusions are therefore valid. Patients 1, 2, and 3 are prepubertal while the others are either within the pubertal period or postpubertal with reference to their chronological age. Apart from Patients 10 and 11 whose bone ages are in the pubertal age, all the others had retarded bone ages suggestive of a prepubertal status. Likewise, apart from Patient 11, all others had a height age and hence a height SDS and bone age indicative

Table IX: The anthropometric data and plasma concentrations of androstenedione and testosterone in Group 1 (eleven) boys after ingestion of 100 mg AD orally.

Pts.	CA yrs	HA yrs	SDS	BA yrs	Time in hours					
					0		1		2	
					nmol/L		nmol/L		nmol/L	
					AD	T	AD	T	AD	T
1.SS	9.25	6.83	-2.23	4.4	0.43	0.35	5.60	4.20	23.70	13.70
2.AG	10.73	8.06	-2.14	9.6	1.30	0.41	8.00	6.67	11.50	9.80
3.SC	11.40	8.50	-2.21	10.1	1.50	0.35	9.00	4.10	18.00	10.70
4.SW	12.18	8.76	-2.47	8.6	0.15	0.57	3.46	4.90	4.89	10.10
5.SD[1]	12.98	10.64	-1.70	9.7	0.14	0.42	5.67	7.00	5.52	6.98
6.TW	13.00	9.41	-2.51	10.3	0.27	0.38	4.40	5.68	4.85	5.62
7.JK	13.14	10.34	-2.01	10.5	0.16	3.34	4.71	7.10	3.84	5.30
8.PM	13.96	9.45	-3.18	8.5	0.14	0.64	4.36	5.10	5.76	8.90
9.SC[2]	15.30	10.79	-3.65	11.9						
10.DC	15.66	11.58	-3.48	13.0	0.17	5.10	5.71	13.70	7.96	16.80
11.GC	15.83	13.59	-1.96	14.5	0.36	16.50	2.02	20.05	7.96	22.50

Pts. = patients. CA = Chronological age.

HA = Height age. BA = Bone age.

SDS = Standard Deviation Score for height. yrs = years.

AD = plasma androstenedione. T = plasma testosterone.

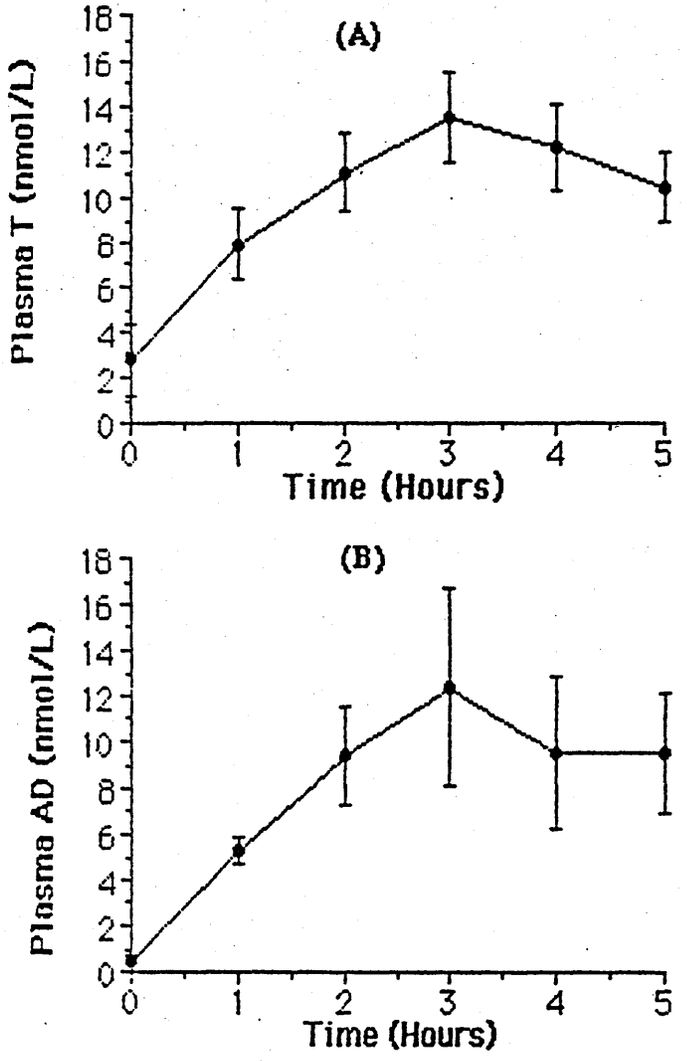
(1) Patient 5 in Table XIII.

(2) Patient 11 in Table XII.

Table IX contd.:

Time in hours									
3		4		5		6		7	
nmol/L		nmo/L		nmo/L		nmo/L		nmol/L	
AD	T	AD	T	AD	T	AD	T	AD	T
34.90	21.5	27.20	20.5	20.90	13.80				
31.40	20.0	27.60	22.9	24.90	20.70				
28.60	20.5	23.70	19.2	11.90	11.20				
4.54	10.5	2.58	7.8	1.15	4.20				
4.71	7.7	3.14	9.5	5.24	8.60				
3.74	7.2	1.57	4.1	1.29	3.57				
2.07	8.8	1.22	6.4	6.98	13.40				
3.60	6.5	3.11	6.6	19.20	10.65				
		9.10	10.3	8.40	6.65	6.80	4.30	6.30	3.56
5.34	13.4	3.11	10.6	2.55	10.00				
4.64	19.2	2.44	16.2	1.90	13.05				

Graphic representation of the means and the standard error of the means of these data is shown overleaf.



Graphic representation of Table (IX) of the means of plasma concentrations of testosterone (A) and androstenedione (B) for Group 1 patients. Bars represent standard errors of the means. The data of the means, standard deviations and standard errors of the means are shown below:

	Plasma concentrations (nmol/L)					
	Testosterone			Androstenedione		
Time (hours)	Mean	SD	SEM	Mean	SD	SEM
0	2.8	5.1	1.6	0.5	0.5	0.16
1	7.9	5.1	1.6	5.3	2.0	0.60
2	11.1	5.3	1.7	9.4	6.6	2.10
3	13.5	6.2	2.0	12.4	13.4	4.30
4	12.2	6.4	1.9	9.5	10.9	3.30
5	10.5	4.9	1.5	9.5	8.6	2.60

SD = Standard deviation; SEM = Standard error of mean.

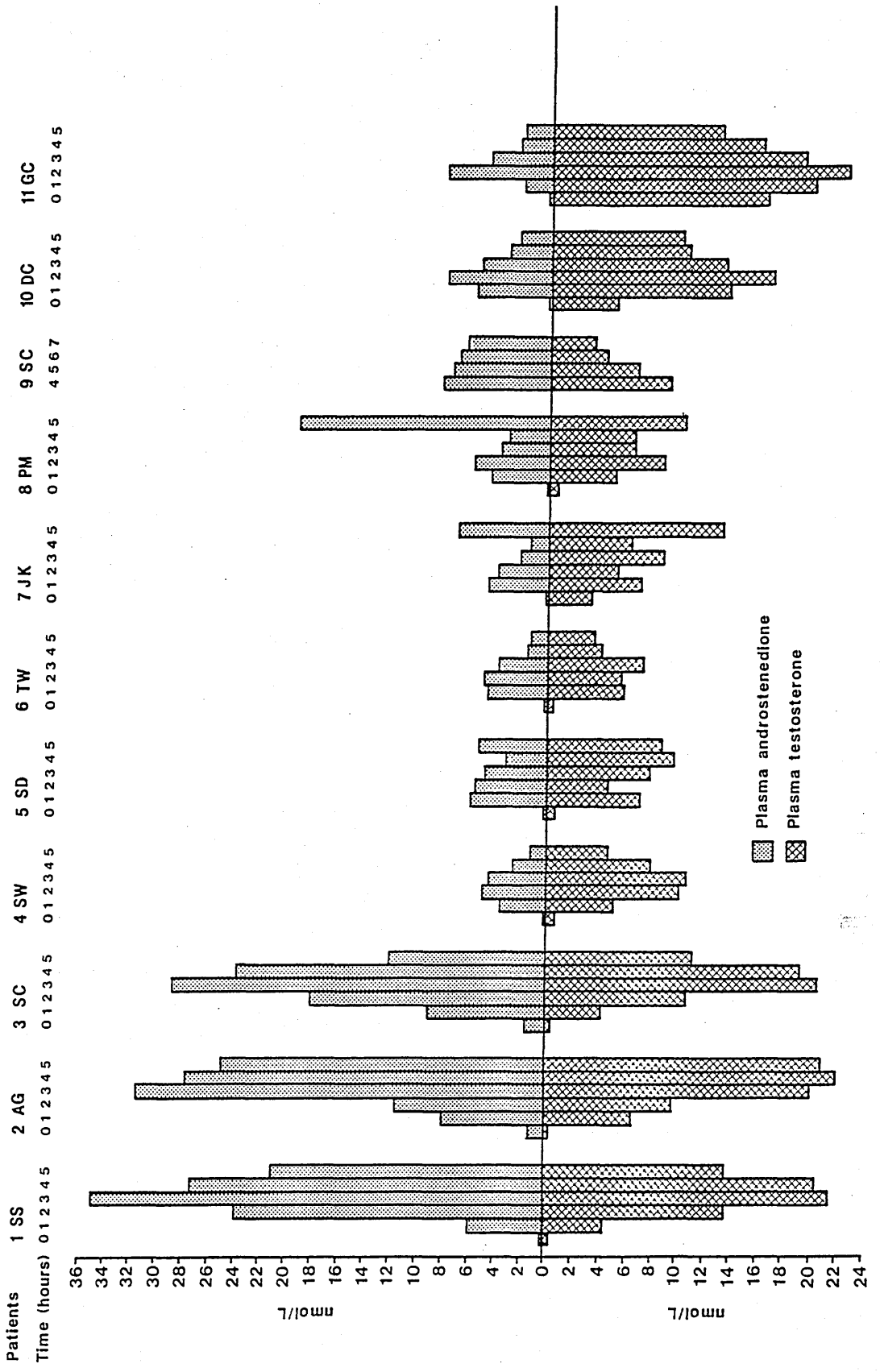


Figure 14 : The histogram of plasma concentrations of AD and testosterone in 11 boys of Group 1 who were given AD orally (100 mg).

of a prepubertal status. Thus the conversion of plasma androstenedione to testosterone is chronologically age-related(i.e AD/T < 1) and neither bone-age nor height-age related (SDS for height-variously negative and BA variously retarded).

In Patient 9, sampling commenced at the 4th hour after androstenedione ingestion and it will be seen that the plasma testosterone concentration exceeded that of androstenedione only in that sample. It may have been, that had sampling continued beyond the 5th hour in the other patients a similar trend would have been observed. This point will be taken up again when the chronic use of androstenedione is considered.

Suggested Explanation.

The conversion of androstenedione to testosterone could take place either in the gonads or in other tissues. If the conversion was by testicular activity it might be expected to be under luteotrophic influence. Table X shows the LH/FSH status of the patients to GnH-RH stimulation. It will be noted that basal concentrations of LH are low in all patients except Patients 4 and 8 (See Table X for normal values). Nonetheless all the patients converted androstenedione to testosterone very adequately in the face of low basal concentrations of LH. It is, therefore, concluded that this conversion is not primarily testicular but rather by other extragonadal tissues.

By the RIA methodology used to assay plasma concentrations of both androgens, however, it is the bound (SHBG) and free fractions which are assayed. It is, therefore, possible to suggest an explanation why Patients 1, 2, 3, 10 and 11 had high plasma concentrations of testosterone while Patients 4-8 had by

Table X : Plasma LH and FSH following GnH-RH test and anthropometric data in Group 1 boys who received AD orally (100 mg).

Pts.	CA years	HA years	SDS*	BA years	LH (IU/L)			FSH (IU/L)		
					0	20	60	0	20	60
1.SS	9.25	6.83	-2.23	4.4	1.6	2.6	2.3	<1.0	<1.0	1.5
2.AG	10.73	8.06	-2.14	9.6	<0.5	1.1	1.9	<1.0	1.2	1.5
3.SC	11.40	8.50	-2.21	10.1	1.1	4.3	3.4	<1.0	2.0	2.0
4.SW	12.18	8.76	-2.47	8.6	4.1	31.0	31.0	3.8	7.6	10.0
5.SD	12.98	10.64	-1.70	9.7	0.8	3.3	3.5	1.5	2.4	2.8
6.TW	13.00	9.41	-2.51	10.3	1.4	4.9	1.2	<1.0	<1.0	<1.0
7.JK	13.14	10.34	-2.01	10.5	1.3	11.0	12.0	1.1	2.1	2.5
8.PM	13.96	9.45	-3.18	8.5	5.4	9.1	8.4	<1.0	<1.0	<1.0
9.SC	15.30	10.79	-3.65	11.9	2.0	20.0	20.0	<1.0	2.0	2.2
10.DC	15.66	11.58	-3.48	13.0	1.0	1.0	9.8	<1.0	<1.0	2.5
11.GC	15.83	13.59	-1.96	14.5	2.6	1.4	1.8	1.4	2.0	1.9

* SDS for height.

Normal values for plasma LH are:

2 - 5 years 0.0 - 2.0 IU/L

5 - 12 years 1.0 - 5.0 IU/L

13 - up years > 5.0 IU/L

Normal incremental increases following GnH-RH = LH x 5 or more.

comparison low plasma concentrations of testosterone.

In these latter patients i.e. Patients 4-8, we must consider the activity of the androgen receptors in the androgen dependent tissues and in other tissues which accept androgens for anabolic purposes. It is clear that from the low basal concentrations of plasma testosterone indicating a lack of testicular contribution, the small increases measured following AD ingestion by these patients represented the balance between that testosterone initially derived from AD and that quantum accepted by the androgen receptors. The younger patients (Patients 1, 2 and 3) must be assumed to have fewer active androgen receptors because of their ages hence the high circulating plasma concentrations of both AD and testosterone. In Patients 10 and 11 high basal level of testosterone were observed and increments above basal values must be seen as the indices of conversion of AD to testosterone, the receptor being already fully saturated by pre-existing testosterone elaborated from adequate gonadal function (note in these two patients the bone ages are within the pubertal range.).

Table XI in a sense presents the same data linked to chronological age (CA), height age (HA), standard deviation score (SDS) for height and bone age (BA) of the eleven boys (Group 1) on whom the kinetics of androstenedione were studied. It will be seen that all patients are short statured showing differences between CA and HA of 2.34 years to 4.51 years or in terms of SDS from -1.7 to -3.65. Observe also that the osseous maturation scores also indicated a retarded BA for all patients. It is of considerable interest that for Patients 1, 2 and 3 the ratio of AD to testosterone is greater than unity while for the other patients

Table XI : The ratios of plasma AD to testosterone and anthropometric data in Group 1 patients following AD (100 mg) ingestion orally.

Pts.	CA yrs	HA yrs	SDS	BA yrs	AD/T Ratios Time in hours 0	1	2	3	4	5	6	7
1.SS	9.25	6.83	-2.23	4.4	1.23	1.33	1.73	1.62	1.33	1.51		
2.AG	10.73	8.06	-2.14	9.6	3.17	1.20	2.40	1.57	1.21	1.20		
3.SC	11.40	8.50	-2.21	10.1	4.29	2.20	1.68	1.40	1.23	1.06		
4.SW	12.18	8.76	-2.47	8.6	0.26	0.71	0.48	0.42	0.33	0.27		
5.SD	12.98	10.64	-1.70	9.7	0.33	0.81	0.79	0.61	0.33	0.61		
6.TW	13.00	9.41	-2.51	10.3	0.71	0.77	0.86	0.52	0.38	0.36		
7.JK	13.14	10.34	-2.01	10.5	0.05	0.66	0.72	0.24	0.19	0.52		
8.PM	13.96	9.45	-3.18	8.5	0.22	0.85	0.65	0.55	0.47	1.80		
9.SC	15.30	10.79	-3.65	11.9					0.88	1.20	1.25	1.58
10.DC	15.66	11.58	-3.48	13.0	0.03	0.42	0.47	0.40	0.29	0.26		
11.GC	15.83	13.59	-1.96	14.5	0.02	0.10	0.35	0.23	0.15	0.15		

CA = Chronological age. HA = Height age.

SDS = Standard deviation score for height. BA = Bone age.

yrs = years.

the ratio is less than unity. This could mean that presented with a bolus of AD (100 mg) the younger patients had an inadequacy of 17β-reductase while that enzyme was more active in the older patients. It is of further interest that in this group of 7 children (4-11 excl. 9) the age of 17β-reductase acquisition was 12.18 year and upwards suggesting that this may be an enzyme which becomes much more active at the age of puberty despite inappropriate osseous maturation. In this respect Patient 9 is of interest for since the ratio becomes greater than unity, after the fourth hour this might suggest exhaustion of an inadequately acquired enzyme-converting ability. The same phenomenon is manifest in Patient 8 at the fifth hour.

While these ratios serve to show trends more information can be derived from absolute values. These are also seen in Table IX where it is clear that there is a marked difference in the plasma concentrations of androstenedione and testosterone achieved by the two sets of patients i.e. Patients 1, 2 and 3 and the others collectively. Patients 1, 2 and 3 have plasma androstenedione concentrations on and after the second hour of 20 nmol/L and upwards with corresponding plasma testosterone concentrations during the same periods of 10 nmol/L and upwards. The fact that plasma testosterone is not being cleared from the circulation suggests that there may be a lack of testosterone receptors in both the androgen dependent tissues and in other tissues. It will be recalled that testosterone is metabolized by different mechanisms in the two types of tissue.

In Patients 4-11 there is a marked fall in the plasma AD concentrations to less than 10 nmol/L but even so the corresponding plasma testosterone concentrations are comparably

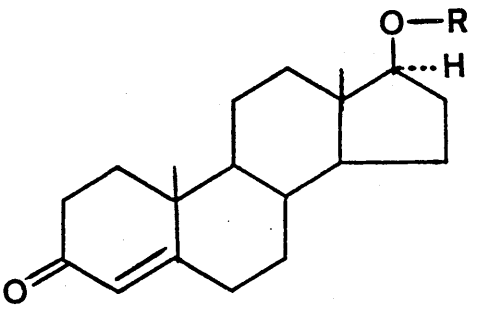
low. This suggest that while 17β-reductase has been active, it also implies that a good uptake by the tissues (i.e. adequate activity of receptors) have served to remove much of the testosterone from the circulation. The question of enzyme conversion versus testosterone metabolism may be answered when the metabolites of AD and testosterone are analysed from the pattern of urinary androgens metabolites.

Group 2 patients plasma concentrations of AD and testosterone.

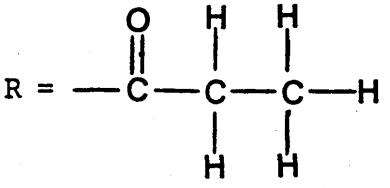
Eleven boys presented with significant short stature and with retarded bone ages. Non-endocrine causes had been excluded and it was judged prudent to perform an insulin hypoglycaemia test on each after their receiving Sustanon (100 mg) intramuscularly three days prior to the hypoglycaemia test. Blood and urine were taken before the testosterone injection and just prior to the injection of the insulin on the third day. Sampling of blood and urine was made at the same hour (10.00 h) on both days.

In Figure 15 is the molecular structures of the three components of Sustanon. The method of plasma testosterone assay utilizes two antibody steps. The first antibody is raised against a C19 hapten, and the second antibody against the first antibody so precipitating the total bound (to the first antibody) testosterone. Thus by the RIA method of assay native testosterone and all three esters of testosterone in circulation will be measured as a group. It is therefore expected that high plasma "testosterone" concentrations will be detected in third day plasma and our interest will centre on the effects of these high values on the plasma AD levels.

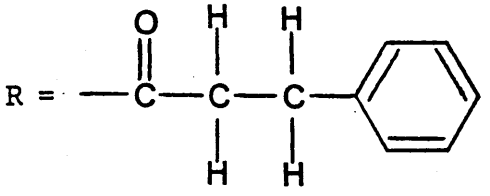
In Table XII and Figure 16 are the plasma concentrations of



T.propionate



T.phenylpropionate



T.isocaproate

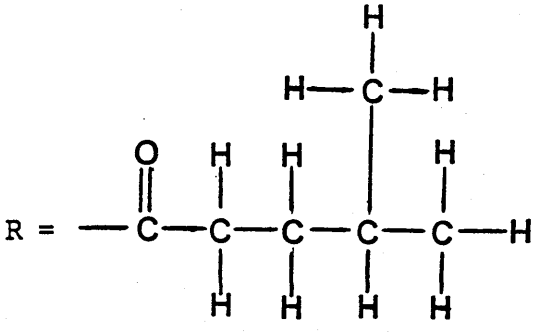


Figure 15: The molecular structure of the three components of Sustanon.

T = Testosterone.

AD and testosterone before Sustanon administration and on the third day post-injection. It will be seen that basal AD plasma concentrations are greater than those of testosterone which themselves are pathologically low (see page 120 for normal values). It might be reasonable therefore to conclude that there is an insignificant testicular contribution to these plasma concentrations of testosterone. Likewise it must be concluded that the increases in the plasma AD concentrations detected after Sustanon administration must be derived from the testosterone esters and not from testicular derived testosterone. In effect the conversion of the total plasma testosterone (as assayed) to AD represents the activity of the 17β-oxido-reductase enzyme.

It is of interest that all subjects received the same mass of testosterone esters yet the plasma concentrations achieved varied widely from 20.45 nmol/L (aged 13.53 year) to 90.0 nmol/L (aged 12.59 year) with a value of 80.0 nmol/L for a boy aged 10.67 year. This suggests a very wide subject capacity in such steps as hepatic hydrolysis, sex hormone protein binding, metabolism and possibly also testosterone receptor activity. Thus it is regrettable that it was not possible to estimate the free and SHBG bound testosterone separately from the triple esters for then a more direct relationship between the total plasma AD concentrations (SHBG bound and free) and the corresponding total plasma testosterone concentrations could have been compared so giving an estimation of the 17β -oxido-reductase enzyme. Much of the total assayed plasma testosterone concentrations is likely to be testosterone esters for it has been already demonstrated that following administration of Sustanon (100 mg) abnormally high plasma testosterone concentrations persist even after one

Table XII: The anthropometric data and plasma concentrations of testosterone (T) and AD in Group 2 patients before and three days after intramuscular Sustanon (100 mg) injection. Also shown: the percentages of testosterone converted to AD (% T to AD).

Pts.	CA years	HA years	SDS*	BA years	Before Sust. nmol/L		3days after Sust. nmol/L		% T to AD
					AD	T	AD	T	
1.SK	10.67	6.74	-3.25	7.2	1.78	0.35	9.00	80.00	8.3
2.DO	11.65	8.32	-2.50	9.5	1.68	0.35	8.00	58.00	9.9
3.AM	11.66	9.02	-1.96	7.7	1.40	0.37	4.50	20.50	13.3
4.MA	12.31	9.50	-2.01	10.7	1.80	0.59	6.60	66.00	6.8
5.NM	12.59	8.82	-2.69	10.7	1.20	2.08	8.40	90.00	7.6
6.SM	12.64	9.66	-2.12	8.3	1.75	0.49	7.86	44.00	12.3
7.JP	12.74	9.89	-2.72	10.8	1.40	0.41	5.30	52.00	7.0
8.DK	13.53	11.04	-1.84	10.0	1.10	0.35	3.00	20.45	8.6
9.SS	14.16	9.86	-3.09	11.2	3.35	0.73	9.40	48.00	11.3
10.AD	14.77	10.91	-3.00	11.3	4.89	4.00	13.27	33.00	22.4
11.SC	15.30	10.79	-3.65	11.9	3.40	2.86	7.70	68.00	6.2

* SDS for height. Sust. = Sustanon.

