ASPECTS OF ADRENAL FUNCTION

IN

CHILDREN

by

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T H E S I S

submitted to the

UNIVERSITY of GLASGOW

for the degree of

DOCTOR of MEDICINE

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PREFACE.

The research work comprising this thesis is partly clinical and partly biochemical.

The clinical aspects concern the diagnosis and treatment of congenital adrenal hyperplasia and an assessment of some children of small stature. Most of these patients have been in Professor J.H. Hutchison's Wards at the Royal Hospital for Sick Children, Glasgow, and I am indebted to him for allowing access to this clinical material. Indebtedness is also due to Dr. James R. Davidson, General Director of Quarrier's Homes, Bridge of Weir, in whose care were some of the children of small stature.

The biochemical aspect of the thesis is a study of urinary steroids in cases of congenital adrenal hyperplasia. While principally to assist in their management, the investigation was extended to urinary aldosterone and 17-oxosteroid studies. In the children of small stature, the metabolism of hydrocortisone has been assessed by the estimation of the urinary metabolites of this compound. From the findings, hepatic reductase activity and 11-oxidation of hydrocortisone have been deduced. In Chapter 4 there is a critical analysis of the value of urinary aldosterone estimation in infancy and /

and childhood.

Dr. J.K. Grant of the Department of Steroid Biochemistry at the Royal Infirmary, Glasgow, taught me the principles of steroid analysis and since my early days in this field, he has many times given me advice on technical problems.

Professor Ivor H. Mills, formerly of St. Thomas' Hospital, London, gave me training in the estimation of aldosterone. To both these colleagues I express gratitude.

While case 4 in Chapter 2 had the clinical and biochemical (block estimations of urinary steroids) features of 3β-hydroxysteroid dehydrogenase deficiency at birth it was Dr. F.L. Mitchell (formerly of Maryfield Hospital, Dundee) who confirmed the diagnosis from urine of the 3rd. day of life.

Dr. Eileen Hill (formerly of the Nuffield Institute of Child Health, Birmingham) estimated some of the Fraction II Fraction III ratios in Case 3 of chapter 2. Dr. Beryl Connor (Imperial Cancer Research Fund, London) estimated the plasma corticotrophin level in this case also. Dr. L. Stimmler (formerly of the Nuffield Institute of Child Health, Birmingham) did the assays of growth hormone.

Technical assistance for parts of this work was given by Mr. Thomas Moodie and Mr. Harry Boyle. The clinical photographs were reproduced by Mr. Joseph Devlin.

Acknowledgments /

Acknowledgments for technical contributions from others are made in the text.

The line drawings and graphs may lack an artist's touch, but to make this work a more personal achievement, they are my own doing.

Parts of this thesis have been published under the following titles.

'Four clinical variants of congenital adrenal hyperplasia'. Archives of Disease in Childhood 1964, 39.66

Aldosterone and 17-hydroxycorticosteroid excretion following Ramstedt's operation.

Archives of Disease in Childhood, 1965, 40, 261.

Finally I wish to express sincere thanks to Professor J.H. Hutchison for his constant encouragement during these research activities and for his stimulus in the writing of this work.

Part of this research has been supported by the Rankin Fund of the University of Glasgow.

NOMENCLATURE

After the announcement of the total synthesis of cortisone by Sarett (1946) the number of published papers on all aspects of steroids, new synthetic pathways, optical rotations, clinical uses, steroid analogues, etc., increased rapidly.

It became necessary, not only for convenience but for mutual understanding, that a standard steroid nomenclature should be accepted. This was achieved at a conference held at the Ciba Foundation, London, in 1950, and was approved by the International Union of Pure and Applied Chemistry (1951).

Since this work is an application of steroid chemical analyses to essentially clinical problems, I shall use trivial names wherever possible for ease of writing and reading. I prefer the terms corticotrophin instead of adrenocorticotrophin and 17-oxosteroid instead of 17-ketosteroid. The tetrahydro- prefixing a compound indicates reduction of the double bond in ring A and this is characteristic of the urinary metabolites of adrenocorticosteroids.

The following trivial names are used:-

Trivial /

Trivial Name	Systematic Name
Adrenosterone	Androst-4-ene-3:11:17-trione.
Etiocholanolone	3α-Hydroxy-5β-androstane-17-one.
Aldosterone	11β:21-Dihydroxy-3:20-dioxopregne 4-en-18-al.
Androstanedione	5α-Androstane-3:17-dione.
Androstenedione	Androst-4-ene-3:17-dione.
Corticosterone	11β:21-Dihydroxypregn-4-ene 3:20-dione.
Cortisone	17α:21-Dihydroxypregn-4-ene 3:11:20-trione.
11-Dehydrocorticosterone	21-Hydroxypregn-4-ene-3:11:20- trione.
Dehydroepiandrosterone	3β-Hydroxyandrost-5-en-17-one.
Desoxycorticosterone	21-Hydroxypregn-4-ene-3:20-dione
Hydrocortisone	11β:17α:21-Trihydroxypregn-4-ene 3:20-dione.
18-Hydroxycorticosterone	11β:18:21-Trihydroxypregn-4-ene- 3:20:dione.
17α-Hydroxy-11-deoxycorti- costerone	17α:21-Dihydroxypregn-4-ene- 3:20:dione.
18-Hydroxy-11-deoxycorti- costerone	18:21-Dihydroxypregn-4-ene-3:20- dione.
17α-Hydroxyprogesterone	17α-Hydroxypregn-4-ene-3:20-dion
21-Hydroxypregnenolone	3β:21-Dihydroxypregn-5-ene-20-on
Pregnanetriol /	