Note that maximum plasma testosterone values assayable accurately by the method used is 34.7 nmol/L. Samples above this concentration were diluted (x3) with 0.0 standard before re-assay.

Patient N^{O} 11 is Patient N^{O} 9 in Table IX. Sustanon injection had been given to him 5 days after AD oral ingestion (100 mg).

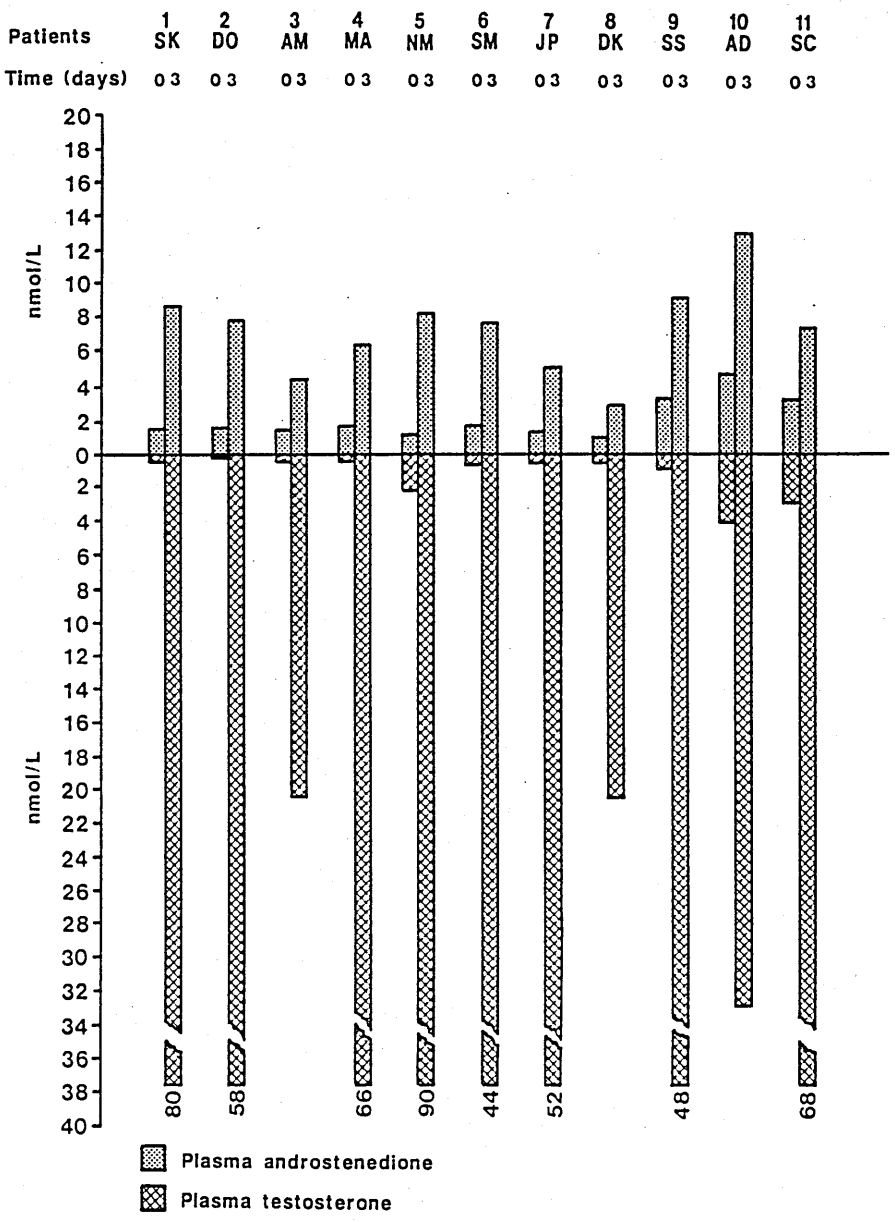


Figure 16 : The histogram of plasma concentrations of AD and testosterone in 11 boys of Group 2 who were given Sustanon I.M. (100 mg).

month[33].

Refer again to Table XII where the plasma AD concentrations achieved from testosterone conversion are expressed as percentages (? conversion) of the total plasma testosterone concentrations at the third day after the Sustanon injection. Subject No 10 is outstanding for being like some others of the group his height SDS was -3.00 yet his "percentage conversion" was greatest being 22.4 per cent while his plasma AD concentration was 13.27 nmol/L (being highest) while the plasma testosterone concentration was third lowest at 33.0 nmol/L. While we must appreciate when taking a single blood or urine sample for assay we are measuring one element of a full sequence of events which must include absorption, hepatic conversion, SHBG-binding, receptor protein binding and degradation, the data for subject No 10 could be interpreted as a failure of the final metabolic degradation of AD so that high plasma concentrations persist due to a slowing of the conversion of AD to its 5ξ-metabolites. This might be borne out when urinary studies are analysed. Nonetheless this patient's endogenous basal plasma testosterone concentration was beginning to rise to nearly 4.0 nmol/L, which level might be regarded as an early pubertal value-thus puberty might be imminent.

Group 3 Patients - plasma concentrations of AD and testosterone.

While formerly I have presented the plasma concentrations of AD and testosterone in acute experimental situations (Group 1 patients over 5 hours and Group 2 patients over 3 days). I now present similar plasma concentrations during the 1 year treatment

period of five growth hormone deficient boys, with AD and growth hormone. Blood and urine sampling was started before treatment and at three monthly intervals during the one year of treatment. These data are presented in Table XIII and Figure 17.

So far I have made only brief reference to normal values (as obtained in the Laboratories of the Department of Child Health) for plasma AD and testosterone concentrations. These values are now given in association with Table XIII. The data in Table XIII indicate that there is no constancy of plasma concentrations of either androgen in relation to the thrice weekly administration of AD (100 mg as a bolus thrice weekly on the days of growth hormone injection). Plasma had been withdrawn at 10.00 hour, i.e. twelve hours after the ingestion of the AD. The shaded areas are those occasions when the highest concentrations of plasma AD and testosterone were found. It will later be shown that these shaded areas correspond exactly with the shaded areas in Table XVI which shows that the greatest growth velocities corresponded on average with the well recognized periods of peak seasonal growth.

Since the dosage schedule was constant during the entire year of treatment the question is therefore posed whether or not there was a seasonal endogenous contribution to these plasma concentrations or whether absorption was seasonally related?. The ultimate effect however cannot be denied that linear growth was greatest when the plasma AD and testosterone concentrations were highest (Table XVI).

It will be seen from Table XIII that at the commencement of treatment the plasma AD concentrations for all patients were either normal (Patients 1, 2 and 3) or low (Patients 4 and 5). After three months of treatment Patients 1, 2 and 3 had increased

beyond normal their plasma concentrations while Patients 4 and 5 had normal values for age. This pattern is on average repeated during the remainder of the year but the general trend is punctuated in Patients 1 and 3 by occasional inexplicably high values. It is difficult to explain the peak concentrations of both AD (34.9 nmol/L) and testosterone (13.5 nmol/L) at 3 months and 34.6 nmol/L of AD and 8.6 nmol/L of testosterone at 9 months in Patient 1 and AD 11.0 nmol/L at 9 months in Patient 3) other than by assuming that the AD had been administered on the morning of blood withdrawal rather than on the previous evening. This thought is endorsed by noting that the plasma testosterone concentrations are correspondingly high at these times (cf. the acute experimental results of Table IX). Otherwise the plasma testosterone concentrations for all patients at the other times indicate subnormality in most of the younger age group and certainly subnormality in the older age group.

This relationship of plasma AD to plasma testosterone is seen better from the histogram of Figure 17. If we accept the exceedingly high concentrations of plasma AD and testosterone in Patients 1 at 3 months and at 9 months as indicative of the possible nearness of AD ingestion to blood sampling and possibly also at 9 months in Patient 3, then the nett effect of chronic AD administration has not been to raise above normal concentrations plasma testosterone which were subnormal in the two older boys and largely normal at 3 months in Patient 3 but otherwise subnormal in the 3 younger boys. It might have been expected that there would have been a steady rise in plasma testosterone concentrations throughout the year but this did not happen. The tendency for plasma AD concentrations to rise as

Table XIII: Plasma concentrations of AD and testosterone (T) in Group 3 patients treated with growth hormone (4 IU thrice weekly) and AD (100 mg orally on same day as GH administered) for one year.

Time (mo.)	0		3		6		9		12	
nmol/L	AD	T	AD	T	AD	T	AD	T	AD	T
Pts.CA(yrs)										
1.AS 6.28	1.1	0.23*	34.9	13.5	2.10	0.35	34.6	8.60	2.27	0.36
2.MR 7.03	1.5	0.31*	2.13	0.68	1.96	0.36	2.5	0.46	1.60	0.30*
3.GY 9.50	1.6	0.33*	4.54	1.05	2.37	0.36	11.0	2.40	2.16	0.37
4.GM 12.80	0.5	0.20*	1.78	0.79	0.98	0.37	1.68	0.71	1.50	0.37
5.SD 13.80	1.4	0.64	3.77	0.64	4.15	0.81	2.52	0.51	3.50	0.72

Pts.CA(yrs) = Patients chronological ages (years).

*The lower limit of detection by the RIA method for testosterone is 0.35 nmol/L. Values obtained below this limit were re-assayed again by using twice the plasma volume used.

-The shaded areas represents maximum concentrations and periods of maximum growth.

-Departmental laboratory normal values in males are for:

Plasma AD: 6 months to 10 years 0.35 - 1.5 nmol/L.

10 years to 20 years 1.50 - 8.5 nmol/L.

Plasma testosterone: 1 year to 10 years 1.0 - 5.0 nmol/L.

10 year to 20 years 5.0 - 20.0 nmol/L.

These values do not differ significantly from recently published data [147].

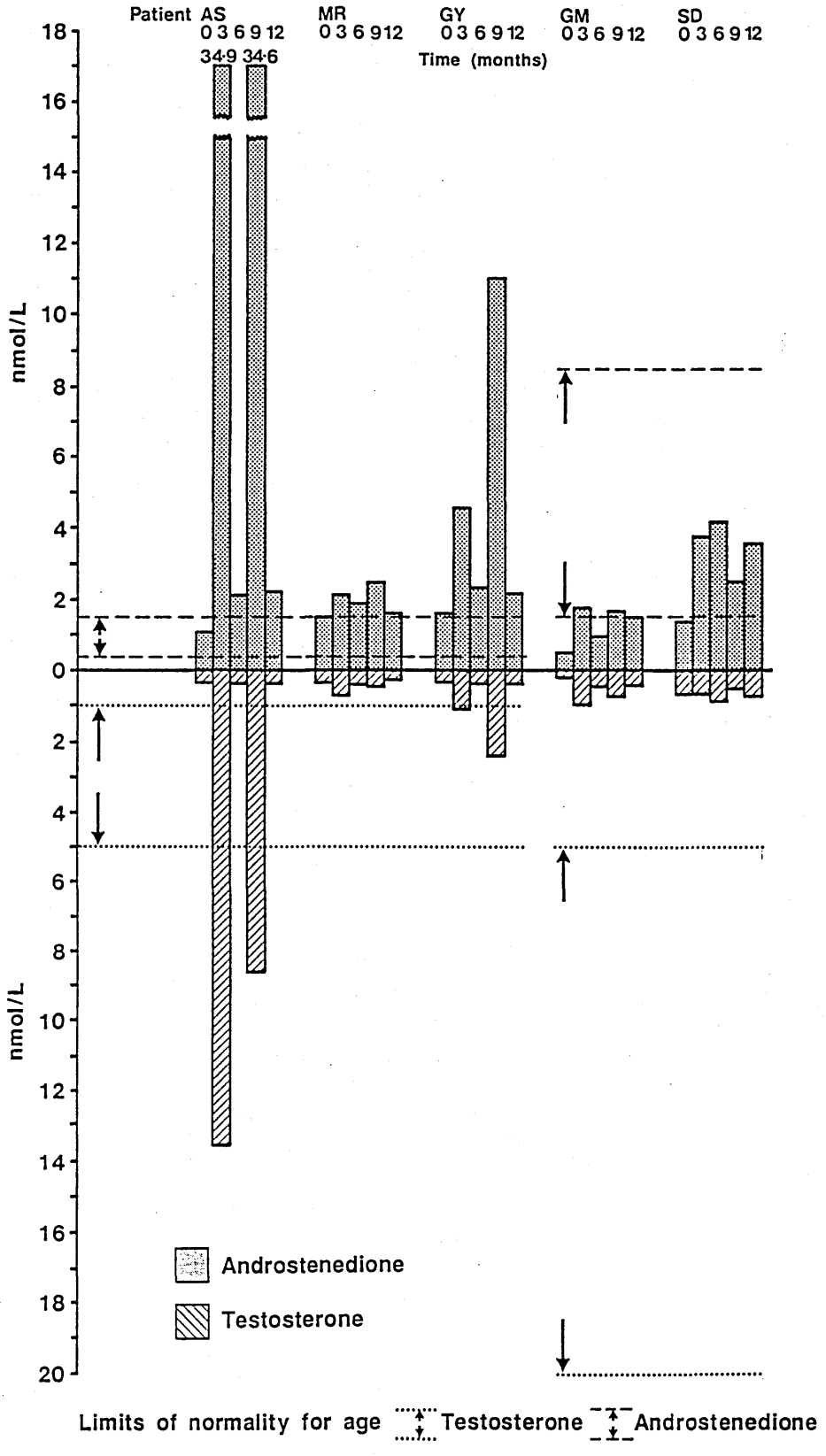


Figure 17: The histogram of plasma concentrations of AD and testosterone in Group 3 patients, with the range of normal concentrations for age.

shown in the histogram need not at this stage create anxiety for its androgenicity is only 12 per cent that of testosterone in the capon's comb test [94].

Intermediate summary of plasma studies.

From the acute studies performed on Group 1 boys AD is readily absorbed from the gut and enters the circulation to result in measurable changes in its concentration certainly after the first hour. A peak concentration is achieved and on average within the experimental period of five hours this peak concentration begins to fall. In the one patient (Patient 9) where sampling commenced at four hours post-ingestion, the concentrations showed a continuous fall but were still above the basal concentration and indeed were generally comparable to the first hour concentrations of the other patients.

Plasma testosterone concentrations followed the same trend as those of AD and showed a certain close relationship even from the first hour samples. This suggests a very active 17β-reductase enzyme and even granting the fact that the methodologies estimated both the bound and free fractions, the removal from the circulation either by the receptor "mop-up" or by metabolism did not frustrate the detection of significant increases in the plasma concentration of both hormones.

When testosterone (Sustanon) (100 mg) was given to the eleven boys of Group 2 there was an undoubted measurable increase in the third day plasma AD concentrations. While as might be expected the plasma testosterone concentrations were "pathologically" high,

the need for such high concentrations in this test situation has already been questioned [33]. Nonetheless the results of the investigation indicate an active 17β-oxidoreductase activity. Thus plasma AD and plasma testosterone, if they have a distinctly separate function in the body, are interconvertible although the current assumption is that the AD exerts its general action by conversion to the more potent testosterone. From the combined studies the data suggest that the acquistion of the enzyme(s) involved is a pubertal phenomenon. The chronic use of oral AD as combined treatment with growth hormone for short stature (growth hormone deficient) did not reveal any constancy in the plasma concentrations of either AD or testosterone.

Analyses of anthropometric changes during treatment with growth hormone and AD in Group 3 patients.

In Table XIV are the anthropometric data of the five boys who were investigated for an endocrine cause of short stature, other causes having been excluded. They were found to be growth hormone deficient. The broadest criteria for considering the need for endocrine investigation of short stature are an SDS for height more negative than -2.5,

(SDS = Height of patient - mean height for patient age / 1.0 SD)

and a bone age one or more years less than the chronological age. Patient No 5 whose height SDS was only -1.96 was investigated because of his markedly retarded bone age of 10.6 year against a chronological age of 13.8 years and a deteriorating height SDS from -1.70 nine months earlier. When patients are assessed at

three monthly intervals it is frequently found that there is a worsening height SDS. It is, therefore, prudent to investigate early rather than waiting until the height SDS reaches the critical point of -2.5 SDS.

Synthetic (recombinant) growth hormone was made avaliable for these children by the Hospital Growth Hormone Committee. The regimen chosen was that they should receive growth hormone 4 IU subcutaneously thrice weekly on the evenings of Mondays, Wednesdays and Fridays and that they should also receive orally AD (100 mg) as a single dose thrice weekly at the same time as the injected growth hormone. Blood and urine sampling was performed at 10.00h on the day following drug administration. These samples plus the recording of the anthropometric data were taken at three-monthly intervals during a one year period. The AD was then discontinued and observations continued at three-monthly intervals until the writing of this work.

In Table XV are the changes in growth parameters for each boy during the first year of combined growth hormone/AD treatment. These achievments are expressed in height age and bone age gains and height SDS.

It will be noted that all patients gained in height reflected by the diminshing SDS (cf. Col. 3 & 7) which represents annual gains of 13.7cm, 15.9cm, 7.6cm, 10.4cm and 8.5cm respectively (Col. 10 i.e Cols. 6-2). Expressed otherwise in one chronological year of treatment all patients experienced gains of 1.3 to 2.2 height age years in one year (Col. 11).

It is of interest to note (Table XVI) the changing rates in linear growth achievement in these patients relative to the seasons. The three monthly gains are expressed as calculated

Table XIV: Basal anthropometric data of Group 3 patients who were to be treated with growth hormone and AD for one year.

Pts.	CA years	HA years	SDS	BA years	Wt. kg
1. AS	6.28	3.68	-3.24	2.9	15.2
2. MR	7.03	4.29	-3.12	6.3	19.6
3. GY	9.50	5.21	-4.05	5.8	18.7
4. GM	12.80	6.74	-4.32	8.8	25.6
5. SD*	13.80	11.19	-1.96	10.6	34.6

* Patient 5 in Table IX.

CA = Chronological age.

HA = Height age.

SDS = Standard deviation score for height.

BA = Bone age.

Table XV : The anthropometric data of Group 3 boys treated with GH and AD for 1 year, basally and a year after the treatment.

	Basal					After one year on GH + AD							
Col.N^{O}	1	2	3	4	5	6	7	8	9	10	11	12	13
Pts.	CA yrs	Ht cm	SDS Ht.	HA yrs	BA yrs	Ht cm	SDS Ht.	HA yrs	BA yrs	6-2 AGV	*HA/ lyr	*BA/ lyr	BA/ HA
1.AS	6.28	99.3	-3.24	3.68	2.9	113.0	-1.65	5.88	6.8	13.7	2.20	3.9	1.77
2.MR	7.03	103.6	-3.12	4.29	6.3	119.5	-1.20	6.49	8.6	15.9	2.20	2.3	1.05
3.GY	9.50	109.6	-4.05	5.24	5.8	117.2	-3.43	6.50	8.8	7.6	1.26	3.0	2.38
4.GM	12.80	119.0	-4.32	6.74	8.8	129.4	-3.61	8.54	11.2	10.4	1.80	2.4	1.33
5.SD	13.80	143.0	-1.96	11.19	10.6	151.5	-1.78	12.70	12.6	8.5	1.41	2.0	1.43

Col. 12 mean = 2.72.

Col.N^{O} = Column numbers. Pts. = Patients. CA = Chronological age.

Ht. = Height. SDS = Standard deviation score (Ht). HA = Height age.

BA = Bone age. yrs = years. AGV = Annual growth velocity (cm/year).

* The increment of HA and BA (years/one year of treatment).

annual growth velocities as though the particular gains had been maintained over the whole year. Greatest growth velocities were recorded during the Spring and Autumn in Patients 1, 2 and 4; Patient 3 Spring and Summer and Patient 5 Summer and Winter. These seasonal increases in height do not strictly conform to reports[148] but that growth was achieved is gratifying.

As might be expected the osseous maturation also advanced from 2.0 to 3.9 bone age years in the treatment year, (Col. 12) in Table XV. But expressed in terms of height achievement these advances in bone age years represent 1.05 to 2.38 bone age years per height age year (Col.13). Expressed otherwise the combined growth hormone/AD therapy advanced the bone age more than the height age by factors ranging from 1.05 to 2.38 years. At present it is not possible to separate the growth promoting effects of growth hormone from the effects of AD. But it may be for the moment assumed that growth hormone would increase linear growth while the anabolic hormone would advance the bone age. The combination of drugs seems therefore to have the disadvantage of accelerating bone maturation more than increasing height velocity.

In Table XVII are some pertinent data from Table XV presented to show that although bone maturation seemed to have advance more rapidly than height increased, the actual status of each child in terms of bone maturation relative to chronological age (except subject No 2), was good in so far as the bone maturation remained less than the chronological ages. The bone maturation is here emphasized for if it advances to the point of epiphyseal fusion linear growth will virtually cease no matter the height. Thus ideal treatment for growth restricted children must not advance the bone maturation beyond the chronological age.

Table XVI: Three-monthly incremental height increases expressed as calculated annual growth velocities (AGV) in Group 3 Patients. Also shown the actual AGV before and one year after combined treatment with growth hormone and AD.

	Annual Growth Velocity (cm/year)					
	Actual	Calculated				Actual
Time (mo.)	0	3mo.	6mo.	9mo.	12mo.	1yr GH+AD
Pts.CA(yrs)						
1.AS 6.28	1.7	24.8 [3]	8.4 [4]	13.6 [1]	8.0 [2]	13.7
2.MR 7.03	1.8	29.6 [3]	13.2 [4]	14.8 [1]	6.0 [2]	15.9
3.GY 9.50	2.6	6.4 [3]	5.6 [4]	10.0 [1]	8.4 [2]	7.6
4.GM 12.80	2.7	22.8 [3]	6.0 [4]	11.6 [1]	1.2 [2]	10.4
5.SD 13.80	3.4	6.4 [1]	14.0 [2]	6.4 [3]	7.2 [4]	8.5
					Mean	11.2

Insert numbers represents seasons:

Spring (1), Summer (2), Autumn (3) and Winter (4).

- Shaded areas are periods of greatest calculated AGV but also periods of highest concentrations of AD and testosterone (see Table XIII).
- Pts.CA(yrs) = Patients chronological ages (years).

Table XVII: Some clinical anthropometric data for Group 3 Patients one year after treatment with growth hormone and AD.

Pts.	CA years	HA years	BA years
1.AS	7.28	5.88	6.80
2.MR	8.03	6.49	8.60
3.GY	10.50	6.50	8.80
4.GM	13.80	8.54	11.22
5.SD	14.80	12.70	12.60

All these data in Tables XIV-XVII are shown for individual patients graphically in the Growth Charts (Figures 18-22). Note that only in Patient 2 (MR) has the bone maturation advanced beyond the chronological age by 0.57 years.

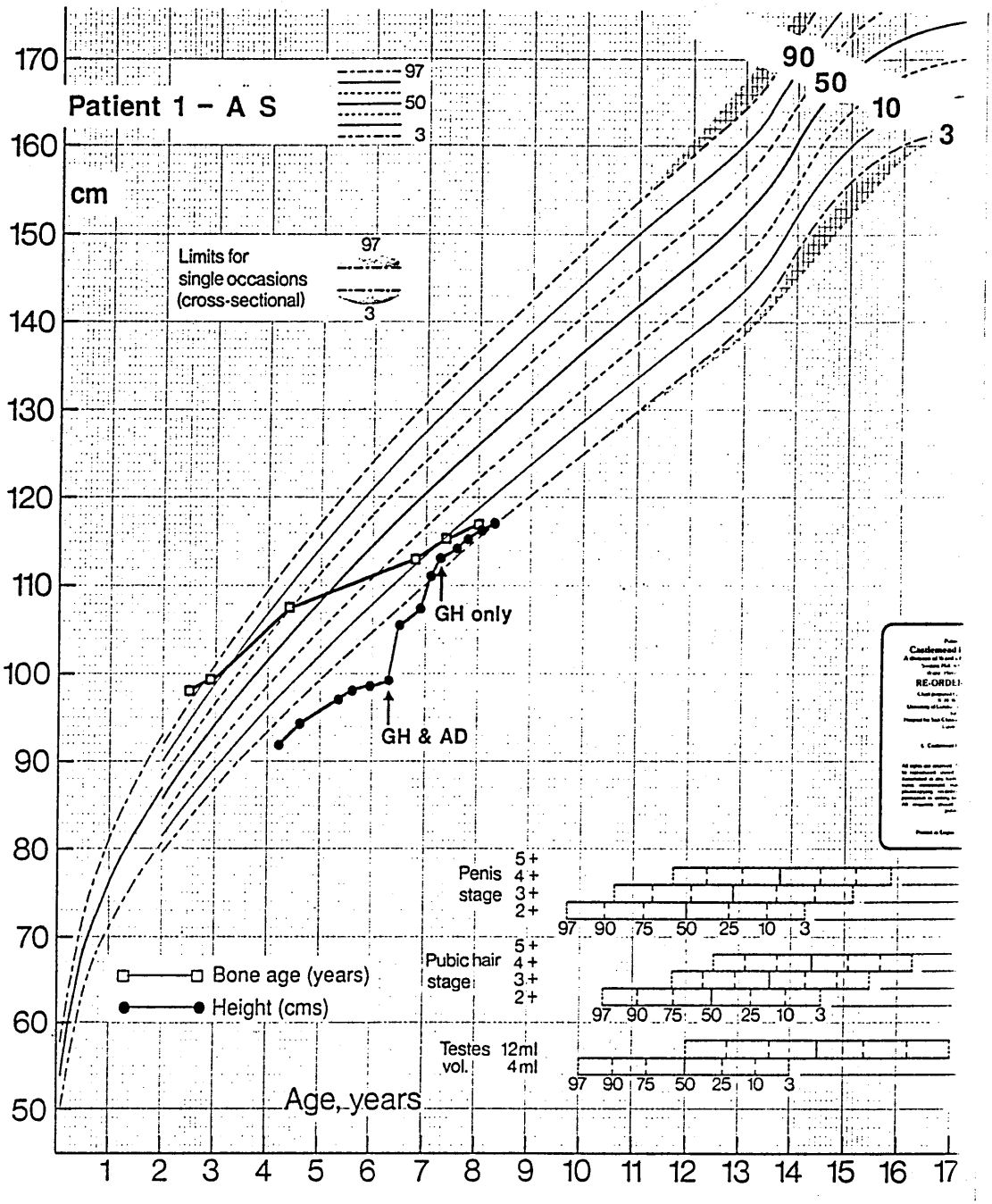


Figure 18: The height growth chart for Patient 1 (AS) in Group 3 patients, showing the growth pattern at three monthly intervals before starting the combined treatment with growth hormone and AD; during the year of this combined treatment and a year after AD was stopped. Also are shown the advances of the bone age in relation to the advances in height age and chronological age.

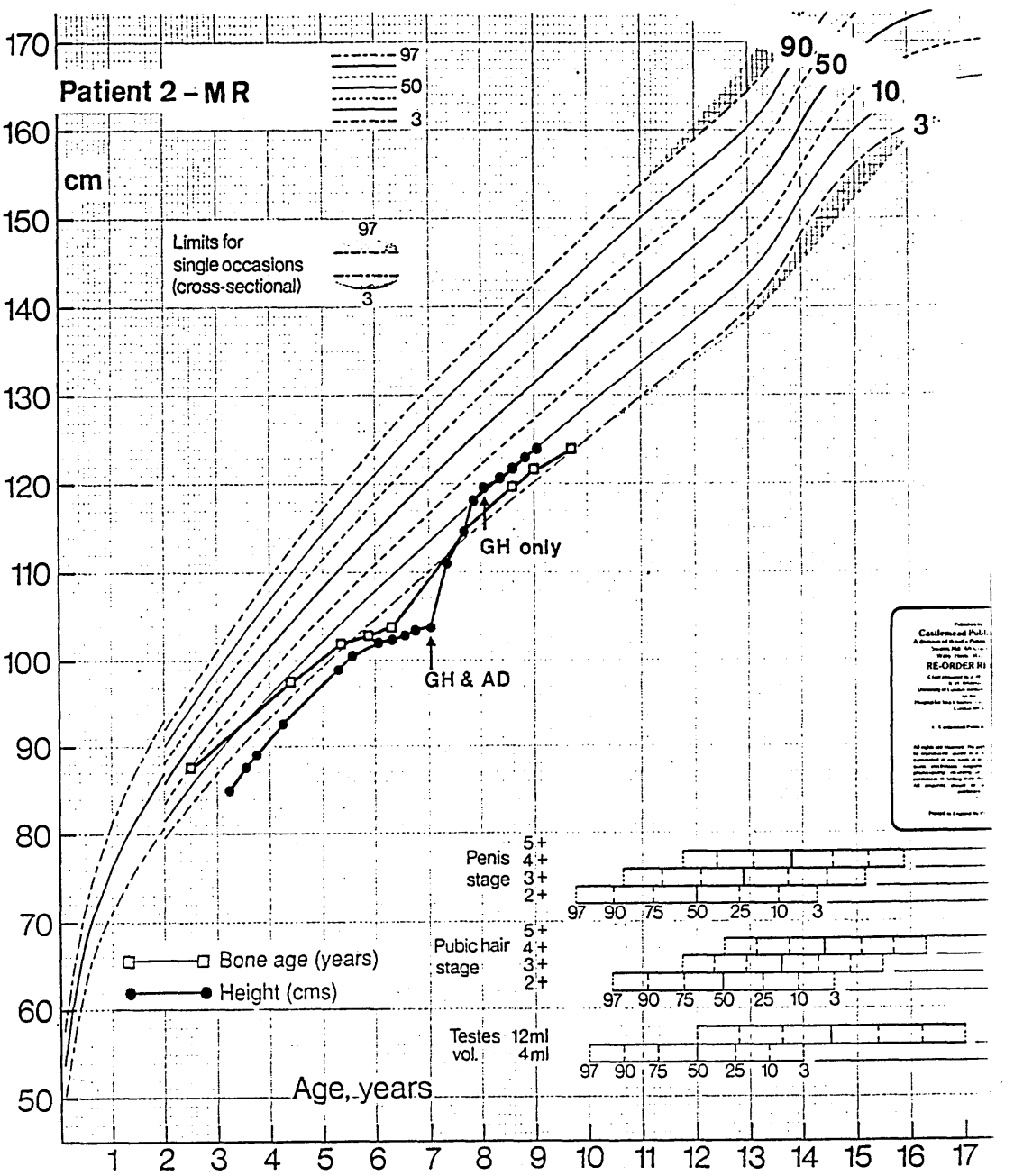


Figure 19: The height growth chart for Patient 2 (MR) in Group 3 patients, showing the growth pattern at three monthly intervals before starting the combined treatment with growth hormone and AD; during the year of this combined treatment and a year after AD was stopped. Also are shown the advances of the bone age in relation to the advances in height age and chronological age.

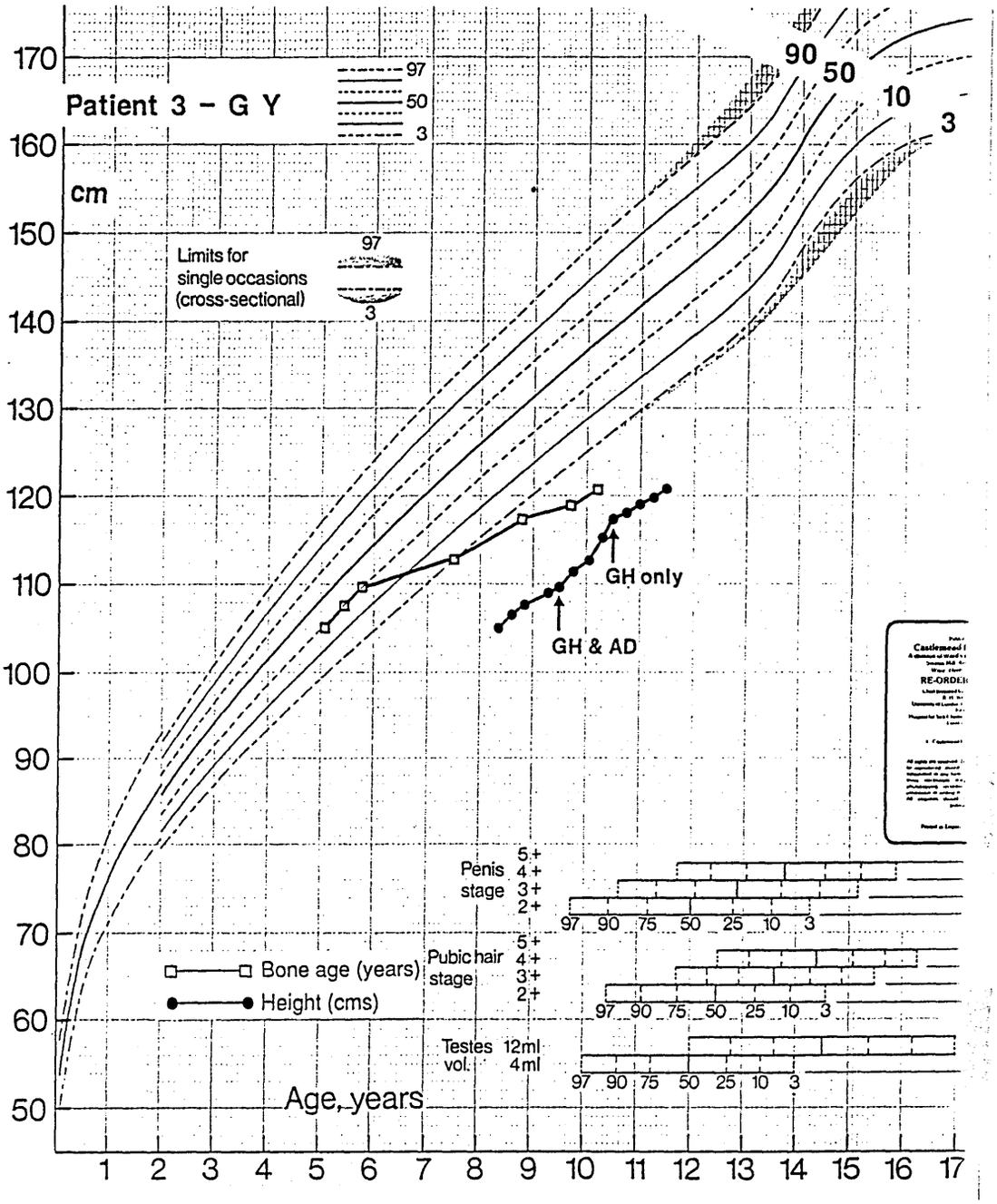


Figure 20: The height growth chart for Patient 3 (GY) in Group 3 patients, showing the growth pattern at three monthly intervals before starting the combined treatment with growth hormone and AD; during the year of this combined treatment and a year after AD was stopped. Also are shown the advances of the bone age in relation to the advances in height age and chronological age.

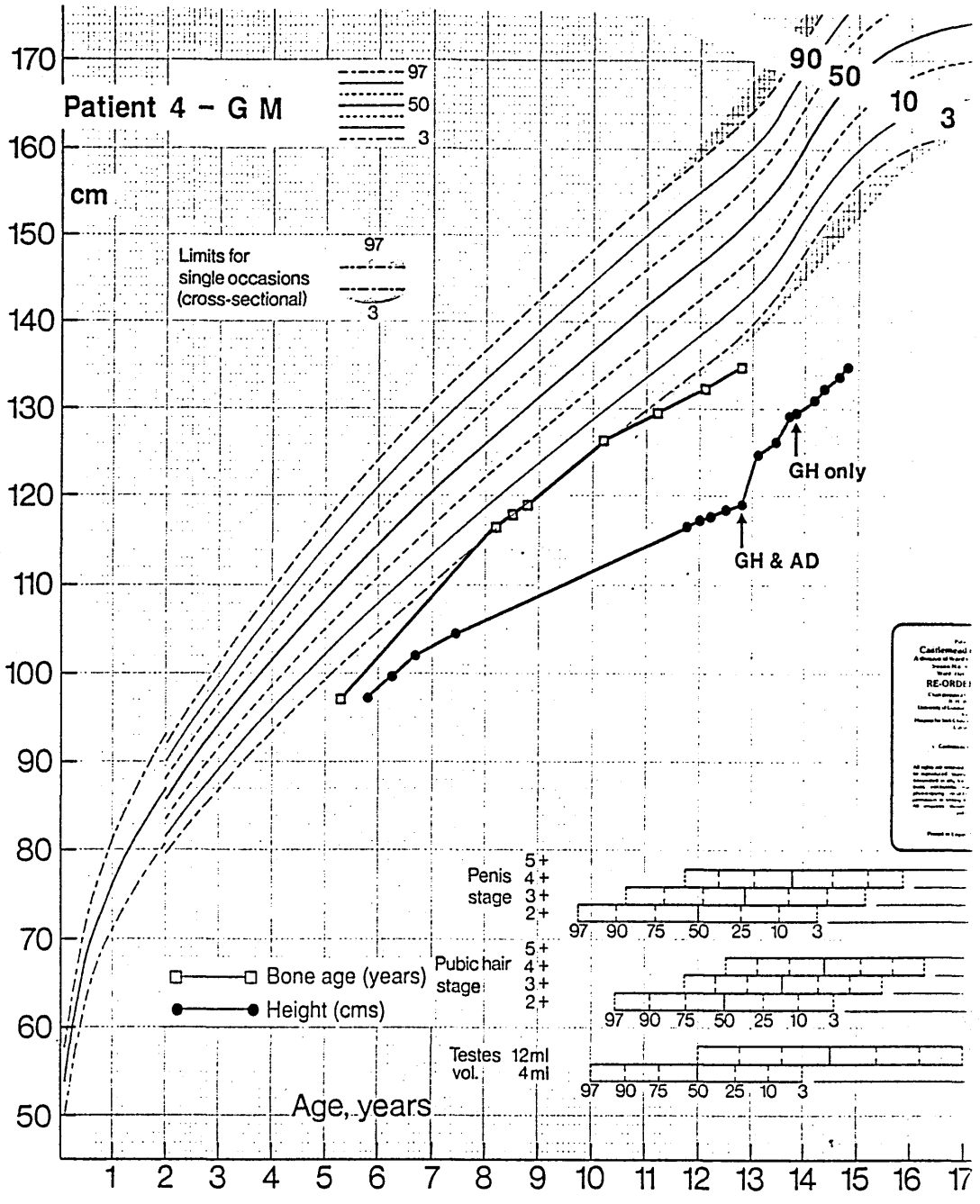


Figure 21: The height growth chart for Patient 4 (GM) in Group 3 patients, showing the growth pattern at three monthly intervals before the combined treatment with growth hormone and AD; during the year of this combined treatment and a year after AD was stopped. Also are shown the advances of the bone age in relation to the advances in height age and chronological age.

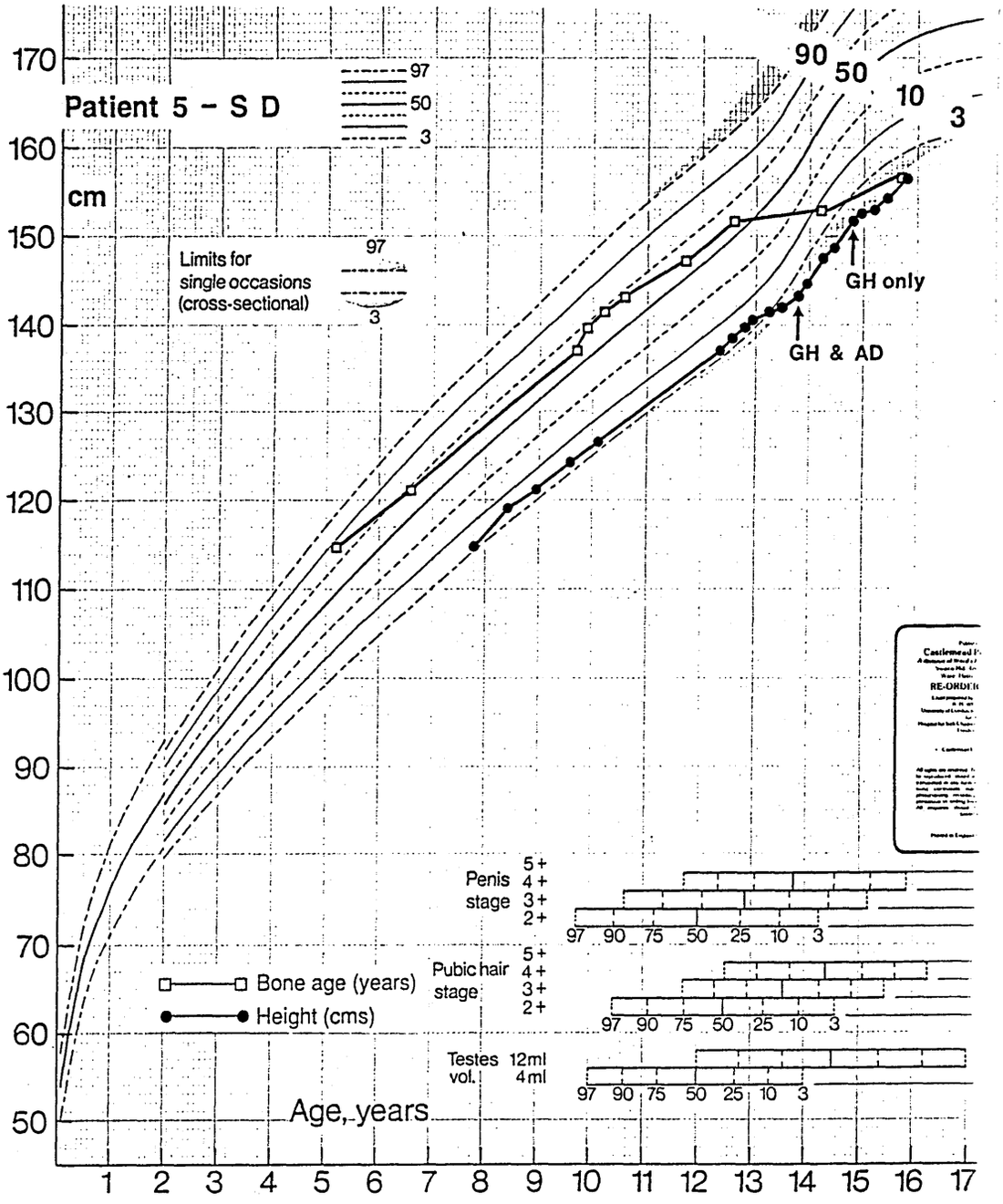


Figure 22: The height growth chart for Patient 5 (SD) in Group 3 patients, showing the growth pattern at three monthly intervals before the combined treatment with growth hormone and AD; during the year of this combined treatment and a year after AD was stopped. Also are shown the advances of the bone age in relation to the advances in height age and chronological age.

We shall now consider briefly the effects of the combined growth hormone/AD treatment on weight. The actual weights and weight trends of the five patients are shown in Figure 23.

While all had increases over their basal weight (i.e. weight at the commencement of combined therapy) during the one year treatment period, the significance of these gains is well shown in Table XVIII where the ratios weight (kg) to height (m) are recorded during the year of combined treatment and one year after the withdrwal of AD. For comparison ideal weight:height ratios of the 50^{th} centiles at the three ages (basal, one year after combined treatment and after one further year on growth hormone alone are shown).

It is of extreme interest that while on combined treatment all patients improved their weight:height ratios to near normal proportions at the end of the year. On withdrawal of AD a similar improvement was not continued on growth hormone alone except in patient MR (2) who during the period under consideration was receiving treatment for asthma which included Becotide (Beclomethasone). The other patients ratios either worsened (Patients 1, 3 and 4) or remained unchanged (Patient 5).

Thus it can be stated confidently that the combined growth hormone/AD treatment tends to normalize weight:height ratio while growth hormone alone is unable to do this. As far as Patient 2 (MR) is concerned a longer survey period might have been required to see if he could have maintained a good ratio even after the withdrawal of Beclomethasone. This drug if in overdose might be expected to increase weight but restrict height.

Table XIX expresses the data of Table XVIII in percentages of the 50^{th} centile weight:height ratios at the three periods of

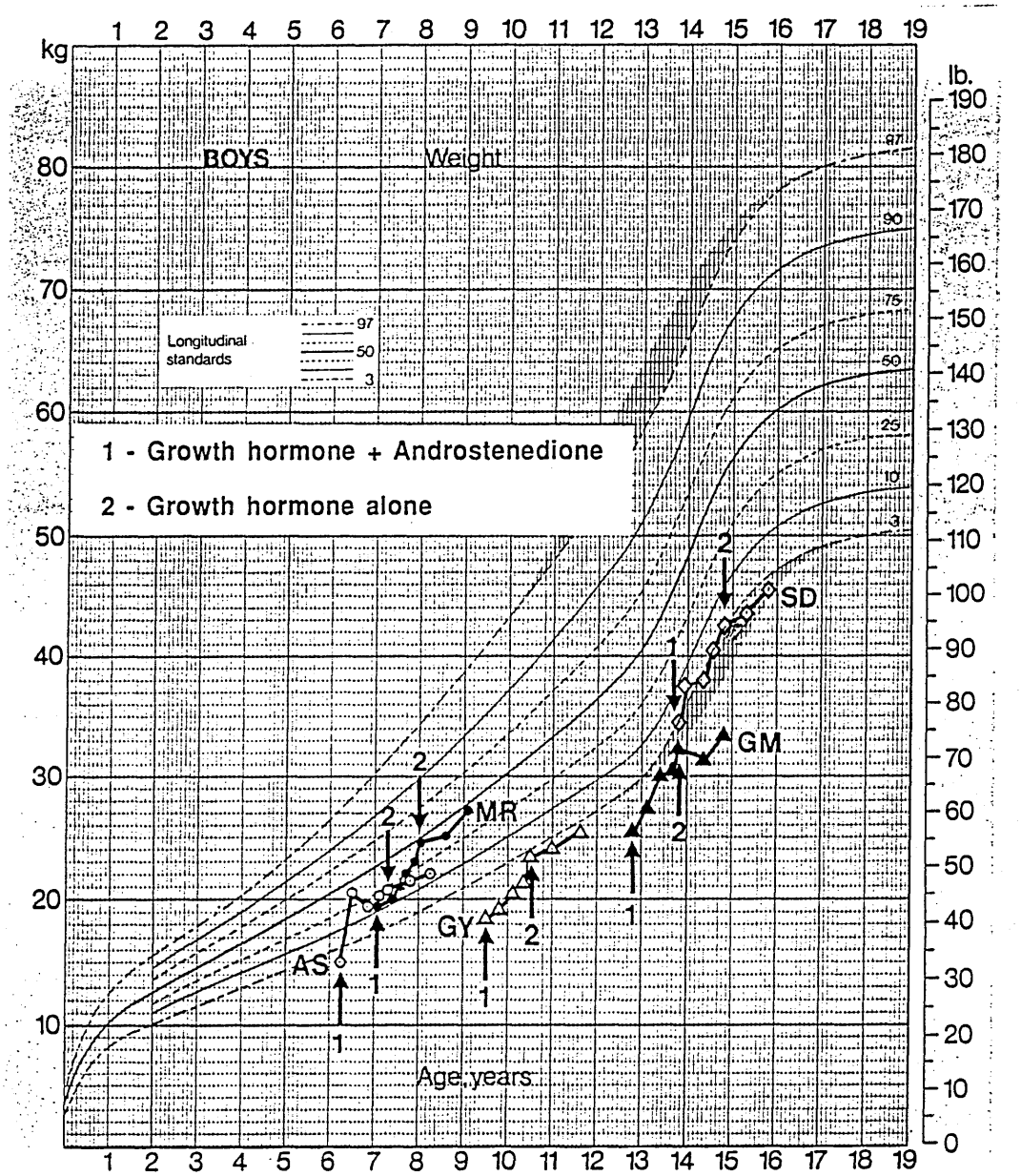


Figure 23: The actual weights (kg) and trends of the weights in Group 3 patients, shown at three monthly intervals during the year of combined treatment with growth hormone and AD and a further year after AD was stopped.