Pregnanetriol	5β-Pregnane-3α:17α:20α-triol.
Pregnenetriol	3β:17α:20-Trihydroxypregn-5-ene
Progesterone	Pregn-4-ene-3:20-dione.
Testosterone	17β-Hydroxyandrost-4-en-3-one.
Tetrahydrocorticosterone	3α:11β:21-Trihydroxy-5β-pregnan-20-one.
Tetrahydrocortisone	3α:17α:21-Trihydroxy-5β-pregnan 11:20-dione.
Tetrahydrocortisol	3α:11β:17α:21-Tetrahydroxy-5β-pregnan-20-one.
Allo-Tetrahydrocortisol	3α:11β:17α:21-Tetrahydroxy-5α-pregnan-20-one.
Betamethasone	9α-Fluoro-16β-methyl-11β:17α:21 trihydroxy-pregna-1:4-diene-3:20-dione.
Dexamethasone	9α-Fluoro-16α-methyl-11β:17α:21 trihydroxy-pregna-1:4-diene-3:20-dione.
Fludrocortisone	9α-Fluoro-11β:17α:21-Trihydroxy pregn-4-ene-3:20-dione.
Triamcinolone	9α-Fluoro-11β:16α:17α:20-tetra-hydroxypregna-1:4-diene-3:20-dione.

Prednisolone /

Prednisolone	11β:17α:21-Trihydroxypregna-1:4-diene-3:20-dione.
Prednisone	17α:21-Dihydroxypregna-1:4-diene-3:11:20-trione.

INTRODUCTION

A review of the literature relating to adrenocortical physiology and pathology.

Leonard Williams, in his Foreword to Biedl's book 'The Internal Secretory Organs' (1912) says that 'the subject of the internal secretions is one which is destined to occupy the attention both of the physiologist and clinician for a long time to come. The study of the internal secretions has already illuminated myxoedema, acromegaly, Addison's disease and some others, and the promise which it holds is as enticing and fascinating as its pursuit is baffling.' Leonard Llewelyn Bulkeley Williams, M.D., Glasg., clinician, linguist and author, died on August 20, 1939 at the age of 77 and he might have been gratified by the truth of his prophecy were he, in the even of his life familiar with the effort of Levy-Simpson (1930 and 1938) who by then was treating cases of Addison's disease with adrenocortical extract and synthetic desoxycorticosterone acetate.

The first accounts however of experiments on animal (rabbits, dogs and cats) survival after extirpation of the adrenal capsules were published in 1856 by Brown-Séquard. He concluded, 'Des expériences extrêmement nombreuses que j'ai faites depuis plus de huit mois concernant ces organes, m'ont conduit à cette conclusion que la fonction des capsules surrénales est non-seulement essentielle a la vie, mais l'une des plus importantes de l'économie'. Harley (1856, 1857 and 1858) refuted Brown-Séquard's conclusion by showing /

showing that his rats lived for weeks or months after extirpation of the suprarenals without showing pathological signs of suppressed suprarenal function.

This controversy continued over the next twenty years or so and was to some extent perpetuated by the work of Nothnagel (1879). He attempted to produce in animals, suprarenal lesions which would result in the clinical complex of Addison's disease as seen in man. His failure to effect total extirpation of suprarenal tissue obscured the true importance of the glands.

Stilling (1890) working on rabbits showed that after extirpation of one suprarenal, the remaining one underwent compensatory hypertrophy and that after bilateral extirpation, remnant suprarenal tissue, impossible to remove, by hypertrophy became as large as a normal suprarenal. Also in 2 out of 40 rabbits he found accessory suprarenals which underwent hypertrophy after extirpation of the normal glands. The hypertrophied tissue had a histological structure resembli cortical substance and was void of medullary elements. He was also first to describe cellular masses in the ganglia of the abdominal sympathetic and in the intercarotid ganglion with a structure similar to the suprarenal medulla. Basing his argument on these two findings he explained the failure to produce Addison's disease in animals. Nothnagel (1879) had not appreciated this possibility.

Abelous and Langlois (1891) removed one suprarenal from /

from frogs and found that this did not result in death while destruction of both invariably did so. Their experiments with partial adrenal destruction indicated that at least one quarter of each gland was required to maintain life in the frogs. These findings were confirmed by similar experiments on guinea pigs and rabbits. Later in 1897 Langlois published his findings in dogs and showed that total extirpation resulted in death within 40 to 52 hours.