Table XVIII: The ratios of weight (kg) to height (m) in Group 3 patients basally, after one year treatment with growth hormone and AD and after one further year on growth hormone alone.

	Wt/Ht Ratios								
Pts. CA years	Basal	$\underline{50^{th}C}$* CA	During 1 yr on GH + AD 3mo.	6mo.	9mo.	12mo.	$\underline{50^{th}C}$* CA+1yr	1yr.GH alone	$\underline{50^{th}C}$* CA+2yr
1.AS 6.28	15.3	18.4	19.6	18.4	18.2	18.5	19.3	18.8	20.2
2.MR 7.03	18.9	19.2	18.0	19.5	19.6	20.8	19.8	22.3	20.8
3.GY 9.50	17.1	21.6	17.3	18.4	18.9	20.1	21.7	21.3	25.2
4.GM 12.80	21.5	27.3	22.1	23.8	23.7	25.0	30.1	25.0	32.5
5.SD 13.80	24.2	30.1	26.0	25.9	27.5	28.0	32.5	29.7	34.2

*The normal ratios of weight:height for the 50^{th} centile at that CA.

Pts.CA = Patients chronological ages. Wt = Weight.

Ht = Height. yr = year.

Table XIX: Percentages of the patients weight:height ratios compared with the 50th centile weight:height ratios for each age at the three periods of observations in Group 3 children.

	% of 50th centile Wt/Ht			% Incr.	% Incr.
Pts.CA(yrs)	Basal	Post lyr GH + AD	Post lyr GH alone	GH + AD	GH alone
Col.N^{O}	1	2	3	2-1	3-2
1.AS 6.28	83.1	96.3	93.3	13.2	-3.0
2.MR 7.03	98.7	104.6	107.1	5.9	2.5
3.GY 9.50	79.0	92.8	84.5	13.8	-8.3
4.GM 12.80	78.8	83.2	76.9	4.4	-6.3
5.SD 13.80	80.4	86.1	86.9	5.7	0.8
			Mean	8.6	-2.9

Pts.CA(yrs) =patients chronological ages (years).

Col.N^{O} = Column numbers.

% Incr. = Percentage increment in the Weight/Height ratios.

observation. It now becomes very clear that on withdrawal of AD Patients 1, 3 and 4 lost ground in terms of weight. Patient 2 (MR) did not keep up the earlier improvment from the combined treatment while Patient 5 (SD) entered puberty in the third phase (clinically) and therefore increased his own anabolic hormone. Androstenedione may therefore have a valuable role to play in the treatment of short stature in terms of weight and height achievement.

Perhaps too much is being claimed here in terms of linear growth for the combined growth hormone/AD treatment. It is well recognized that usually the greatest growth spurts are seen in the first year of growth hormone treatment. The question now is " Has the additional AD made any great improvment over growth hormone alone?". In Table XX are growth response data from five aged-matched children whose other anthropometric measurements approximated to the five patients of this survey. These control patients had been investigated and their endocrine status accepted by the MRC Growth Hormone Committee. Their treatment was cadaveric growth hormone 4 IU intramuscularly three times weekly.

The maximal seasonal increments achieved are as shown in the shaded areas and occurred mainly in the Spring but also in Autumn and Winter. Clearly larger series of patients would be required to prove the literature claims that maximual growth occurs in Spring. But more important, the actual and mean annual growth velocities during this first year of treatment compared very unfavourably with those on the combined growth hormone/AD treatment, (cf. Tables XX and XVI; page 140). The mean of the combined treatment group was 11.2 cm per year while for

Table XX : The anthropometric data of Group 4 patients (controls) over the first year of treatment with cadaveric growth hormone alone.

	Basal					After one year on GH alone							
	CA yrs	HA yrs	SDS	BA yrs	AGV	Inc HA*	SDS	Inc BA*	Calculated AGV cm/yr 3mo	6mo	9mo	12mo	AGV
Col.	1	2	3	4	5	6	7	8	9	10	11	12	13
Pts.													
1.RB	6.06	2.06	-4.65	2.9	2.4	1.4	-3.77	0.8	12.0^{4}	12.8^{1}	6.4^{2}	5.6^{3}	9.2
2.JC	7.38	4.34	-3.37	6.7	2.6	1.6	-2.40	1.3	18.0^{2}	10.4^{3}	8.8^{4}	4.0^{1}	10.3
3.JA	9.62	6.30	-3.01	8.7	2.9	0.8	-2.07	0.8	5.6^{3}	8.0^{4}	4.8^{1}	4.0^{2}	5.6
4.NM	12.20	6.88	-3.89	10.6	3.2	0.9	-3.27	0.6	8.4^{4}	6.0^{1}	2.4^{2}	2.0^{3}	4.7
5.BD	13.90	10.6	-2.45	10.3	3.0	1.5	-1.50	1.3	10.4^{1}	5.6^{2}	8.8^{3}	5.2^{4}	7.5
												Mean	7.5

Col. 8 mean = 0.96.

Seasons: Spring (1), Summer (2), Autumn (3) and Winter (4).

*The increment of height age and bone age (years) one year post treatment.

Col.= Column numbers. yrs = years.

CA = Chronological age. HA = Height age.

SDS = Standard deviation score for height. BA = Bone age.

AGV = The annual growth velocity (cm/year).

those treated with growth hormone alone, the mean was 7.5 cm per year. These findings again suggest that androstenedione may have a role in the treatment of growth hormone-deficient patients. However the data for the annual growth velocities for Groups 3 and 4 patients were statistically analysed by the Wilcoxon test ($p = 0.1$) and t-test ($0.2 > p > 0.1$). This indicates that more patients could be required for the treatment programme to be shown to be significant at the 5 per cent level.

It is necessary also to consider the changes in the weight: height ratios in these five control patients during their first year of treatment. Table XXI shows the changes in the ratios basally and at three monthly intervals during the first year of growth hormone treatment. Also are shown the percentages of the basal and final weight:height ratios when compared with the weight:height ratios of the 50^{th} centile for age. Probably the best way to compare these with the corresponding changes in the combined treatment group is to note the percentage change between basal ratios and that at the end of one year treatment (cf. Table XIX, Col. 2-1 and Table XXI, Col. 8 (7-2); page 142). It will immediately be seen that in the combined treatment group the mean improvement was 8.6 percent while in the control group there was only a 4.4 percent improvement, These data give considerable support to the claim that AD has a definite role to play in the treatment of children with growth hormone deficiency. However for every advantage there may be an accompanying disadvantage. If one now compares the advances in bone maturation of these two groups of patients (Table XV Col. 12 p. 126 cf. Table XX Col. 8; p. 140) it will be appreciated that during one year treatment with growth hormone alone the mean increase in bone maturation for the control

five boys was 0.96 years per chronological year while for those five boys on the combined growth hormone/AD treatment the corresponding value was 2.72 years per chronological treatment year. This disadvantage will be discussed later.

Table XXI : The ratios of weight (kg) to height (m) in Group 4 boys basally and after one year treatment with growth hormone alone.

	Basal	% $50^{th}C$*	Wt/Ht during 1 yr on GH				%$50^{th}C$*	% Incr
	Wt/Ht	Wt/Ht	3mo.	6mo.	9mo.	12mo.	Wt/Ht	col.7-2
Col. N^{O}	1	2	3	4	5	6	7	8
Pts.								
1.RB	15.3	88.6	15.4	16.8	16.3	17.1	91.9	3.3
2.JC	19.0	98.4	19.3	20.7	21.3	21.9	107.9	9.5
3.JA	17.2	80.0	17.5	16.8	17.0	19.1	83.4	3.4
4.NM	25.6	98.0	25.8	26.0	27.7	28.2	98.6	0.1
5.BD	24.7	81.3	25.7	27.6	27.7	28.9	86.8	5.5
							Mean	4.4

Col.N^{O} = Column numbers. Pts. = Patients.

Wt = Weight (kg). Ht = Height (m).

% Incr = Percentage increment.

* Percentage of actual weight:height ratios compared with the 50^{th} centile weight:height ratios for each age.

Analyses of the Urinary Androgen Metabolites.

Up till now we have been concerned with the plasma concentrations of AD and testosterone, their interconvertibility and their achieved concentrations by the administration of each separately. Equally important in this work is the nature of their metabolism for if the patients are to benefit from improved plasma concentrations of each or both, then there must be evidence of their metabolic products. Important to this consideration is that the weak androgen AD, was administered in the hope that the untoward side-effects of an equivalent "mass" of therapy with testosterone or other synthetic anabolic hormone would be avoided. Nonetheless it was hoped that the endogenous conversion of AD to testosterone would be essentially the controllable event from which would be seen good and improved linear growth following administration of AD. Thus to begin with the plasma concentrations of testosterone achieved and the direct urinary metabolic products will be considered.

Before doing so it must be restated which urinary metabolites shall be sought and the reason for their presence in the urine. Testosterone has one action through its conversion to 5α-dihydrotestosterone. This takes place via the hormone-receptor complex within the cell cytoplasm and the so-formed 5α-dihydrotestosterone stimulates RNA and DNA synthesis. By this action the 5α-dihydrotestosterone is reduced to 5α- and 5β-androstanediol which appear in the urine. This process is a particular feature of the testosterone-dependent tissues. Interestingly AD by conversion to androstanedione or to testosterone participates in this effect. The other function

of testosterone which is of interest here is its ability to increase IGF-I, but this stimulation at present seems to be linear only up to plasma testosterone concentrations of 6.9 nmol/L[149]. Higher plasma concentrations are associated with a plateauing of IGF-I. It can, therefore, be expected that when the studies involving prolonged AD administration are made the excretion rates of androsterone and aetiocholanolone will be important evidence to suggest possible IGF-I stimulation. In summary then I am studying the effect of testosterone on bone plate cells in so far as there are receptors for testosterone on the osteoblasts[150] and so 5α-dihydrotestosterone is a necessary by product of RNA and DNA synthesis at that level. Moreover testosterone acts probably by virtue of its Δ4-3oxo function and will stimulate both hepatic and local IGF-I and which in conjunction with growth hormone will accelerate linear growth.

Group 3 patients Chronic use of AD and the urinary metabolites.

Let us now apply this logic to the five patients who received growth hormone (4 IU thrice weekly) and AD (100 mg thrice weekly) as combined therapy for their isolated growth hormone deficiency. In Table XXII are presented the plasma concentrations of testosterone achieved at three-monthly intervals during the year of combined treatment along with the respective urinary metabolites (5α-androstane-3α,17β-diol: 5α-ASD; 5β-androstane-3α,-17β-diol: 5β-ASD and of adrenal origin Δ5-androstene-3β,17β--diol: Δ5-diol). It will be observed that on several occasions 5α-ASD and 5β-ASD could not be detected in the urine. These urine

Table XXII: Plasma concentrations of testosterone and the corresponding urinary metabolites in Group 3 patients.

			Pts.CA (yrs)	1.AS 6.28	2.MR 7.03	3.GY 9.5	4.GM 12.8	5.SD 13.8
nmol/L	Plasma T	Time (months)	0	0.23 *@	0.31	0.33 *	0.20 *@	0.64
			3	13.50	0.68	1.05	0.79	0.64
			6	0.35 *@	0.36	0.36	0.37	0.81
			9	8.60	0.46	2.40	0.71	0.51
			12	0.36	0.30 *	0.37	0.37	0.72
µg/100 mg urinary creatinine	Urinary 5α-ASD	Time (months)	0	ND	1.0	ND	ND	0.1
			3	24.0	197.0	2.0	10.0	0.3
			6	ND	0.2	0.2	0.1	1.0
			9	2.0	1.0	20.0	0.2	0.1
			12	0.2	ND	0.1	0.1	0.1
	Urinary 5β-ASD	Time (months)	0	ND	1.0	0.1	ND	0.3
			3	48.0	138.0	1.0	11.0	0.2
			6	ND	1.0	0.2	0.1	2.0
			9	3.0	4.0	43.0	0.3	0.1
			12	1.0	0.1	0.1	0.2	0.1
	Urinary Δ5-diol	Time (months)	0	ND	ND	ND	ND	0.1
			3	ND	ND	ND	ND	1.0
			6	ND	ND	0.1	0.1	ND
			9	8.0	ND	ND	0.2	ND
			12	ND	ND	0.1	0.1	ND

Pts.CA (yrs) = Patients chronological ages (years).

5α-ASD = 5α-androstane-3α,17β-diol; 5β-ASD = 5β-androstane-3α,17β-diol; Δ5-diol = Δ5-androstene-3β,17β-diol. ND = Not detected.

Periods of non-detections, (*) for 5α-ASD and (@) for 5β-ASD.

samples had been taken at the same time as the plasma and so represented on average a five-hour collection up to the time of sampling. It will be noticed further that when either metabolite was not detected the plasma testosterone concentrations were less than unity. Indeed the mean concentrations of the plasma testosterone for these occasions of non-detection of urinary 5ε-ASD was 0.28 nmol/L for 5α-ASD and 0.26 nmol/L for 5β-ASD (see Table XXII). Thus it can be concluded from these results that at plasma concentrations of testosterone between 0.2 and 0.35 nmol/L the concentration within the cell to facilitate conversion to 5α-dihydrotestosterone is inadequate and therefore RNA and DNA synthesis dose not take place.

The compound Δ5-androstene-3β,17β-diol did not significantly change in any subject throughout the test period. Thus it can be stated that plasma testosterone does not stimulate the adrenals to secret this substance for it is of adrenal origin. Alternatively part of what may have been secreted may have been metabolized to testosterone and hence contributed to both the plasma concentrations of testosterone and to the urinary 5α-ASD and 5β-ASD. A further point mentioned but to be considered later is that the shaded areas in Table XXII are the periods of maximum growth velocity (calculated as cm per year-see Table XVI p. 128). (In all patients the periods of maximum linear growth corresponded to the highest plasma testosterone concentrations and the highest output of these urinary metabolites. The conclusion would therefore be that at the periods of greatest linear growth there is the greatest activity of RNA and DNA synthesis).

The question now is, "Is the increased linear growth due entirely to these events?". Let us now consider the other

aspect of plasma testosterone, that of increasing circulating IGF-I. It has been pointed out that an increasing plasma testosterone concentration increases the circulating IGF-I concentration up to maximum of 6.9 nmol/L testosterone. Therefore the IGF-I plasma concentration forms a plateau. It will be seen that during the year of treatment with oral AD (100 mg thrice weekly) the plasma testosterone concentrations increased above basal even although they did not reach normal concentrations (Figure 17 p. 121). Nonetheless the data from the literature[149] indicate that even as the plasma concentrations of testosterone are increasing, so is the circulating concentration of IGF-I. By this mechanism therefore are the good effects of this combined treatment first, to raise indirectly through plasma AD, the concentrations of plasma testosterone which in turn increase the synthesis of RNA and DNA and the circulating concentrations of IGF-I. The administered growth hormone has, therefore, the stage set for its own maximum action.

We now turn to Table XXIII where the data concerning AD and its urinary metabolites are shown. Regrettably I was not able to estimate urinary AD (see Discussion Chapter). In a sense our interests lie in its conversion to testosterone. If this conversion could have been influenced we might have attempted to normalize plasma testosterone concentrations. The risk here however would have been to advance further osseous maturation as we have noted from Table XV. Thus the patients' own converting ability was the rate-limiting factor. The AD had therefore completed its task and was therefore to be cleared by metabolism and excretion of metabolites. Androsterone, aetiochanolone and epiandrosterone are the recoginized urinary metabolites of AD. It

Table XXIII: Plasma concentrations of AD and the corresponding urinary metabolites in Group 3 patients.

Pts.CA (yrs)			1.AS 6.28	2.MR 7.03	3.GY 9.5	4.GM 12.8	5.SD 13.8
nmol/L	Plasma AD	Time (months) 0	1.10	1.50	1.60	0.50	1.40
		3	34.90	2.13	4.54	1.78	3.77
		6	2.10	1.96	2.37	0.98	4.15
		9	34.60	2.50	11.00	1.68	2.52
		12	2.27	1.60	2.16	1.50	3.50
µg/100 mg urinary creatinine	Urinary A	Time (months) 0	3.0	4.0	1.0	2.0	4.0
		3	8471.0	594.0	4.0	120.0	6.0
		6	2.0	7.0	4.0	8.0	205.0
		9	675.0	7.0	976.0	11.0	4.0
		12	7.0	3.0	0.2	3.0	3.0
	Urinary AE	Time (months) 0	3.0	5.0	1.0	3.0	5.0
		3	5170.0	566.0	6.0	174.0	4.0
		6	3.0	7.0	3.0	8.0	230.0
		9	455.0	10.0	1592.0	12.0	4.0
		12	9.0	7.0	0.3	4.0	2.0
	Urinary Epi.A	Time (months) 0	1.0	1.0	0.2	1.0	1.0
		3	639.0	63.0	1.0	29.0	1.0
		6	12.0	1.0	1.0	1.0	163.0
		9	31.0	8.0	11.0	5.0	0.4
		12	6.0	0.4	0.2	1.0	1.0

Pts.CA (yrs) = Patients chronological ages (years).

A = Androsterone . AE = Aetiocholanolone.

Epi.A = Epiandrosterone.

will be seen that over the year of treatment the mass of urinary metabolites excreted were in direct relationship to the plasma concentrations of AD so that there was no cumulative effects from the thrice weekly administration of AD (100 mg thrice weekly orally). The same can be said for plasma testosterone. It is of interest to note from Table XXIII that seasonal linear growth was greatest corresponding to the peaks of plasma AD. Interestingly in deer[151] peaks of plasma AD correspond to the induction of mating potential and to antler growth.

Plasma Testosterone to AD and their urinary metabolites in Group 2 boys.

We will now consider the effects on the system which we are studying of testosterone (Sustanon) 100 mg intramuscularly given as a single dose. Note in Table XXIV that the plasma testosterone concentrations achieved on the third day after injection reached supraphysiological concentrations. This phenomenon has already been reported and even after one month plasma concentrations still may be above normal concentrations[33]. However note that the third day values for urinary 5α- and 5β-androstane-3α,17β--diols are low and correspond in this respect with those quoted by Chapman[152] (Protocols 24, 29, 35, 45, 47, 50, 52, 57, 64, 73, 74 and 79). These latter patients were also short statured but not growth hormone deficient. If the postulate of this present thesis is accepted namely that these 5ε-metabolites represent the end products the testosterone ⟶ 5α-dihydrotestosterone pathway then it can be concluded that there is probably a rate-limiting phenomenon for the utilization of free testosterone characteristic

Table XXIV : Plasma testosterone concentrations and the corresponding urinary metabolites in Group 2 boys given Sustanon (100 mg) I.M.

	nmol/L		µg/100 mg urinary creatinine					
	Plasma T		Ur. 5α-ASD		Ur. 5β-ASD		Ur. Δ5-diol	
Time (days)	0	3rd	0	3rd	0	3rd	0	3rd
Pts.CA (yrs)								
1.SK 10.67	0.35	80.00	ND	1.0	0.3	4.0	ND	1.0
2.DO 11.65	0.35	58.00	ND	8.0	ND	10.0	ND	ND
3.AM 11.66	0.37	20.50	0.3	3.0	0.4	1.0	ND	ND
4.MA 12.31	0.59	66.00	2.0	3.0	ND	3.0	ND	1.0
5.NM 12.59	2.08	90.00	ND	11.0	0.2	9.0	ND	ND
6.SM 12.64	0.49	44.00	ND	ND	33.0	51.0	ND	ND
7.JP 12.74	0.41	52.00	1.0	17.0	ND	11.0	ND	6.0
8.DK 13.53	0.35	20.45	3.0	14.0	4.0	15.0	2.0	3.0
9.SS 14.16	0.73	48.00	ND	2.0	ND	3.0	ND	ND
10.AD 14.77	4.00	33.00	ND	2.0	ND	2.0	ND	ND
11.SC 15.30	2.86	68.00	ND	6.0	2.0	9.0	ND	ND

5α-ASD = 5α-androstane-3α, 17β-diol.

5β-ASD = 5β-androstane-3α, 17β-diol.

Δ5-diol = Δ5-androstene-3β, 17β-diol.

Pts.CA (yrs) = Patients chronological ages (years).

Ur. = Urinary. ND = Not detected.

of children with short stature. This phenomenon involves the testosterone receptor protein and the intracellular conversion to 5α-dihydrotestosterone before the action of the nuclear material converts 5α-DHT to the 5α- and 5β-androstane-3α,17β--diols. It might be attractive to consider this primarily as a receptor protein phenomenon. Since it is also generally accepted that synthetic anabolic hormones in non-growth hormone deficient states can increase linear growth, this then is probably effected by increasing plasma concentrations of IGF-I so that normally present growth hormone will combine to give increased linear growth although osseous maturation advances disproportionately faster. The two modes of action of anabolic hormones on linear growth, RNA and DNA synthesis and osseous maturation are difficult to separate in research work of this nature.

In Table XXV are the plasma AD concentrations obtained on the third day following Sustanon injection. It will be seen that normal or slightly above normal concentrations (Patients 1 and 9) are achieved by all patients. Patient 10 (AD) had an abnormally high plasma concentration. Nonetheless all patients showed a vastly increased excretion of androsterone, aetiocholanolone and epiandrosterone on the third day over the basal excretion rates. This supports the thesis that the small part played by plasma AD to maintain plasma testosterone levels, is short-lived and thereafter the plasma AD is rapidly cleared from the system. It will be recalled that Patient N° 10 (AD) had the highest percentage conversion of plasma testosterone to AD (p.117). It was there suggested that he might have had a deficiency in clearing the AD from the system. This could now be borne out for his increases over basal urinary excretion rates of 5ξ-metabolites are

Table XXV : Plasma AD concentrations and the corresponding urinary metablites in Group 2 patients given Sustanon (100 mg) I.M.

	nmol/L		µg/100 mg ur. creatinine					
	Plasma AD		Ur.A		Ur.AE		Ur.Epi.A	
Time (days)	0	3rd	0	3rd	0	3rd	0	3rd
Pts.CA(yrs)								
1.SK 10.67	1.78	9.00	2	108	13	128	8	51
2.DO 11.65	1.68	8.00	15	373	17	289	11	29
3.AM 11.66	1.40	4.50	15	51	17	72	18	34
4.MA 12.31	1.80	6.60	20	92	26	67	13	36
5.NM 12.59	1.20	8.40	4	106	3	227	4	18
6.SM 12.64	1.75	7.86	60	245	90	209	42	80
7.JP 12.74	1.40	5.30	16	215	16	133	29	73
8.DK 13.53	1.10	3.00	37	130	32	129	52	125
9.SS 14.16	3.35	9.40	18	79	15	64	12	70
10.AD 14.77	4.89	13.27	34	85	36	84	15	39
11.SC 15.30	3.40	7.70	21	111	40	200	26	80

A = Androsterone. AE = Aetiocholanolone.

Epi.A = Epiandrosterone. Ur.= Urinary.

Pts.CA(yrs) = Patients chronological ages (years).

in the region of only two fold while most others have increased urinary execretion rates more than three fold over basal values.

Urinary excretion of testosterone and AD metabolites in Group 1 boys.

The data in Table XXVI are those of the eleven boys on whom the acute studies with oral AD were performed. Allusion has already been made to the relationships between the plasma concentrations of the two related compounds. The considerable interest in the 5ε-metabolites of testosterone is that all patients except Patient 11 (GC) had low plasma concentrations of testosterone, in keeping with the respective basal urinary excretion values. Thus it might be concluded that the systemic effects of the plasma testosterone concentrations achieved are complete by the fifth hour post-ingestion of AD. However since the 5ε-androstane-3α,17β-diols in the urine are in low concentrations it might be thereby concluded that much of the plasma testosterone in Patients 4-11 has been bound to non-androgen dependent tissues, (see page 111 bottom data). In Patient 9 (SC) whose sampling carried on to the seventh hour, there was a slight intermediate increase (probably due to ingestion of food) before its falling again to basal excretory values. Thus it might be concluded that any testosterone derived from orally ingested AD has a short period of systemic activity being complete with reference to its urinary metabolites, by the fifth to seventh hour.

A study of Table XXVII shows again that the plasma AD is

steadily and increasingly metabolized for excretion, the values for urinary androsterone, aetiocholanolone and epiandrosterone continuing high through to the seventh hour and probably beyond. Thus it is concluded that the period of efficacy of the orally ingested AD as mediated through plasma testosterone, is probably not greater than seven hours. Interestingly if the AD is given concurrently with the injected growth hormone in the treatment Group (3), there is maximum synergism, both hormones having a similar period of maximum activity.

In summary it is argued from the study of the urinary metabolites of the plasma AD and testosterone that there is a plasma testosterone concentration above which there is no evidence that it is being utilized as far as the testosterone ⟶ 5α-dihydrotestosterone ⟶ 5ε-androstane-3α,17β-diols are concerned (? receptor-binding). If this argument is accepted then the correctness of raising plasma testosterone concentrations above normal and into supranormal levels must be questioned. At any rate the urinary excretion rates of the 5ε-androstanediols do indicate that the action of oral AD (100 mg) is short lived and even although plasma concentrations of testosterone may be grossly elevated by Sustanon no benefit is derived if these 5ε-ASD excretions values noted in Group 2 boys are significant.

Table XXVI: The plasma concentrations of testosterone and the corresponding urinary metabolites in Group 1 patients.

Pts.CA			1.SS 9.25	2.AG 10.73	3.SC 11.4	4.SW 12.18	5.SD 12.98
Plasma T (nmol/L)	Time (hours)	0	0.35	0.41	0.35	0.57	0.42
		1	4.20	6.67	4.10	4.90	7.00
		2	13.70	9.80	10.70	10.10	6.98
		3	21.50	20.00	20.50	10.50	7.70
		4	20.50	22.90	19.20	7.80	9.50
		5	13.80	20.70	11.20	4.20	8.60
		6					
		7					
Urinary 5α-ASD (μg/hr)	Time (hours)	0	ND	0.2	0.2	ND	ND
		1	1.0	1.0	1.0	0.2	3.0
		2	1.0	2.0	2.0	1.0	6.0
		3	2.0	3.0	2.0	2.0	4.0
		4	3.0	2.0	3.0	0.2	3.0
		5	1.0	1.0	2.0	1.0	2.0
		6					
		7					
Urinary 5β-ASD (μg/hr)	Time (hours)	0	ND	ND	ND	ND	ND
		1	1.0	1.0	0.2	0.1	3.0
		2	1.0	3.0	3.0	1.0	5.0
		3	2.0	4.0	3.0	1.0	5.0
		4	3.0	3.0	4.0	0.1	4.0
		5	2.0	2.0	4.0	0.1	3.0
		6					
		7					

Table XXVI: contd.

6.TW 13.0	7.JK 13.14	8.PM 13.96	9.SC 15.3	10.DC 15.66	11.GC 15.83
0.38	3.34	0.64		5.10	16.50
5.68	7.10	5.10		13.70	20.05
5.62	5.30	8.90		16.80	22.50
7.20	8.80	6.50		13.40	19.20
4.10	6.40	6.60	10.30	10.60	16.20
3.57	13.40	10.65	6.65	10.00	13.05
			4.30		
			3.56		
ND	ND	ND		ND	12.0
1.0	1.0	I.0		1.0	10.0
4.0	4.0	1.0		7.0	23.0
5.0	3.0	1.0		7.0	17.0
3.0	4.0	1.0	12.0	4.0	13.0
1.0	3.0	1.0	4.0	4.0	12.0
			4.0		
			1.0		
ND	ND	2.0		ND	10.0
1.0	2.0	5.0		1.0	7.0
5.0	3.0	4.0		7.0	18.0
7.0	2.0	6.0		7.0	14.0
6.0	3.0	4.0	21.0	4.0	15.0
2.0	2.0	5.0	5.0	4.0	13.0
			8.0		
			6.0		

continued.....

Table XXVI : contd.

Urinary Δ5-diol (µg/h) Time (hours)		Pts.CA 1.SS 9.25	2.AG 10.73	3.SC 11.4	4.SW 12.18	5.SD 12.98
	0	ND	ND	0.1	ND	ND
	1	ND	0.6	1.0	ND	1.2
	2	ND	0.7	2.4	ND	2.2
	3	ND	1.4	2.1	ND	0.2
	4	ND	0.9	1.6	ND	0.4
	5	ND	0.5	1.2	ND	2.3
	6					
	7					

5α-ASD = 5α-androstane-3α, 17β-diol.

5β-ASD = 5β-androstane-3α, 17β-diol.

Δ5-diol = Δ5-androstene-3β, 17β-diol.

Pts.CA = Patients chronological age (years).

Table XXVI: contd.

6.TW 13.0	7.JK 13.14	8.PM 13.96	9.SC 15.3	10.DC 15.66	11.GC 15.83
ND	ND	ND		ND	ND
0.5	ND	3.6		0.9	ND
2.3	ND	1.1		2.3	8.7
3.4	ND	1.0		3.4	1.5
4.1	ND	1.0	2.9	ND	ND
1.6	ND	2.2	ND	ND	ND
			ND		
			ND		

Table XXVII: The plasma cocentrations of AD and the corresponding urinary metabolites in Group 1 boys received AD (100 mg) orally.

	Pts.CA	1.SS 9.25	2.AG 10.73	3.SC 11.4	4.SW 12.18	5.SD 12.98
Plasma AD (nmol/L) Time (hours)	0	0.43	1.30	1.50	0.15	0.14
	1	5.60	8.00	9.00	3.46	5.67
	2	23.70	11.50	18.00	4.89	5.52
	3	34.90	31.40	28.60	4.54	4.71
	4	27.20	27.60	23.70	2.58	3.14
	5	20.90	24.90	11.90	1.15	5.24
	6					
	7					
Urinary A (µg/hr) Time (hours)	0	16	22	16	10	18
	1	184	I88	49	21	144
	2	447	508	254	59	448
	3	349	789	476	160	105
	4	572	222	908	18	472
	5	502	180	329	16	485
	6					
	7					
Urinary AE (µg/hr) Time (hours)	0	12	17	9	7	16
	1	112	142	34	11	144
	2	301	273	181	57	406
	3	277	416	257	155	81
	4	361	166	507	7	380
	5	270	134	226	7	412
	6					
	7					

Table XXVII: contd.

6.TW 13.0	7.JK 13.14	8.PM 13.96	9.SC 15.3	10.DC 15.66	11.GC 15.83
0.27	0.16	0.41		0.17	0.36
4.40	4.71	4.36		5.71	2.02
4.85	3.84	5.76		7.96	7.96
3.74	2.07	3.60		5.34	4.64
1.57	1.22	3.11	9.10	3.11	2.44
1.29	6.98	19.20	8.40	2.55	1.90
			6.80		
			6.30		
33	25	23		28	192
90	305	228		197	201
403	542	300		997	812
362	563	463		639	370
246	344	244	898	556	324
120	530	308	547	452	300
			527		
			280		
29	30	33		28	55
72	143	250		130	73
344	173	317		665	781
302	168	342		628	315
173	176	219	972	276	268
76	237	495	710	254	260
			704		
			297		

continued.....

Table XXVII : contd.

Urinary Epi.A (ug/hr)	Time (hours)	Pts.CA	1.SS 9.25	2.AG 10.73	3.SC 11.4	4.SW 12.18	5.SD 12.98
		0	1	3	3	3	7
		1	10	18	12	4	9
		2	30	50	93	5	29
		3	43	78	106	11	9
		4	72	39	152	3	29
		5	40	20	91	6	31
		6					
		7					

A = androsterone.

AE = aetiocholanolone.

Epi.A = epiandrosterone.

ND = Not detected.

Pts.CA = Patients chronological age (years).

Table XXVII: contd.

6.TW 13.0	7.JK 13.14	8.PM 13.96	9.SC 15.3	10.DC 15.66	11.GC 15.83
4	8	2		7	24
14	67	4		30	50
32	87	36		82	68
33	148	68		46	49
14	113	7	229	25	45
5	128	88	57	25	40
			68		
			46		

CHAPTER FOUR

DISCUSSION

Introduction

The data presented here cannot be confirmed, corroborated nor compared with similar in the literature for such does not exist as far as my search is concerned. Chapman in his Ph.D, thesis has studied the effect of human chorionic gonadotrophin on the plasma concentrations of testosterone (presumably elaborated by the testes) and its urinary metabolites. I have used his data but there is no available information on plasma testosterone concentrations consequent on oral administration of free androstenedione (AD) nor on their respective metabolites. Thus what is applied here is a reasonable, logical thesis based on clinical, biochemical and physiological principles. Before embarking on this discussion there are some points in the thesis which perhaps require explanation or amplification.

First reference is made to the biochemical and technological steps required for the separation and quantitation of the urinary androgen metabolites. The standard procedure for hydrolysis of urinary steroid conjugates proved trouble free. The enzyme β-glucuronidase contained 25,000 sulphatase units per gram compared with 1.47×10^6 β-glucuronidase units per gram. Most of the urinary metabolites were considered as glucuronides although it is conceivable that sulphates and perhaps disulphates of steroid metabolites were present. It is likely that this is the reason why the 5α-dihydrotestosterones were not detected. Free AD might well have been a urinary product but a corresponding peak was not detected in the g.l.c tracing. Some workers[153] have said that AD may be detected by g.l.c without derivative

formation. There is however an RIA method for estimating urinary AD[154]. AD is a diketone and therefore apart from the possible formation of two isomers from the Δ4-3-oxo structure of Ring A, the best chance of the g.l.c detection would be by oximation followed by halidization with say heptafluorobutyric anhydride. This method proved entirely unsuccessful similar to others[155] in detecting urinary AD by g.l.c. However an "unknown" peak was observed in the g.l.c tracing of urinary extracts which was not present in the tracing of a mixture of standards. A quantity of urinary extract was saved and subjected to GC-MS and regrettably this peak had the MS configuration of a phthalic acid diester. (I am indebted to Dr. Robert Anderson of the Department of Forensic Medicine and Science of Glasgow University for his technical help and to Prof. Charles J. W. Brooks for his helpful discussion on the subject). Interestingly this problem has already been encountered [156]. The phthalate is most likely to have come from the plastic urine containers.

It is a great pity that urinary androstenedione was not estimated since along with the plasma concentrations, it would have been possible to calculate a clearance rate and tubular reabsorption rate and from these possibly to determine whether or not the kidney metabolized AD. This is very likely.

Secondly there is a paucity of published work on the daily urinary excretion of the metabolites of 5α-dihydrotestosterone i.e. of the 5ξ-androstane-3α, 17β-diols in children. The data for adults are few but a figure of 133 ± 25 μg per day for 5α-androstane-3α,17β-diol is quoted [157] and 50 μg per day as the lower limit for 5β-androstane-3α, 17β-diol is recorded [158]. Chapman [159] whose work in the DCH Laboratories has not been equalled,

stated that for children the daily urinary excretion rate for both these diols is greater than 50 μg per day. The point of interest in this work is that the excretion rate of 5α-androstane-3α,-17β-diol represents extrahepatic metabolism of testosterone through 5α-dihydrotestosterone and therefore reflects the androgenicity of testosterone [157]. This is the important point here since testosterone to 5α-dihydrotestosterone and hence to 5α-androstane-3α, 17β-diol reflects osteoblastic activity. Indeed it has been shown [160] from well designed experiments using rabbit articular cartilage and growth plate cartilage that testosterone is converted by these tissues to androstenedione, dihydro- testosterone and the 5ε-androstanediols. This conversion was greater in articular cartilage than in growth plate cartilage. Also in this cartilage at almost end-point incubation the concentration of testosterone and 5α-dihydrotestosterone were similar. But interestingly the resultant AD and the 5ε-androstanediols (99 per cent of both) were found in the incubation effluent, which suggested complete usage of the 5α-dihydrotestosterone, its being metabolized fully and excreted by the articular cartilage cells. This may to some extent explain the rapid increase in osseous maturation observed in this study.

Thirdly a comment on the use of anabolic hormones is necessary. The general advantages and disadvantages of these compounds are well known in the endocrine field. Of considerable interest is the fact that the achievements claimed for them in terms of clinical advantage is not in the literature supported with plasma concentrations. Attempts to estimate the free steroid in plasma even by HPLC have failed and RIA methods have been frustrated by lack of an antibody to the specific anabolic hormone

used. The Olympic Committee [161] in their testing of athletes use a GC-MS system not usually freely available in clinical practice. Further, the urinary excretion of these synthetic anabolic hormones is slow hence accummulation takes place presumably in the liver. There is some comparatively recent attempts to estimate urinary levels of these substances in low nanogram amounts [162] by isotope dilution-mass fragmentation and by an integrated approach to GC-MS analysis [163].

Fourthly it is now necessary to consider AD in its anabolic role and with particular reference to its osteoblastic activity. This action it shares with the synthetic anabolic steroids to which I now refer. Stanozolol (17β-hydroxy-17α-methyl-androstane (3,2-C) pyrazole) has been shown to have osteoblastic activity on human bone cells and it has largely been used to prevent bone loss in postmenopausal osteoporotic subjects. It increases total body calcium and circulating alkaline phosphatase in long-term use and these together with its osteoblastic cell proliferative action makes its use in linear growth disorders undesirable in so far as the above biochemical and mitogenic actions will advance osseous maturation [164]. Interestingly stanozolol has been reported to lower the concentrations of plasma testosterone in normal males [165] and yet to increase RNA concentrations [166] as well as to increase nitrogen retention [167]. However it is not at all clear from the literature what is its fine mode of action by which the effects on bone are obtained whether or not it is through an intracellualr dihydrotestosterone-receptor protein mechanism and final cellular RNA and DNA synthesis. In none of the published work are effective plasma levels recorded. An extensive literature on 19-nortestosterone, its esters, 2-hydroxymethylene-

-17β-hydroxy-17α-methyl-5α-androstane-3-one and others (pharmacologically methyltestosterone, methandrostenolone, noreth-androlone, oxymetholone and stanozolol) now exist but in none of the clinically oriented published work is reference made to either the effective plasma concentrations at which wanted and unwanted effects were obtained nor to their intimate modes of action despite an excellent paper by Bertrand and his colleagues from Lyon and Canada (168).

As far as androstenedione is concerned there is an abundant literature on its interconversion with testosterone by the enzyme 17β-oxidoreductase as shown schematically in Figure 24.

Testosterone is not an adrenal-produced androgen. Largely in the prepubertal period it is elaborated by hepatic conversion of AD. In the testes, testosterone can be elaborated from acetate in which pathway AD is an intermediary. Also as early as at six to eight fetal weeks the testes can convert both AD and dehydroepian-drosterone to testosterone (169). Testosterone is the most potent androgen in terms of nitrogen retention (anabolic) and as a stimulator of sexual development (androgenic and mitogenic). While AD has also these potentials its anabolic potency varies with the mode of application. For example if applied locally say to the Capon's Comb it has an androgenic potency 80 per cent that of testosterone. But if applied indirectly (systemically) the androgenic potency falls to 12 per cent that of testosterone (94). Clearly the explanation of this phenomenon is that following absorption, degradation of the free hormone commences immediately and so less is avaliable for systemic action. This is an important factor in our decision to use AD systemically for the end result is attenuated by metabolic degradation as well as

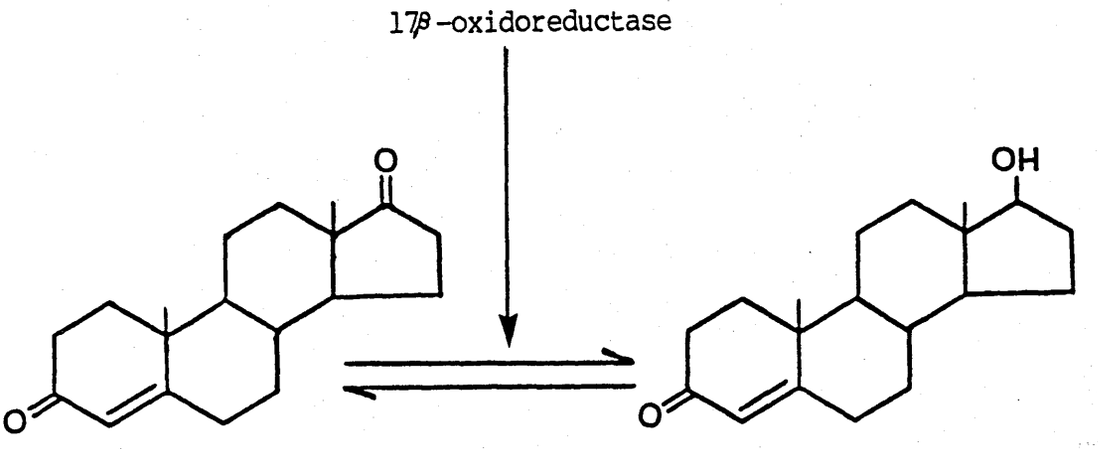


Figure 24: Digramatic representation of androstenedione and testosterone interconversion relationship.

conversion to testosterone. Whether or not the greater potency of the so-formed testosterone more than compensates for the AD potency loss, will be seen when the total effects of the therapeutic experiments are analysed.

In terms of the anabolic (nitrogen retention) efficiency of AD relative to testosterone in a scale of potency 1 to 5 with testosterone being 1, AD ranks 3 presumably also because of concomitant conversion to testosterone and degradation to metabolites for excretion. The so-formed testosterone is presumably therefore, likely to be the mode of action for the major part of the anabolic effects of AD. This anabolic effect will be discussed later in relation to the androgenic effects.

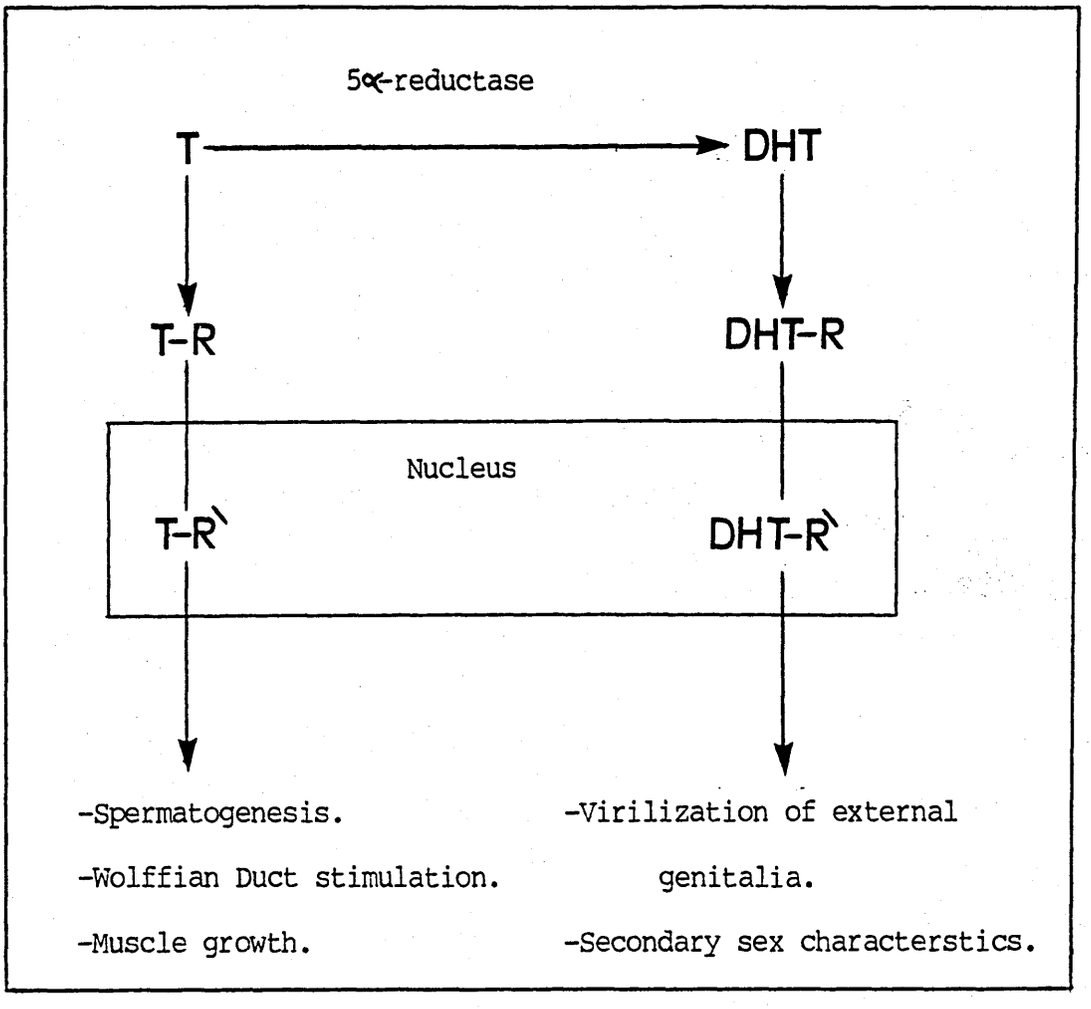
As far as absorption of AD from the gut is concerned, reliable published data has not been found. I can therefore only argue that following a bolus dose of AD significantly high plasma levels of both AD and testosterone were recorded and therefore the absorption from the gut of AD and its conversion to testosterone are highly efficient mechanism. In this I agree with Horton and Tait [107] although the substance of that paper I regarded as highly suspect because of the paucity of specificity in their data and faulty logic.

It is of interest that there is a circadian rhythm for plasma AD. From integrated plasma AD studies there are peak plasma concentrations at 0530h and 0930h with troughs between 1900h and 0230h[99]. Also there are plasma peaks associated with lunch and dinner due to endogenous production. This phenomenon was observed in several of the patients in Group 1 who had snacks or lunches during the five hours of acute experiments. The higher plasma concentrations in these patients could be interpreted as food

facilitating AD absorption or an endogenous secretion stimulated by food complementing the high plasma concentrations due to gut absorption. Note here that in this work the free hormone is being used and not an ester. The AD is therefore subjected immediately to all the body mechanisms unprotected by esterification.

Fifthly we come now to a brief consideration of the role of testosterone in the human organism as far as this thesis is concerned. It is generally understood that testosterone is the most powerful anabolic substance and in this respect linear growth, organ growth and muscle development are important. We have seen in this work how that the small incremental increases in plasma testosterone following AD administration correlated with both linear growth (as well as growth hormone) and with an improvement in body proportions (weight/height). This latter must have been the result of protein accummulation from aminoacids as well as an improvement in linear growth. But to be active at cellular level we know that there must be testosterone receptors at its site of action. The well recognized receptor on the androgen-dependent tissues has been much studied and while deficiency receptor states have been described for example in the testicular feminization syndrome, problems relative to total utilization of free plasma testosterone are still not fully understood. The mechanism of testosterone action at cell level has been well summarized [170] as shown (modified) in Figure 25.

The concept of two receptors, one in the cell membrane taking the testosterone into the cell cytoplasm for conversion by 5α-reductase into a dihydrotestosterone-protein complex, and one at nuclear level to facilitate the action of dihydrotestosterone on the nucleus (RNA and DNA replication) has a certain credence.



T = testosterone. DHT = dihydrotestosterone. R = receptor.

Figure 25: The mechanisim of testosterone action at cell level and the resultant effects[170].

It is interesting to speculate on the possiblity of there being a clinical condition associated with an excess of receptors and were this so maybe such a condition as Leydig cell hyperplasia (non LH-dependent) could be explained. Similarly androgen dependent tumours eg. prostatic cancer and breast cancer may also be rationalized. As will be seen from Figure 25 muscle growth is included in this testosterone-dihydrotestosterone mechanism although the general action of the Δ4-3-oxo function must also play a part. This type of action could be the protein synthesizing mechanism either locally at muscle level or centrally at hepatic level with the subsequent distribution of protein via plasma proteins to muscle. It is known that the body at all costs attempts to maintain normal plasma protein concentrations and that muscle protein is not a static accummulation of protein. There is a continuous withdrawal and replenishing of muscle protein to satisfy the liver's constant activity of transamination, deamination and reamination of aminoacids for protein synthesis.

Recent published work suggests that testosterone whether acting directly through the liver or at appropriate sites does raise the plasma IGF-I concentrations[149] and thus in conjunction with growth hormone linear growth is stimulated. In summary then the interest in this work relative to the action of androstenedione derived-testosterone is firstly its 5α-dihydrotestosterone mechanism and its mitogenic activity at the cartilage growth plate, then its action on the osteoblasts in the general bone substance (probably incorporating a calcium depositing mechanism), then in its action on the myoblasts incorporating thymidine, an action not shared by androstenedione nor dihydrotestosterone [56] and finally its potential to increase

plasma concentration of IGF-I.

Finally and important to this work are the effects or lack of these above effects when testosterone circulating concentrations are reduced. The kinetics of testosterone transport across the cell and nuclear membranes are different in that the nuclear membrane transport appears to be a limited process compared to membrane transport into the cell cytoplasm. The activity of a variety of cellular and nuclear biosynthetic processes to be induced would be limited by the restriction of transport. The quantitative conversion of testosterone to dihydrotestosterone which takes place in the cytoplasm does not reduce transport and very likely only affects the ratio of testosterone and dihydrotestosterone transferred into the nucleus. Most important here is that small changes in the cytoplasmic concentration of dihydrotestosterone within the range 0-10 nM will produce much greater changes in the nuclear concentration of dihydrotestosterone spanning the range 0-120 nM with an upper limit of 250 nM in the absence of testosterone. When plasma testosterone concentrations are subphysiological the cytoplasmic concentration of dihydrotestosterone greater than 1-2 nM cannot be maintained. Thus the nuclear concentration of dihydrotestosterone will fall to a level which cannot maintain full cell differentiation. Thus in all our subjects whose basal plasma testosterone concentrations were below the lower range of normality for their ages, it is easy to understand both their reduced height and poor physical development.

But the effects of low plasma testosterone concentrations may be even far more far reaching as will be appreciated from further analyses of the data obtained during the clinical "trials".

Analysis and Discussion of Data

Group 1 Patients who received oral androstenedione.

The plasma concentrations of testosterone and AD basally and over the 5h test period are given in Table IX. The younger patients under 11.4 years were found to have plasma comcentrations of AD higher than that of testosterone, while the reverse was found for patients over that age. The reason for this could be explained by the activity of 17β-oxidoreductase enzyme which increases with age and reaches full maturity at puberty [171]. Nonetheless the plasma concentrations of testosterone and AD were found to be higher in the younger patients. These findings strongly suggest that the receptors in the younger patients for testosterone and AD were unable to remove these hormones from the blood.

It is now accepted that the steroid hormone receptors have three domains. The first domain is central and specifically binds to DNA. The other two domains are, one (carboxy terminal) to bind with the respective steroid hormone and the other (N-terminal) for transcription regulation. This provides another possible explanation for the higher plasma concentrations of testosterone and AD in the younger patients. Although the total mass of receptors was present the receptor domains may have been immature, so were unable to remove the steroids from the circulation. On the other hand if the plasma concentrations of these compounds are maintained at higher than the patients normal concentrations over a period of time, then activation of these

domains may well take place. This would lower the plasma concentrations of testosterone and AD and the evidence of their utilization would appear both clinically and in their urinary metabolite excretions.

The principal metabolites of AD are androsterone and aetiocholanolone. The urinary excretions of these two metabolites were increased (Table XXVII) above the basal excretion rates throughout the five hour periods of the test. This phenomenon was also evident for 5α- and 5β-androstanediols (Table XXVI) as the representative metabolites of testosterone. The total excretion rates for the five hour periods and the means of these totals were calculated and brought together in Table XXVIII. The means of the total excretions of androsterone and aetiocholanolone are greater for the two older patients (10 and 11) (2427.0 µg per 5h androsterone and 1825.0 µg per 5h aetiocholanolone) than for the younger patients (1-8) (1617.0 µg per 5h androsterone and 1100.5 µg per 5h aetiocholanolone). The corresponding values for 5α-androstanediol are 10.4 µg per 5h for the younger and 49.0 µg per 5h for the older patients and for 5β-androstanediol are 14.4 µg per 5h for the younger and 45.0 µg per 5h for the older patients. It is clear from the foregoing data that the two older patients increased their metabolism of androstenedione to androsterone by 150.0 per cent and to aetiocholanolone by 165.8 per cent compared to that of the younger patients. They also increased their metabolism of testosterone to 5α-androstanediol by 471.2 per cent and to 5β-androstanediol by 312.5 per cent in comparison to the younger patients. In contrast there was no significant difference between the older patients (10 and 11) and the younger patients (1 - 8) in the excretion of

Table XXVIII: Group 1 patients: the total and the means of urinary androgen metabolites.

Pts.	CA(yrs)	BA(yrs)	µg per total test period (5-hours)*				
			A	AE	5α-ASD	5β-ASD	Epi.A
1.SS	9.25	4.4	2054	1321	8.0	9.0	195
2.AG	10.73	9.6	1887	1131	9.0	13.0	205
3.SC	11.40	10.1	2016	1205	10.0	14.2	454
4.SW	12.18	8.6	274	237	4.4	2.3	29
5.SD	12.98	9.7	1654	1423	18.0	20.0	107
6.TW	13.00	10.3	1221	967	14.0	21.0	98
7.JK	13.14	10.5	2284	897	15.0	12.0	543
8.PM	13.96	8.5	1543	1623	5.0	24.0	203
9.SC	15.30	11.9	2252	2683	21.0	40.0	400
10.DC	15.66	13.0	2841	1953	23.0	23.0	208
11.GC	15.83	14.5	2007	1697	75.0	67.0	252
1-8 @	12.1	9.0	1617.0	1100.5	10.4	14.4	229.3
10+11@	15.7	13.8	2424.0	1825.0	49.0	45.0	230.0
% of 10+11 cf. 1-8 *			150.0	165.8	471.2	312.5	100.3

* For Patient 9 (SC) total test period was 4-hours.

Pts. = Patients. CA(yrs) = Chronological age (years).

BA(yrs) = Bone age (years). A = Androsterone.

AE = Aetiocholanolone. 5β-ASD = 5β-androstanediol.

5α-ASD = 5α-androstanediol. Epi.A = Epiandrosterone.

@ Means of patients 1-8 and 10+11. * cf. = Compared of with.

epiandrosterone. It is therefore hard to believe that epiandrosterone is a metabolite of testosterone.

But there is one further and perhaps most important fact to be derived from Table XXVIII namely that when these changing values are considered in relation to the respective bone ages of the patients, those with bone ages under 11.9 years are (precisely) in the chronologically younger age group although some are between 11.4 years and 13.96 years and their's is the low excretion rates of the metabolites while in those older patients with bone maturation scores of 13.0 and 14.5 years the excretion of the metabolites rises steeply. Patient 9 intermediate between the two groups with a bone maturation score of 11.9 years, shows an excretion rate for the metabolites 5α- and 5β-androstanediols double those of the younger patients but less than the older patients with excretion rates for androsterone and aetiocholanolone better than the younger and for aetiocholanolone even than the older groups. It is regrettable that more patients were not available for this part of the study but as far as the metabolism of testosterone is concerned there is a "take off" in utilization of plasma testosterone at a bone maturation score of 12.0 years approximately, an age which might be regarded as important for the start of puberty. Of course this begs the question as to which comes first, bone maturation or the ability to metabolize testosterone to the 5ϵ-metabolites?. Clearly some symphonic cadence reverberates through the organism to create a harmony. But this deduction may be important when considering the administration of an anabolic hormone to patients, for the bone maturation score at the time of commencement of the therapy is likely to be of prime importance. My argument, therefore, to

explain the data presented is that while 17β-oxidoreductase does increase in activity with age, increased urinary excretion of the 5ξ-androstanediols does point to a maturing steroid domain on the androgen receptors as well as or as a result of increased intracelluar testosterone for conversion to dihydrotestosterone. This is almost certainly confirmed by published work.

Group 2 Patients who had received IM. Sustanon.

Let me continue the argument as to which mechanism is most relevant in the maturing androgen-handling phenomenon. The three aspects: a maturing 17β-oxidoreductase enzyme, maturation of the androgen receptor moiety or intracellular conversion of testosterone to dihydrotestosterone are still under consideration. In Tables XXIV and XXV are shown the data concerning the plasma concentrations of testosterone and androstenedione and their urinary metabolites 5ξ-androstanediols and 5ξ-androstanolones following the administration of Sustanon (100 mg IM.).

To compare these data with those of Group 1 patients it is necessary to recalculate plasma concentrations and urinary excretion rates so as to achieve means for each over the same period of time. Thus from Table XXVI and XXVII are calculated the mean bone maturation score for the nine patients whose score was less than 12.0 years, the mean plasma testosterone for each patient and the mean of these means for the group of nine patients. Likewise was calculated the urinary excretion rates of 5ξ-androstanediols and 5ξ-androstanolones. These were values for a 5h acute assay. Similar means were calculated for plasma

androstenedione and its corresponding metabolites. These new data I present for ease in Table XXIX. From Tables XXIV and XXV (Group 2 patients) I have calculated precisely the same information bearing in mind that those data for the urinary metabolites related to 100 mg of excreted creatinine. To obtain comparable values I have adjusted the urinary excretion values to a 5h period taking 1000 mg as an average daily creatinine output for the age group concerned. These calculated data are also collected to Table XXIX.

Immediately it will be seen that for both groups the osseous maturation scores are similar being for Group 1 9.3 years and for Group 2 9.9 years. Thus data from the two Groups of patients may be compared. On oral androstenedione (100 mg bolus) a mean plasma concentration of 10.1 nmol/L was achieved with conversion to testosterone of 9.7 nmol/L. This suggests for Group 1 patients an active 17β-reductase i.e androstenedione to testosterone.

A vastly different picture is seen for Group 2 wherein a high mean plasma testosterone concentration of 52.7 nmol/L was associated with a low mean plasma concentration of androstenedione of 7.5nmol/L. It might be naive to conclude that 17β-oxidase is not at all active i.e testosterone to androstenedione, for one might have expected that after three days following Sustanon injection higher plasma androstenedione concentrations would have been achieved. On the other hand the testosterone may still have been in its esterified form and not available for conversion to androstenedione. We know that the RIA method for testosterone assay measures also esterified materials as well as bound (protein) and free.

When we consider the urine excretion rates for the

5ξ-androstandiols it must be remembered that on g.l.c. tracings of all urine samples, 5ξ-dihydrotestosterones were not detected. I, therefore, assume that the 5ξ-androstanediols represent the excretory products of corresponding 5ξ-dihydrotestosterones. It will therefore be seen that despite the remarkable differences in the mean plasma testosterone concentrations, the excretion of 5α-androstanediol is remarkably similar in the two Groups. Could this fact lead us to conclude that intracellular 5α-reductase for testosterone in these two groups of patients is the rate-limiting factor determining in these acute experiments, the biochemical response to testosterone either derived from androstenedione or from the injected Sustanon?.

In Group 2 patients the mean urinary excretion of 5β-androstanediol is a little higher than that of Group 1 patients but this can be accounted for by a contribution from hepatic metabolism of the higher plasma testosterone values.

It is of interest from Table XXIX that the urinary excretion values for androsterone and aetiocholanolone are so high for Group 1 boys when compared to the corresponding values for Group 2 patients. Clearly the orally administered androstenedione was rapidly metabolized at the A ring to 5ξ-,3α-hydroxyandrostanes and this phenomenon clearly restricted the rise in plasma testosterone in Group 1 boys. This was indeed one of the hopes for the use of androstenedione as a therapeutic anabolic substance. Whether or not the conversion of androstenedione to testosterone as seen here is sufficiently low as it dose not appear to be, will be further assessed when we discuss Group 3 patients who received androstenedione orally in addition to growth hormone.

Finally from Table XXIX it will be noted that the excretion

Table XXIX: The means of plasma levels of androstenedione and testosterone and the corresponding urinary metabolites in Group 1 (1-9 patients) and Group 2 (1-11 patients), presented with the means of their bone ages.

Means of		Group 1	Group 2
BA (years)		9.3	9.9
Plasma AD	nmol/L	10.1	7.5
Urinary A	µg/5h	1687.2	302.0
Urinary AE	µg/5h	1276.3	303.4
Plasma T	nmol/L	9.7	52.7
Urinary 5α-ASD	µg/5h	11.6	12.7
Urinary 5β-ASD	µg/5h	17.3	22.3
Urinary Epi.A	µg/5h	248.2	120.3

AD = Androstenedione. A = Androsterone.

AE = Aetiocholanolone. T = Testosterone.

5α-ASD = 5α-androstanediol. 5β-ASD = 5β-androstanediol.

Epi.A = Epiandrosterone. BA = Bone age.

NB: Plasma androstenedione and testosterone in Group 1 patients are the mean of the means of 5h periods.

rate of epiandrosterone for Group 2 patients is almost half that of Group 1 patients despite the gross disparity of the corresponding plasma testosterone concentrations. Here again it is hard to accept that epiandrosterone is a metabolite of testosterone and not of another Δ5-3β-hydroxy-compound.

It may now be concluded as part of the argument that while 17β-reductase is more active than 17β-oxidase, plasma testosterone must enter the cell for full utilization. The data suggest that at this stage, in so far as 5α-androstanediol excretion was similar in both Groups, that we must first consider that the steroid domain on the androgen receptor, especially when the bone maturation score is less than 12.0 years, is immature and therefore is not capable of transporting the testosterone within the cell. But one point that should be made is that the high plasma testosterone concentrations noted after testosterone administration could have blocked the receptors for testosterone simply by the surfeit creating a down regulation of the efficiency of these receptors.

Group 3 Patients on low dose androstenedione and growth hormone.

We have seen that when the children are given a bolus of oral androstenedione there is a rapid rise in plasma testosterone concentrations but these high values are not associated with a corresponding high urinary output of 5α-androstanediol, a compound which for the purposes of this thesis is regarded as evidence of the testosterone/dihydrotestosterone intracellular mechanisms for testosterone utilization. We have also noted that when children

have been injected with a high stat dose of testosterone (Sustanon) there is a grossly and unphysiologically high plasma concentration of testosterone (as free, protein bound and ester) yet there is no corresponding evidence for its utilization over a short period (i.e three days after the injection) as judged by the urinary 5α-androstanediol excretion values. What then are the effects of low dose chronic administration of oral androstenedione? Tables XXII and XXIII show the plasma and urinary androgens data of five boys who were proven to have idiopathic growth hormone deficiency and who received growth hormone (12 units weekly) and oral androstenedione (100 mg thrice weekly on the days of growth hormone administration). I have adjusted the urinary biochemical data to correspond with the data already presented in Table XXIX and for ease these new calculated data are shown for each patient in Table XXX. The means are shown, one for the basal concentrations, one to include all five patients and one to include four patients, for as will be seen Patient 1 had high plasma concentrations of both androstenedione and testosterone suggesting that administration of the androstenedione had been given on the morning of blood sampling. The reader will recall that blood and urine sampling in this Group of patients was on the morning after the evening of the administration of androstenedione (orally) and growth hormone (sc).

It will immediately be seen that the means for all compounds in plasma and urine after treatment exceeded the basal means but the "treatment" means of the plasma concentrations of AD and testosterone were more in the physiological range (especially for the four patients) than was noted in the Groups 1 and 2 patients

Table XXX: The means of plasma concentrations of androstenedione and testosterone and of the corresponding urinary metabolites in Group 3 patients.

Pts.	CA	BA	nmol/L	µg/5h		nmol/L	µg/5h		
			AD	A	AE	T	5α-ASD	5β-ASD	Epi.A
1.AS	6.28	2.9	18.50	4768	2936	5.7	14.0	27.0	358
2.MR	7.03	6.3	2.00	318	307	0.5	103.0	75.0	38
3.GY	9.50	5.8	5.00	513	834	1.0	12.0	23.0	7
4.GM	12.80	8.8	1.20	74	103	0.6	5.0	6.0	19
5.SD	13.80	10.6	3.50	114	125	0.7	0.8	1.3	86
Mean 5 Pts.		6.9	6.00	1157	861	1.7	27.0	26.5	102
Mean 4 Pts.		7.9	2.90	255	342	0.7	30.2	26.3	38
Mean Basal			1.22	5.8	7.1	0.34	0.46	0.58	1.7

Pts. = Patients. CA = Chronological age (years).

BA = Bone age (years). AD = Androstenedione.

A = Androsterone. AE = Aetiocholanolone.

T = Testosterone. 5α-ASD = 5α-androstanediol.

5β-ASD = 5β-androstanediol. Epi.A = Epiandrosterone.

Mean 4 Pts. = Patients 2-5.

NB: CA and BA are of the data at the begining of treatment.

of Table XXIX. The most interesting and important point of note is that the increase in the mean urinary 5α-androstanediol rose to 30.2 μg per 5h (for four patients) or 27.0 μg per 5h (for five patients). Again to facilitate comparison, in Table XXXI these means have been placed alongside those of patients in Groups 1 and 2. Similar conclusions are now evident but it is important to highlight the fact that the excretion rate of 5α-androstanediol has risen significantly by 246.0 per cent (for four patients) or 221.3 per cent (for five patients) when compared with the mean of 12.2 μg per 5h in the other two Groups of patients. Chapman [159] in his study of testing for testicular function in boys stated that urinary 5α-androstanediol levels greater than 30.0 μg/24h indicated normal plasma testosterone concentrations and its appropriate utilization through the 5α-dihydrotestosterone mechanism. Thus the addition of androstenedione (100 mg orally thrice weekly) has corrected by exogenous means the endogenous lack of both adequate plasma testosterone production and its adequate utilization. Clearly the small increases in the plasma testosterone concentrations maintained over the one year of androstenedione therapy, has up-regulated the androgen domains on the receptors as well as increasing the activity of intracellular 5α-reductase for the cytoplasm/testosterone complex with subsequent formation of 5α-dihydrotestosterone (fully metabolizable to 5α-androstanediol). This, therefore, concurs with the fact that small changes in the cytoplasmic concentration of 5α-dihydrotestosterone (receptor and 5α-reductase activity) produce a much greater change in the nuclear concentration of 5α-dihydrotestosterone to facilitate cell multiplication and differentiation.

Table XXXI: The means of plasma concentrations of androstenedione and testosterone and the corresponding urinary metabolites in Group 1 (1-9 patients), Group 2 (1-11 patients) and Group 3 (5patients) presented with means of bone ages.

Means of		Group 1	Group 2	Group 3	
				4 Pts*	5 Pts
BA (years)		9.3	9.9	7.9	6.9
Plasma AD	nmol/L	10.1	7.5	2.9	6.0
Urinary A	µg/5h	1687.2	302.0	255.0	1157.0
Urinary AE	µg/5h	1276.3	303.0	342.0	861.0
Plasma T	nmol/L	9.7	52.7	0.7	1.7
Urinary 5α-ASD	µg/5h	11.6	12.7	30.0	27.0
Urinary 5β-ASD	µg/5h	17.3	22.3	26.3	26.5
Urinary Epi.A	µg/5h	248.2	120.3	38.0	102.0
% Incr. of 5 -ASD of Group3 from Groups 1&2				246.0	221.3
Mean 5 -ASD for Groups1 & 2 patients			12.2		

* 4 patients = patients 2-5.

Pts. = Patients. BA = Bone age.

AD = Androstenedione. A = Androsterone.

AE = Aetiocholanolone. T = Testosterone.

5α-ASD = 5α-androstanediol. 5β-ASD = 5β-androstanediol.

Epi.A = Epiandrosterone.

NB: Plasma androstenedione and testosterone in Group 1 patients are the mean of the means of 5h periods.

We have already seen the effects of this "internal" improvement in biochemical efficiency in the five treated patients for their annual growth velocities increased remarkably beyond those of a comparable group of age matched controls who received during the first year only growth hormone as therapy (Tables XIX and XX). Table XXXII has brought together the clinical effects of the combined treatment and comparable data for one year on GH alone.

The considerable advantages of the combined therapy on linear growth are evident on all patients. The Table shows their individual response by accelerating linear growth well beyond their pretreatment achievements with the means of 2.4 cm per year (pretreatment) accelerating to a mean of 11.2 cm per year. But after one year with growth hormone alone as treatment the annual growth velocities decelerated individually to a mean for the group of 4.4 cm per year which fortunately was still almost twice that of the mean pretreatment annual growth velocity.

This linear growth must surely represent cell multiplication at the growth plates of the long bones and as has been stated earlier, can be in some part related to the dihydrotestosterone activity on osteoblastosis and chondroblastosis.

The question, however, is whether or not the deceleration in annual growth velocity is to be accounted for by the withdrawal of androstenedione? It is well known that all the synthetic anabolic hormones are stored in the liver and continue to have their effects long after their withdrawal. At least on linear growth the withdrawal of androstenedione is both sudden and quite remarkable and this testifies to its non-accumulation a distinct advantage of androstenedione as an anabolic hormone.

Table XXXII: The clinical anthropometric data in Group 3 patients during the pretreatment period, after one further year of combined treatment with growth hormone and androstenedione (GH + AD) and after another year of treatment with growth hormone alone (GH alone).

Pts.	Pretreatment			GH + AD			GH alone		
	CA	BA	AGV	CA	BA	AGV*	CA	BA	AGV*
1.AS	6.28	2.9	1.7	7.28	6.8	13.7	8.28	8.0	4.0
2.MR	7.03	6.3	1.8	8.03	8.6	15.9	9.03	9.7	4.5
3.GY	9.50	5.8	2.6	10.50	8.8	7.6	11.50	10.2	3.3
4.GM	12.80	8.8	2.7	13.80	11.2	10.4	14.80	12.8	5.4
5.SD	13.80	10.6	3.4	14.80	12.6	8.5	15.80	15.7	4.6
Mean	9.90	6.9	2.4	10.90	9.6	11.2	11.90	11.3	4.4

Pts = Patients. CA = Chronological age in years.

BA = Bone age in years. AGV = Annual growth velocity (cm/year).

Table XXXII also reveals the effects of treatment on the advancing bone age of each patient. These are seen for the individuals but for the group there is an advance of 2.7 bone age years per treatment year. While it is true that the starting point for all patients was a retarded bone age and only in Patient 2 did it exceed the chronological age, such a continued rate of increase would certainly be disadvantageous if the combined treatment continued at the same dosage level.

However during the year of treatment with growth hormone alone the mean rate of increase in bone maturation fell to 1.7 bone age years per year (i.e 11.3 - 9.6 BA years) so that perhaps it would be permissible to conclude that only one bone age year per year during the treatment period was due to the androstenedione and 1.7 bone age years per year due to the growth hormone.

This logic raises some interesting thoughts. Firstly the weekly dosage level of androstenedione could be reduced say by half and/or 3 - 6 months "on-off" treatment with AD. Secondly the androstenedione weekly dosage level could be maintained and the actual growth hormone dosage reduced by half, for it seems to contribute more to bone maturation than does androstenedione. Thirdly both dosage schedules could be reduced by half or by any proportion agreed, for an experimental period. This is a very important consideration for there is a current concept advantageous only to drug companies producing the recombinant growth hormone, that high dosage growth hormone therapy is necessary to maintain annual growth velocities optimally to give an acceptable adult height.

The avenues opened up by this work undoubtedly mean that

clinicians cannot afford to treat growth hormone deficient patients monitoring only anthropometric data but must include in all their evaluations a biochemical study of the type presented here.

One possibility remains to explain the continuing bone maturation during the year of treatment with growth hormone alone. The androstenedione (oral) may have up-regulated receptor activity as well as enzymic activity in the areas under discussion here. If this were so-how long does the upregulation persist? Or once upregulated will the improved activities remain.? and could it be that the androstenedione therapy has stimulated endogenous production of androgens to result in small but effective increases in plasma testosterone concentrations. Could it be that this is required to intiate androgen-dependent chondroblastic and osteoblastic activity to a level which will normalize linear growth?.

PROTOCOLS

The following protocols relevant to the clinical and biochemical data of Group 3 patients are presented here for the interested reader. Group 3 patients were treated with growth hormone and androstenedione for one year and with growth hormone alone for a further year. Those patients are presented individually on the following pages.

Protocol	1
Name	Alan S.
Hospital No	310356

Age at investigation 6.28 to 8.28 years.
Diagnosis: Complete growth hormone deficiency (isolated).

The triple test:-*

Time (min.)	0	5	10	15	20	30	60	90	120
GH (mU/L)	4.7	2.5	2.1	1.9	2.8	4.0	2.6	12.0	9.5
TSH (mU/L)	1.7				7.9		5.4		
LH (U/L)	1.5				2.3		2.4		
FSH (U/L)**	<1.0				2.0		2.3		
Cortisol	490	420	360	330	320	420	750	670	500
T4 (nmol/L)	83.0								
T3 (nmol/L)	2.2								

Anthropometric data:

	Pretreatment	1yr GH+AD	1yr GH only
Height (cm)	99.3	113.0	117.0
SDS for height	-3.24	-1.65	-1.85
AGV (cm)	1.7	13.7	4.0
Weight (kg)	15.2	20.9	22.0
Bone age (yr)	2.9	6.8	8.0
Pubertal stage	Pl Gl	Pl Gl	Pl Gl

Biochemical data over 1yr treatment (GH+AD):

	Basal	3/12	6/12	9/12	12/12
Plasma AD (nmol/L)	1.1	34.9	2.1	34.6	2.27
Plasma T (nmol/L)	0.23	13.5	0.35	8.6	0.36
Urinary Creatinine (mg/urine vol.)	27.3	47.4	132.0	23.7	28.1
Urinary Androgens: (µg/100 mg creatinine)					
1. 5α-androstanediol	ND	24	ND	2	0.2
2. 5β-androstanediol	ND	48	ND	3	1
3. Δ5-androstenediol	ND	ND	ND	ND	ND
4. 5α-3β,17β-androstanediol	ND	ND	ND	ND	ND
5. androsterone	3	8471	2	675	7
6. aetiocholanolone	3	5170	3	455	9
7. dehydroepiandrosterone	ND	ND	ND	ND	ND
8. epiandrosterone	1	639	12	31	6
9. 5α-dihydrotesosterone	ND	ND	ND	ND	ND

* Triple test consist of the insulin hypoglycaemia test; GnH-RH test and TRH test which were done in one test. This test was done 3 days after a single priming injection of Sustanon (100 mg) I.M..
** Plasma concentrations in nmol/L.
T4 = thyroxine. T3 = triiodothyronine.
Plasma T = plasma testosterone.
ND = not detected.

Protocol 2
Name Ryan M.
Hospital No 286904
Age at investigation 7.03 to 9.03 years.
Diagnosis: Complete growth hormone deficiency (isolated).

The triple test:*

Time (min.)	0	5	10	15	20	30	60	90	120
GH (mU/L)	4.4	2.3	1.9	2.3	1.5	1.0	3.1	5.1	6.3
TSH (mU/L)	2.3				2.9		1.4		
LH (U/L)	1.6				3.4		3.7		
FSH (U/L)	1.6				3.4		3.7		
Cortisol**	300	340	300	280	400	580	650	420	360
T4 (nmol/L)	41.0								
T3 (nmol/L)	2.1								

Anthropometric data:

	Pretreatment	1yr GH+AD	1yr GH only
Height (cm)	103.6	119.5	124.0
SDS for height	-3.12	-1.2	-1.3
AGV (cm)	1.8	15.9	4.5
Weight (kg)	19.6	24.8	27.7
Bone age (yr)	6.3	8.6	9.7
Pubertal stage	Pl Gl	Pl Gl	Pl Gl

Biochemical data over 1yr treatment (GH+AD):

	Basal	3/12	6/12	9/12	12/12
Plasma AD (nmol/L)	1.5	2.13	1.6	1.96	2.5
Plasma T (nmol/L)	0.31	0.68	0.3	0.35	0.46
Urinary Creatinine (mg/urine vol.)	42.0	71.6	58.0	45.0	62.6
Urinary Androgens: (µg/100 mg creatinine)					
1. 5α-androstanediol	1	197	0.2	1	ND
2. 5β-androstanediol	1	138	1	4	0.1
3. Δ5-androstenediol	ND	ND	ND	ND	ND
4. 5α-3β,17β-androstanediol	ND	ND	ND	0.1	ND
5. androsterone	4	594	7	7	3
6. aetiocholanolone	5	566	7	10	7
7. dehydroepiandrosterone	ND	ND	ND	ND	ND
8. epiandrosterone	1	63	1	8	0.4
9. 5α-dihydrotesosterone	ND	ND	ND	ND	ND

* Triple test consist of the insulin hypoglycaemia test; GnH-RH test and TRH test which were done in one test. This test was done 3 days after a single priming injection of Sustanon (100 mg) I.M..
** Plasma concentrations in nmol/L.
T4 = thyroxine. T3 = triiodothyronine.
Plasma T = plasma testosterone.
ND = not detected.

Protocol 3
Name Gary Y.
Hospital No 328740
Age at investigation 9.5 to 11.5 years.
Diagnosis: Complete growth hormone deficiency (isolated).

The triple test:*

Time (min.)	0	5	10	15	20	30	60	90	120
GH (mU/L)	4.8	4.2	3.7	3.5	2.7	2.7	9.0	2.1	1.2
TSH (mU/L)	2.6				10.0		6.7		
LH (U/L)	1.0				1.8		2.3		
FSH (U/L)**	1.2				1.5		2.4		
Cortisol	600	430	460	510	530	560	740	810	420
T4 (nmol/L)	113.0								
T3 (nmol/L)	2.4								

Anthropometric data:

	Pretreatment	1yr GH+AD	1yr GH only
Height (cm)	109.6	117.2	120.5
SDS for height	-4.05	-3.43	-3.48
AGV (cm)	2.6	7.6	3.3
Weight (kg)	18.7	23.6	25.7
Bone age (yr)	5.8	8.8	10.2
Pubertal stage	Pl Gl	Pl Gl	Pl Gl

Biochemical data over 1yr treatment (GH+AD):

	Basal	3/12	6/12	9/12	12/12
Plasma AD (nmol/L)	1.6	4.54	2.37	11.0	2.16
Plasma T (nmol/L)	0.33	1.05	0.36	2.4	0.37
Urinary Creatinine (mg/urine vol.)	32.3	224.0	35.5	46.6	24.2
Urinary Androgens: (µg/100 mg creatinine)					
1. 5α-androstanediol	ND	2	0.2	20	0.1
2. 5β-androstanediol	0.1	1	0.2	43	0.1
3. Δ5-androstenediol	ND	ND	0.1	ND	0.1
4. 5α-3β,17β-androstanediol	ND	ND	ND	0.2	ND
5. androsterone	1	4	4	976	0.2
6. aetiocholanolone	1	6	3	1592	0.3
7. dehydroepiandrosterone	ND	ND	ND	ND	ND
8. epiandrosterone	0.2	1	1	11	0.2
9. 5α-dihydrotesosterone	ND	ND	ND	ND	ND

* Triple test consist of the insulin hypoglycaemia test; GnH-RH test and TRH test which were done in one test. This test was done 3 days after a single priming injection of Sustanon (100 mg) I.M..
** Plasma concentrations in nmol/L.
T4 = thyroxine. T3 = triiodothyronine.
Plasma T = plasma testosterone.
ND = not detected.

Protocol 4
Name George M.
Hospital No 252489
Age at investigation 12.8 to 14.8 years.
Diagnosis: Complete growth hormone deficiency (isolated).

The triple test:*

Time (min.)	0	5	10	15	20	30	60	90	120
GH (mU/L)	3.2	3.7	4.0	2.8	4.2	4.0	9.7	8.8	3.9
TSH (mU/L)	1.7				11.2		8.3		
LH (U/L)	1.4				3.5		3.1		
FSH (U/L)**	<1.0				1.2		1.6		
Cortisol	350	450	480	560	610	720	850	920	550
T4 (nmol/L)	95.0								
T3 (nmol/L)	3.2								

Anthropometric data:

	Pretreatment	1yr GH+AD	1yr GH only
Height (cm)	119.0	129.4	134.8
SDS for height	-4.32	-3.61	-3.84
AGV (cm)	2.7	10.4	5.4
Weight (kg)	25.6	32.4	33.7
Bone age (yr)	8.8	11.2	12.8
Pubertal stage	P1 G1	P1 G1	P2 G2

Biochemical data over 1yr treatment (GH+AD):

	Basal	3/12	6/12	9/12	12/12
Plasma AD (nmol/L)	0.5	1.78	0.98	1.68	1.5
Plasma T (nmol/L)	0.2	0.49	0.37	0.71	0.37
Urinary Creatinine (mg/urine vol.)	72.4	91.5	55.8	37.8	67.2
Urinary Androgens: (µg/100 mg creatinine)					
1. 5α-androstanediol	ND	10	0.1	0.2	0.1
2. 5β-androstanediol	ND	11	0.1	0.3	0.2
3. Δ5-androstenediol	ND	ND	0.1	0.2	0.1
4. 5α-3β,17β-androstanediol	ND	ND	ND	ND	ND
5. androsterone	2	120	8	11	3
6. aetiocholanolone	3	174	8	12	4
7. dehydroepiandrosterone	ND	ND	ND	ND	ND
8. epiandrosterone	1	29	1	5	1
9. 5α-dihydrotesosterone	ND	ND	ND	ND	ND

* Triple test consist of the insulin hypoglycaemia test; GnH-RH test and TRH test which were done in one test. This test was done 3 days after a single priming injection of Sustanon (100 mg) I.M..
** Plasma concentrations in nmol/L.
T4 = thyroxine. T3 = triiodothyronine.
Plasma T = plasma testosterone.
ND = not detected.

Protocol 5
Name Steven D.
Hospital No 201059
Age at investigation 13.8 to 15.8 years.
Diagnosis: Complete growth hormone deficiency (isolated).

The triple test:*

Time (min.)	0	5	10	15	20	30	60	90	120
GH (mU/L)	<1.0	<1.0	<1.0	<1.0	<1.0	3.1	3.5	1.5	5.2
TSH (mU/L)	1.6				10.0		7.0		
LH (U/L)	1.1				2.3		2.1		
FSH (U/L)**	<1.0				1.3		1.3		
Cortisol	250	160	170	200	360	360	240	230	210
T4 (nmol/L)	84.0								
T3 (nmo/L)	1.7								

Anthropometric data:

	Pretreatment	1yr GH+AD	1yr GH only
Height (cm)	143.0	151.5	156.1
SDS for height	-1.96	-1.78	-2.12
AGV (cm)	3.4	8.5	4.6
Weight (kg)	34.6	42.4	46.4
Bone age (yr)	10.6	12.6	15.7
Pubertal stage	P1 G1	P1 G2	P2 G3

Biochemical data over 1yr treatment (GH+AD):

	Basal	3/12	6/12	9/12	12/12
Plasma AD (nmol/L)	1.4	3.77	4.15	2.52	3.5
Plasma T (nmol/L)	0.64	0.64	0.81	0.51	0.72
Urinary Creatinine (mg/urine vol.)	24.1	269.0	220.7	53.5	62.5
Urinary Androgens: (µg/100 mg creatinine)					
1. 5α-androstanediol	0.1	0.1	1	0.1	0.1
2. 5β-androstanediol	0.3	0.2	2	0.1	0.1
3. Δ5-androstenediol	0.1	1	ND	ND	ND
4. 5α-3β,17β-androstanediol	ND	ND	ND	ND	ND
5. androsterone	4	6	205	4	3
6. aetiocholanolone	5	4	230	4	2
7. dehydroepiandrosterone	ND	ND	ND	ND	ND
8. epiandrosterone	1	1	163	0.4	1
9. 5α-dihydrotesosterone	ND	ND	ND	ND	ND

* Triple test consist of the insulin hypoglycaemia test; GnH-RH test and TRH test which were done in one test. This test was done 3 days after a single priming injection of Sustanon (100 mg) I.M..
** Plasma concentrations in nmol/L.
T4 = thyroxine. T3 = triiodothyronine.
Plasma T = plasma testosterone.
ND = not detected.

CHAPTER FIVE

REFERENCES

1. Marie P. Surdeux cas d'acromégalie. Hypertrophie Singulière non congenitale des extremities superieures, inferieures, et cephalique.
   Rèv de Mèd 1886; 6: 297-333.
   Quoted from: Medvel VC.,ed. A History of Endocrinology. MTP Press ltd., Lancaster and Boston, 1982; 289-351.
2. Minkowski O. Ueber einen fall von akromegalie.
   Berl Klin Wschr 1887; 24: 371-374.
   Quoted from: Medvel VC.,ed. A History of Endocrinology. MTP Press ltd., Lancaster and Boston, 1982; 289-351.
3. Cushing H. Partial hypophysectomy for acromegaly: With remarks on the function of the hypophysis.
   Ann Surg 1909; 50: 1002-1017.
4. Isovesco H. Contribution a La physiologie Du Lobe Anterieur De L'hypophyse. Le Lipoide Du Lobe Anterieur.
   C R Soc Biol (Paris) 1913; 75: 450-452.
5. Evans HM and Long JA. The effect of the anterior lobe of the hypophysis administered intraperitoneally upon growth and the maturity and oestrus cycles of the rat.
   Anat Rec 1921; 21: 61-63.
6. Johns WS, O'Mulvenny TO, Potts EB and Laughton NB. Studies on the anterior lobe of the pituitary body.
   Amer J Physiol 1927; 80: 100-106.
7. Houssay BA and Biasotti A. The hypophysis, Carbohydrate metabolism and diabetes.
   Endocrinology 1931; 15: 511-523.

8. Smith PE. Hypophysectomy and a replacement therapy in the rat.
Amer J Anat 1930; 45: 205-273.

9. Fraenkel-Conrat HL, Meamber DL, Simpson ME and Evans HM. Further purification of the growth hormone of the anterior pituitary.
Endocrinology 1940; 27: 605-613.

10. Evans HM, Simpson ME, Marx W and Kilricke E. Bioassy of pituitary growth hormone. Width of proximal epiphyseal cartilage of tibia in hypophysectomized rats.
Endocrinology 1943; 32: 13-16.

11. Li CH and Evans HM. The isolation of pituitary growth hormone.
Science 1944; 99: 183-184.

12. Li CH and Papkoff H. Preparation and properties of growth hormone from human and monkey pituitary glands.
Science 1956; 124: 1293-1294.

13. Knobil E and Greep RO. The physiology of growth hormone with particular reference to its action in the rhesus monkey and the "species specificity" problem.
Rec Progr Horm Res 1959; 15: 1-69.

14. Raben MS. Preparation of growth hormone from pituitaries of man and monkey.
Science 1957; 125: 883-884.

15. Wilhelmi AE. Fractionation of human pituitary glands.
Canad J Biochem Physiol 1961; 39: 1659-1668.

16. Li CH, Liu WK and Dixon JS. Human pituitary growth hormone. XII: The amino acid sequence of the hormone.
J Amer Chem Soc 1966; 88: 2050-2051.

17. Li CH, Dixon JS and Liu WK. Human pituitary growth hormone.

XIX: The primary structure of the hormone.

Arch Biochem Biophysics 1969; 133: 70-91.

18. Simpson JA. Diseases of the nervous system. In: Macleod J., ed. Davidson's principles and practice of medicine. Churchill Livingstone. Edinburgh, London, Melbourne and NewYork, 1984; 591-669.

19. Underwood LE, Fisher DA, Fraiser SD, Gertner JM, Kaplan SL, Kirkland RT, Lippe BM and Ratti S. Degenerative neurologic diseases in patients formerly treated with human growth hormone.

J Pediatr 1985; 107: 10-12.

20. Duffy P, Wolf J, Collins J, DeVoe AS, Streeten B and Cowen D. Possible person-to-person transmission of Creutzfeldt-Jakob disease.

N Engl J Med 1974; 290: 692-693.

21. Bernoulli C, Siegfried J, Baumgartner G, Regli F, Rabinowicz T, Cajdnsek DC and Gibbs C J-JR. Danger of accidental person to person transmission of Creutzfeldt-Jakob disease by surgery.

Lancet 1977; 1: 478-479.

22. Goeddel DV, Heyneker HL, Hozumi T, Arentzer R, Itakura K, Yansura DG, Ross MJ, Miozzari G, Grea R and Seeburg PH. Direct expression in Escherchia Coli of a DNA sequence coding for human growth hormone.

Nature 1979; 281: 544-552.

23. Flodh H. Human growth hormone produced with recombinant DNA technology: Development and production.

Acta Paediatr Scand (Suppl) 1986; 325: 1-9.

24. Takano K and Shizume K. Current clinical trial with authentic

recombinant human growth hormone in Japan.

Acta Paediatr Scand (Suppl) 1986; 325: 93-97.

25. Salmon WD Jr and Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage In Vitro.

J Lab Clin Med 1957; 49: 825-836.

26. Almqvist S. Studies on sulfation factor (SF) activity of human serum. Effect of human growth hormone on (SF) levels in pituitary dwarfism.

Acta Endocrinol (Copenh) 1960; 35: 381-396.

27. Almqvist S and Rune I. Studies on sulfation factor (SF) activity of human serum. The variation of serum (SF) with age.

Acta Endocrinol (Copenh) 1961; 36: 566-576.

28. Grant DB, Hambley J, Becker D and Pimstone BL. Reduced sulfation factor in under nourished children.

Arch Dis Child 1973; 48: 596-600.

29. Daughday WH, Hall K, Raben MS, Salmon WD Jr, Vanden Brande JL and Van Wyk JJ. Somatomedin: Proposed designation for sulfation factor.

Nature 1972; 235: 107-107.

30. Rinderknecht E and Humbel RE. The amino acid sequence of human insulin-like growth factor 1 and its structural homology with proinsulin.

J Biol Chem 1978; 253: 2769-2776.

31. Rinderknecht E and Humbel RE. Primary structure of human insulin like growth factor 2.

FEBS Lett 1978; 89: 283-286.

32. Hunter WM and Greenwood FC. A radioimmuno electrophoretic

assay for human growth hormone.

Biochem J 1962a; 85: 39p-40p.

33. Hamilton W and Khattab MM. Sex hormone priming prior to the combined hypoglycaemia test.

Acta Endocrinol (Copenh) (Suppl) 1986; 279: 60-65.

34. Hindmarsh BC and Brook CGD. Auxological and biochemical assesment of short stature.

Acta Paediatr Scand (Suppl) 1988; 343: 73-76.

35. Hunter WM and Rigal WM. The diurnal pattern of plasma growth hormone concentration in children and adolescents.

J Endocrinol 1966; 34: 147-153.

36. Finkelstein JW, Roffwarg HP, Boyar RM, Kream J and Helman L. Age-releated changes in the twenty four hours spontaneous secretion of growth hormone.

J Clin Endocrinol Metab 1972; 35: 665-670.

37. Buckler JMH. Exercise as screening test for growth hormone release.

Acta Endocrinol (Copenh) 1972; 69: 219-229.

38. Weber B, Helge H and Quabbe H-J. Glucagon induced growth hormone release in children.

Acta Endocrinol (Copenh) 1970; 65: 323-341.

39. Hayek A and Crawford JD. L-DOPA and pituitary hormone secretion.

J Clin Endocrinol Metab 1972; 34: 764-766.

40. Parker ML, Hamond JM and Daughaday W. The arginine provocative test: An aid in the diagnosis of hyposomatotropism.

J Clin Endocrinol Metab 1967; 27: 1129-1136.

41. Lanes R and Hurtado E. Oral clonidine an effective growth hormone-releasing agent in prepubertal subjects.

J Pediatr 1982; 100: 710-714.

42. Sandahl-Christiansen J, Ørskov H, Binder C and Kastrup KW. Imitation of normal plasma growth hormone profile by subcutaneous administration of human growth hormone to growth hormone deficient children.
Acta Endocrinol (Copenh) 1983; 102: 6-10.

43. Albertsson-Wikland K, Westphal O and Wevtgren U. Daily subcutaneous administration of human growth hormone in growth hormone deficient children.
Acta Paediatr Scand 1986; 75: 89-97.

44. Eastman CJ, Lazarus L, Stuart MC and Casy JH. The effect of puberty on growth hormone secretion in boys with short stature and delayed adolescence.
Aust N Z J Med 1971; 2: 154-159.

45. Sar M and Stumpf WE. Autoradiographic localization of radioactivity in the rat brain after the injection of 1,2-^{3}H-Testosterone.
Endocrinology 1973; 92: 251-256.

46. Plapinger L and McEwen BS. Ontogeny of oestradiol-binding sites in rat brain. I: Appearance of presumptive adult receptors.
Endocrinology 1973; 93: 1119-1128.

47. Naftolin F, Ryan KJ and Petro Z. Aromatization of androstenedione by the diencephalon.
J Clin Endocrinol Metab 1971; 33: 368-370.

48. Weisz J and Gibbs C. Conversion of testosterone and androstenedione to oestrogens In Vitro by the brain of female rats.
Endocrinology 1974; 94: 616-620.

80. Martin LG, Grossman MS, Connor TB, Levitsky LL, Clark JW and Camitton FD. Effect of androgen on growth hormone secretions and growth in boys with short stature.
Acta Endocrinol (Copenh) 1979; 91: 201-212.

81. Rosenfeld RG, Northcraft BA and Hintz RL. A prospective randomized study of testosterone treatment of constitutional delay of growth and development in male adolescents.
Pediatrics 1982; 69: 681-687.

82. Martin MM, Martin ALA and Mossman KL. Testosterone treatment of constitutional delay in growth and development: Effect of dose on predicted versus definitive height.
Acta Endocrinol (Copenh) (Suppl) 1984; 279: 147-152.

83. Kaplowitz PB. Diagnostic value of testosterone therapy in boys with delayed puberty.
Amer J Dis Child 1989; 143: 116-120.

84. Whitelaw MJ, Thomas SF, Graham W, Foster TN and Brook CGD. Growth response in gonadal dysgenesis to the anabolic steroid norethandrolone.
Amer J Obst Gynecol 1962; 84: 501-504.

85. Prader A. The influence of anabolic steroids on growth.
Acta Endocrinol (Copenh) (Suppl) 1961; 63: 78-88.

86. Johanson AJ, Brasel JA and Blizzard RM. Growth in patients with gonadal dysgenesis receiving fluoxymesterone.
J Pediatr 1969; 75: 1015-1021.

87. Rosenbloom AL and Frias JL. Oxandrolone for growth promotion in Turner's syndrome.
Amer J Dis Child 1975; 125: 385-387.

88. Moore DC, Tattoni DS, Ruvalcaba RHA, Limbeck GA and Kelley V C. Studies on anabolic steroids. VI: Effect of prolonged administration of oxandrolone on growth in children and adolescents with gonadal dysgenesis.
J. Pediatr 1977; 90: 462-466.

89. Urban MD, Lee PA, Dorst JP, Poltnick LP and Mingeon CJ. Oxandrolone therapy in patients with Turner's syndrome.
J Pediatr 1979; 94: 823-827.

90. Rudman D, Goldsmith M, Kutner M and Blackston D. Effect of growth hormone and oxandrolone singly and together on growth rate in girls with X chromosome abnormalities.
J Pediatr 1980; 96: 132-135.

91. Joss EE and Zuppinger K. Oxandrolone in girls with Turner's syndrome, a pair-matched controlled study up to final height.
Acta Paediatr Scand 1984; 73: 674-679.

92. Stanhope N, Lingstaedt K, and Willig RP. Oxandrolone increased final height in Turner's syndrome.
Pediatr Res 1985; 19: 620-620.

93. Joss EE. Anabolic steroids in girls with Turner's syndrome.
Acta Paediatr Scand (Suppl) 1988; 343: 38-42.

94. Dorfman R and Shipley RA. Androgens. Biochemistry, Physiology and clinical significance. John Wiley and sons, INC., NewYork and Chapman and Hall Ltd., London, 1956; 116-128.

95. Wieland RG, Courcy C, Levy RP, Zala AP and Hirschmann H. $C_{19}O_2$ Steroids and some of their precursors in blood from normal human adrenals.
J Clin Invest 1965; 44: 159-168.

96. Savard K, Dorfman RI and Poutasse E. Biogenesis of androgens in human testis.

J Clin Endocrinol Metab 1952; 12: 935-935.

97. Kase N, Forchielli E and Dorfman RI. In Vitro production of testosterone and androst-4-ene-3,17-dione in a human ovarian homogenate.

Acta Endocrinol 1961; 37: 19-23.

98. Baulieu E-E and Robel P. Catabolism of testosterone and androstenedione. In: Eik-Nes KB, ed. The androgens of the testis. Marcel and Dekker, INC., NewYork, 1970; 49-71

99. Goldman J, Wajchenberg BL, Liberman B, Nery M, Achando S and Germek OA. Contrast analysis for the evaluation of the circadian rhythms of plasma cortisol, androstenedione and testosterone in normal men and the possible influence of meals.

J Clin Endocrinol Metab 1985; 60: 164-167.

100. Khammar F and Brudieux R. Seasonal changes in testicular contents and plasma concentrations of androgens in desert gerbil (Gerbillus gerbillus).

J Reprod Fertil 1987; 80: 589-594.

101. Dell´Aquila S, Crasto A, Alberico G, Varriale B, Pelosi A and Pierantoni R. Seasonal plasma profile of testosterone and androstenedione in the Gentile dipuglia ram in southern Italy .

J Endocrinol Invest 1985; 8: 263-264.

102. Cohn GL and Mulrow PJ. Androgen release and synthesis In Vitro by human adult adrenal gland.

J Clin Invest 1963; 42: 64-78.

103. McShan WH and Perdue JF. Gonadotropic hormones and end site

metabolism. In: Litwack G and Kritchevsky D, eds. Actions of hormones on molecular process. John Wiley and sons INC., NewYork, London and Sydney, 1964; 172-208.

104. Forest MG. Age related response of plasma testosterone, delta-4-androstenedione and cortisol to adrenocorticotropin in infants, children and adults.
J Clin Endocrinol Metab 1978; 47: 931-937.

105. Forest MG, Sizonenko PC, Cathiard AM and Bertrand J. Hypophyso-gonadal function in humans during the first year of life. I: Evidence for testicular activity in early infancy.
J Clin Invest 1974; 53: 819-828.

106. Forest MG and Cathiard AM. Pattern of plasma testosterone and Δ4-androstenedione in normal newborns: Evidence for testicular activity at birth.
J Clin Endocrinol Metab 1975; 41: 977-980.

107. Horton R, and Tait JF. Androstenedione production and interconversion rates measured in peripheral blood and studies on the possible site of its conversion to testosterone.
J Invest 1966; 45: 301-313.

108. Milewich L, Whisenant MG and Sawyer MK. Androstenedione metabolism by human lymphocytes.
J Steroid Biochem 1982; 16: 81-85.

109. Mulder E, Lamers-Stanhlhofen GJN and Vander Molen HJ. Isolation and characterization of 17β-hydroxy-steroid dehydrogenase from human erthrocytes.
Biochem J 1972; 127: 649-659.

110. Milewich L and Whisenant MG. Metabolism of androstenedione

by human platlates: A source of potent androgens.

J Clin Endocrinol Metab 1982; 54: 969-974.

111. Perel E, Wilkins D, Killinger DW. The conversion of androstenedione to estrone, estradiol and testosterone in breast tissue.

J Steroid Biochem 1980; 13: 89-94.

112. Milewich L, Winter AJ, Stephens P and Macdonald PC. Metabolism of dehydroisoandrosterone and androstenedione by the human lung In Vitro.

J Steroid Biochem 1977; 8: 277-284.

113. Sevensson J, Eneroth P, Gustafsson J-A, Ritzen M and Stenberg A. Metabolism of androstenedione in skin and serum levels of gonadotrophins and androgens in prepubertal boys with hypospadias.

J Endocrinol 1978; 76: 399-409.

114. Silva PD, Geutzschein EE and Lobo RA. Androstenedione may be more important precursor of tissue dihydrotestosterone than testosterone in women.

Fertil Steril 1987; 48: 419-422.

115. Baulieu EE. On the metabolism of testosterone and its compartmentalization. In: Tamm J, ed. Testosterone. Proceeding of workshop conference held from April 20th. to 22nd., 1967 at Tremsbuttel. George Thieme, Verlag and Stuttgart, 1968; 68-75.

116. Harper MJK. Effect of androstenedione on pre-implantation stages of pregnancy in rats.

Endocrinol 1967; 81: 1091-1098.

117. Bonlanger P, Somma M, Chevalier S, Bleau G, Roberts KD and Chapdelaine A. Elevated secretion of androstenedione in a

patient with a leydig cell tumour.

Acta Endocrinol (Copenh) 1984; 107: 104-109.

118. Wellen JJ, Smals AG, Rijken JC, Kloppenborg PW and Benrand T J. Testosterone and delta-4-androstenedione in the saliva of patients with Klinefelter's syndrome.

Clin Endocrinol 1983; 18: 51-59.

119. Ross EJ. Symptomatology in adrenal diseases. In: Keynes WM and Fowler PBS, eds. Clinical Endocrinology. William Heinemann Medical Books, London, 1984; 148-194.

120. Ando S, Giacchetto C, Canonaco M, Aquila S, Valenti A, Braldi E, Piro A and Dessi-Fulgheri F. Effects of castration on androstenedione, testosterone and dihydrotestosterone plasma levels in adult male rats.

Horm Res 1986; 23: 122-127.

121. Vermeulen A. Postmenopausal ovarian function. In: James VHT, Serio M and Giusti G, eds. The Endocrine Function of the Human Ovary. Academic Press. London, NewYork and SanFrancisco, 1976; 237-244.

122. Gustafsson J-A, Pousette A, Stenberg A and Wrange O. High affinity binding of 4-androstene-3,17-dione in rat liver.

Biochem 1975b; 14: 3942-3948.

123. Tanner JM. Physical growth and development. In: Forfar JO and Arneil GC, eds. Text book of Paediatrics. Churchill Livingstone, Edinburgh, London, Melbourne and NewYork, 1984; 278-330.

124. Tanner JM, Whitehouse RH and Marshall WA. Assessment of skeletal maturity and prediction of adult height (TW2 Method). Academic press London, NewYork and SanFrancisco, 1975.

125. Oertel GW. Determination of plasma testosternoe.
Acta Endocrinol (Copenh) 1961; 37: 237-240.

126. Horning EC. Gas phase analytical methods for the study of the steroid hormones and their metabolism. In: Eik-Nes KB and Horning EC, eds. Gas Phase Chromatography of Steroids. Springer-Verlag, Berlin-Heidelberg and NewYork, 1968; 11-11.

127. Horning EC. Gas phase analytical methods for the study of the steroid hormones and their metabolism. In: Eik-Nes KB and Horning EC, eds. Gas Phase Chromatography of Steroids. Springer-Verlag, Berlin-Heidelberg and NewYork, 1968; 42-42.

128. Folin O. Approximately complete analyses of thirty "normal" urines.
Amer J Physiol 1905a; 13: 45-65.

129. Jaffe M. Ueber den Niederschlag, welchen pikrinsaure in normalem Harn erzengt und über eine neue Reaction des Kreatinins.
Ztschr F Phsiol Chemie 1886; 10: 391-400.

130. Bonsnes RW and Taussky HH. On the colorometric determination of creatinine by the Jaffe reaction.
J Biol Chem 1945; 158: 581-591.

131. Folin O. Laws governing the chemical composition of urine.
Amer J Physiol 1905b; 13: 66-115.

132. Smith OW. Creatinine excretion in women; data collected in course of urinalysis for female sex hormones.
J Clin Endocrinol Metabol 1942; 2: 1-12.

133. Wary PM and Scott Russell S. The value of the creatinine estimation as a gauge of the completeness of the 24-hour

specimen.

J Obst Gynecol Brit Emp 1960; 67: 623-626.

134. Paterson N. Relative constancy of 24-hour urine volume and 24-hour creatinine output.

Clin Chem Acta 1967; 18: 57-58.

135. Kirschner MA, Zuker IR and Jespersen DL. Ovarian and adrenal vein catheterization studies in women with idiopathic hirsutism. In: James VHT, Serio M and Giasti G, eds. The Endocrine Function of the Human Ovary. Academic press, London, NewYork and SanFrancisco, 1976; 443-456.

136. Vande Wiele RL, Macdonald PC, Gurpide E and Lieberman S. Studies in the secretion and interconversion of the androgens.

Rec Progr Horm Res 1963; 19: 275-310.

137. Okamoto M, Setaiski C, Nakagama K, Honiuchi Y, Monya K and Itoh S. Diurnal variations in the levels of plasma and urinary androgens.

J Clin Endocrinol Metab 1971; 32: 846-851.

138. Boon DK, Keenan RE and SlannWhite WR. Plasma testosterone variation in men: Variation but not circadian rhythm.

Steroid 1972; 20: 269-278.

139. Evans JI, MaeLean AW, Ismail AA and Love D. Concentration of plasma testosterone in normal men during sleep.

Nature 1971; 229: 261-262.

140. Vermeulen A and Verdonck L. Radioimmunoassay of 17β-hydroxy-5α-androstan-3-one, 4-androstene-3,17-dione, dehydroepiandrosterone, 17-hydroxyprogesterone and progesterone, and application to human male plasma.

J Steroid Biochem 1976; 7: 1-10.

141. Dufau ML, Catt KJ, Tsuruhara T and Ryan D. Radioimmunoassay of plasma testosterone.
Clin Chim Acta 1972; 37: 109-116.

142. Gandy HM and Peterson RE. Measurement of testosterone and 17-ketosteroids in plasma by the double isotope dilution derivative technique.
J Clin Endocrinol Metab 1968; 28: 949-977.

143. Mauvais-Jarvis P and Baulieu EE. Studies of testosterone metabolism. IV. Urinary 5α- and 5β-androstanediols and testosterone glucuronide from testosterone and dehydroisoandrosterone sulfate in normal people and hirsute women.
J Clin Endocrinol Metab 1965; 25: 1167-1178.

144. Vierhapper H, Nowotny P and Waldhausl W. Estimation by gas chromatography-mass spectrometry with selected ion monitoring of urinary excretion rates of 3α-androstanediol during/after I.V. administration of ^{13}C-labelled testosterone in man.
J Steroid Biochem, 1988; 29: 105-109.

145. Voigt W, Fernandez EP and Hsia SL. Transformation of testosterone into 17β-hydroxy-5α-androstan-3-one by microsomal preparations of human skin.
J Biol Chem 1970; 245: 5594-5599.

146. Finkelstein M, Forchielli E and Dorfman RI. Estimation of testosterone in human plasma.
J Clin Endocrinol Metabol 1961; 21: 98-100.

147. Styne DM. Paediatric Endocrinology for the house officer. William and Wilkins. Baltimore, Hong Kong, London and Sydney, 1988; 178-178.

148. Tanner JM. Physical growth and development. In: Forfar JO

and Arneil GC, eds. Text Book of Paediatrics. Churchill Livingstone. Edinburgh, London, Melbourne and NewYork, 1984; 322-322.

149. Mitamura R, Itoh Y, Suzuki N, Yano K and Okuno A. Relationship of Insulin-like growth factor 1 level to height velocity, testosterone and oestradiol in healthy children.
Acta Paediatr Scand (Suppl) 1989; 356: 127-127.

150. Kasperk CH, Wergedal JE, Farley JR, Linkhart TA, Turner RT and Baylink DJ. Androgens directly stimulate proliferation of bone cells In Vitro.
Endocrinology 1989; 124: 1576-1578.

151. Bubenik GA, Pomerantz DK, Schams D and Smith PS. The role of androstenedione and testosterone in the reproduction and antler growth of a male white-tailed deer.
Acta Endocrinol (Copenh) 1987; 114: 147-152.

152. Chapman PH. The urinary metabolites of testosterone an index of testicular function in children.
A Ph.D Thesis submited to Glasgow University, Department of Child Health, 1976; Chapt. 5; 240-321.

153. Horning EC. Gas phase analytical methods for the study of steroid hormones and their metabolites. In: Eik-Nes KB and Horning EC, eds. Gas phase chromatography of steroids. Springer-Verlag, Berlin-Heidelberg and NewYork, 1968; 1-71.

154. Andino N, James VHT, Parker V and Rippon AE. Excretion of non-conjugated androstenedione and testosterone in human urine.
Steroids 1976; 28: 837-846.

155. Taylor NF, Wallace AM, Rodriguez M, Liedo G and Seara G.

Comparison of blood spot androstenedione and 17α-hydroxyprogesterone with urinary steroid profiling for monitoring 21-hydroxylase deficiency.

Br Soc Paed Endocrinol Meeting held in Leicester, Sep. 28th-29th 1989. Paper N_o 8.

156. Masse R, Laliberte C, Tremblay L and Dugal R. Gas chromatographic/mass spectrometric analysis of 19-nortestosterone urinary metabolites in man.

Biomed Mass Spectro 1985; 12: 115-121.

157. Mauvis-Jarvis P, Charransol G and Bobas-Masson F. Simultaneous determination of urinary androstanediol and testosterone as an evaluation of human androgenicity.

J Clin Endocrinol Metab 1973; 36: 452-459.

158. Berthou FL, Bardon LG and Floch HH. Measurement of 5α-androstan-3α,17β-diol and 5β-androstan-3α,17β-diol in the urine of healthy men and women.

J Steroid Biochem 1971; 2: 141-153.

159. Chapman PH. The urinary metabolites of testosterone an index of testicular function in children.

A Ph.D Thesis submited to Glasgow University, Department of Child Health, 1976; 106-207.

160. Takahashi Y, Corvol MT, Tsagris L, Carrascosa A, Bok S and Rappaport R. Testosterone metabolism in prepubertal rabbit cartilage.

Mol Cell Endocrinol 1984; 35: 15-24.

161. Report of the International Olympic Committee Medical Commission. Innsbruck. (1974). Quoted by: Masse R, Ayotte C and Dugal R. Studies on anabolic steroids. I. Integrated methodological approach to the gas chromatographic-mass

spectrometric analysis of anabolic steroids metabolites in urine.

J Chromatogr 1989; 489: 23-50.

162. Lantto O, Bjorkhem I, Ek H and Johnston D. Detection and quantitation of stanozolol (stromba) in urine by isotope dilution-mass fragmentography.

J Steroid Biochem 1981; 14: 721-727.

163. Masse R, Ayotte C and Dugal R. Studies on anabolic steroids. I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine.

J Chromatogr 1989; 489: 23-50.

164. Vaishnav R, Beresford JN, Gallagher JA and Russell RGG. Effects of anabolic steroid stanozolol on cells derived from human bone.

Clin Science 1988; 74: 455-460.

165. Small M, Beastall GH, Semple CG, Cowan RA and Forbes CD. Alteration of hormone levels in normal males given anabolic steroid stanozolol.

Clin Endocrinol 1984a; 21: 49-55.

166. Edwards RH, Dowrzak F, Gerber RP, Griggs RC, Ford C, Halliday D and Rennie MJ. Stanozolol in patients with muscular dystrophy increases muscle protein synthesis measured In Vivo with stable isotopes.

B J Clin Pharmacol 1985; 19: 124-125.

167. Garden OJ, Blamey SL, Shenkin A and Carter DC. Reduced nitrogen excretion with an anabolic steroid in the postoperative period.

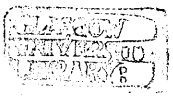
Proc Nutr Soc 1984; 43: 81A.

168. Dugal R, Dupuis C and Bertrand MJ. Radioimmunoassay of anabolic steroids: An evaluation of three antisera for the detection of anabolic steroids in biological fluids.
B J Sports Med 1977; 11: 162-169.

169. Hamilton W. The action of oxymetholone on human foetal testes.
Acta Paed Scand 1971; 60: 604-604.

170. Brooks RV. Androgens: physiology and pathology. In: Makin HLJ, ed. Biochemistry of steroid hormones. BlackWell Scientific publication. Oxford, London and Edinburgh, 1984; 591-591.

171. Snipes CA, Becker WG and Migbon CJ. The effect of age on the In Vitro metabolism of androgen by guinea pig testis.
Steroids 1965; 6: 771-776.



ON THE SYNERGISM OF GROWTH HORMONE AND ANDROSTENEDIONE IN CHILDREN

BY

DR. SAMI NOOH HASSAN

M.B., Ch. B., D.C.H., (GLASGOW)

AN ABSTRACT OF

THE THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

FROM THE

DEPARTMENT OF CHILD HEALTH, FACULTY OF MEDICINE

MAY 1990

ABSTRACT

The origin of thinking regarding growth hormone, in health, in disease and therapy was reviewed. The factors involved in the human growth process were enumerated with particular references to the insulin-like growth factors (IGF-I and IGF-II) and the anabolic hormones-natural and synthetic.

The subject of the thesis was to test the naturally occurring anabolic hormone androstenedione (AD) as therapy in conjunction with standard doses of growth hormone (GH).

First to study the mechanism of action of AD, 11 boys were given a bolus oral dose of AD (100mg). The conversion to plasma testosterone (T) and to metabolites of both AD and T was studied. Both were in the plasma in supraphysiological concentrations. The principal metabolite of T was 5α-androstane-3α,17β-diol (5α-ASD) and low excretion rates were observed despite high plasma T concentrations (mean 11.6 μg per 5h).

Secondly plasma AD and T concentrations were noted in 11 boys who received T (100 mg IM) as part of an ITT. Again despite there being high plasma T concentrations, the urinary excretion rates of 5α-ASD were low, comparable to those of the AD (oral) group (mean 12.7 μg per 5h).

Growth hormone (GH) (4 IU subcutaneously) and AD (100 mg orally) both thrice weekly were give to 5 boys with idiopathic growth hormone deficiency (IGHD) age range 6.28 to 13.8 years and plasma concentrations of AD and T and urinary metabolites including 5α-ASD were studied. Both plasma AD and T concentrations were maintained in their physiological range and urinary 5α-ASD increased (mean 27.0 μg per 5h).

Linear growth in these 5 boys increased to a mean of 11.2 cm per year compared to that of a control group of boys on growth hormone alone of 7.5 cm per year. During the second year of treatment on growth hormone alone the AGV fell to 4.4 cm per year.

Bone maturation during the first year of treatment of the 5 IGHD boys increased by a mean of 2.7 years compared with a mean for the control group of 5 IGHD boys of 0.96 years (GH alone).

It is, therefore, concluded that oral AD (100 mg) and GH (4 IU SC) both thrice weekly had increased the AGV in 5 IGHD boys approximately twice the AGV in 5 IGHD boys (control) treated by GH alone during the first year of treatment in both Groups. This was supported when AD therapy was stopped, the AGV during 1 further year's treatment on GH alone fell to 1/3 of that on combined treatment. As far as bone maturation score is concerned, further assays should be done using 25 to 50 mg AD orally thrice weekly and/or 3 - 6 months "on-off" AD therapy. The dose of GH could also be modified since it had been shown that it contributed to an increment of 1.7 years bone maturation per 1 year of treatment while AD contributed only to 1.0 year of osseous maturation during the treatment year. The other interesting conclusion is that the therapy had activated the androgen receptors or specific steroid domains on the receptors so that optimum concentrations of 5α-DHT were achieved in the cell cytoplasm and hence at nuclear level. This was supported by the increased excretion rates of 5α-ASD.