Hultgren and Andersson (1898) considered the problem of how much suprarenal tissue was required to maintain life. In cats, extirpation of one suprarenal with partial destruction of the other resulted only in transient emaciation but removal of both suprarenals was followed by death within 68 hours. Strehl and Weiss (1901) working with nine species involving 114 animals came to the conclusion that all animals die after removal of both suprarenals.

By the turn of the century the evidence from the early experiments led to the following conclusions.

1. Signs of suprarenal suppression do not follow the removal of one suprarenal. The remaining organ invariably undergoes hyperplasia and this hyperplasia is shared by any accessory suprarenal cortical tissue which may be present.

2. Death follows extirpation of both suprarenals within a very short time - a few hours to a few days and is not due to the operation or subsequent infection but to suppression /

suppression of suprarenal function. 3. When the suprarenals are removed separately, the interval between each operation being days or weeks, life is prolonged beyond the expectancy of that when both glands are removed at once. This is due to compensatory hypertrophy in the interval of the remaining gland and any accessory suprarenal tissue present. The final attempt by the accessory tissue alone to maintain life is necessarily insufficient. 4. If survival follows extirpation of both suprarenals, then it is due to the presence of accessory suprarenal tissue. 5. Partial removal of both suprarenals may be borne without sequelae but this depends on the amount of tissue left.

It is clear from the literature that during the first decade of the twentieth century many workers were engaged in repeating the early animal experiments, the results of which had excited so great an interest. The volume of published work on suprarenal physiology increased rapidly from 1914 to 1939.

To Elliot (1914) must be attributed the first detailed description of the nature of death following excision of the adrenal glands. (From this time onwards the word 'adrenal' replaced suprarenal). His work was on cats and after excision of the glands all the cats lost weight but only partly due to loss of appetite. There was no relish in eating. The approach of death was heralded by characteristic /

characteristic weakness, the animals being bothered by the difficulty of standing. Soon they lay down, chest and belly on the ground and shortly fell on to their sides, limbs, legs and tail fully extended - an attitude of sheer weakness. Emotionally there was no annoyance, the eyeballs lost their tension and became soft, the blood pressure fell as did also the rectal temperature to 85°F before death ensued. Alarm would rouse them to co-ordinated and fairly strong muscular movements indicating that there was no paralysis of the skeletal muscles or their nerves.

The way in which Elliot conducted his experiments led him to conclude that life depended either on a continual slow excretion from the adrenal medullary cells or on some activity of the cortical tissues. He favoured the second possibly because he observed that accessory glands which hypertrophied after removal of the adrenals were composed almost entirely of cortical cells.

The next advance in the understanding of adrenal physiology came from the work of Rogoff and Stewart. In a series of papers (1926a,b; 1927a,b; 1928a,b,c,d) on well-planned experiments, they confirmed that removal of both adrenals from dogs resulted in death and went on to show that death could be delayed by the intravenous administration of Ringer's solution. After total adrenalectomy the blood urea and serum calcium rose and the specific gravity of the blood increased. Heat and pregnancy in /

in operated bitches tended to prolong life. But their main contribution was their proof that a substance could be extracted from the adrenal cortex of dogs and sheep which prolonged life in dogs after total adrenalectomy. It is not to be thought however that Rogoff and Stewart scored firsts but rather that they put to scientific testing much of the earlier statements and theories. After all, Marshall and Davis (1916) had shown a rise in blood urea in cats following complete removal of the adrenals and Vulpain and Cloez (1857) had been interested in extracts from the adrenal glands from which they isolated among other things taurocholic acid. So early in 'steroid' chemistry this finding in adrenal extracts of a lipophilic hydrocarbon foreshadowed a greater truth.

Hartman and Hartman (1928) with various colleagues were undoubtedly the first to publish in detail a method for extraction of the active substance from the adrenal cortex. They used beef adrenals which had been chilled immediately after removal. From their preparation they first removed epinephrine and then by salting out with sodium chloride they obtained a substance which when injected prolonged the life of adrenalectomised cats. They proposed that this substance be named Cortin. The adrenals thereby attained endocrine status.

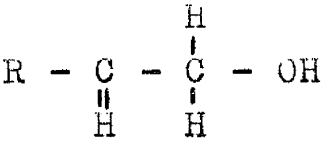
Heretofore in the adrenal field the facts had been established /

established by physiologists but from the partnership of Swingle and Pfiffner, (1930, 1934) an amalgam of physiologist and biochemist came the next advance. They succeeded in preparing an aqueous extract from beef adrenals which had physiological activity when administered subcutaneously to cats. Their cats lived in excellent health for more than 80 days. Further, their experiments indicated that the hormone was not stored in the body but metabolised and excreted. During the next two years Pfiffner in conjunction with Wintersteiner (Pfiffner and Wintersteiner 1935, Wintersteiner and Pfiffner 1935, 1936) showed that the active fraction in alcohol absorbed ultra-violet light maximally at 236 mμ. indicating that the substance possessed in its molecule an αβ-unsaturated ketone. The active fractio they found, could be rendered inactive by treatment with benzoyl chloride in pyridine indicating further the presence of acylable hydroxyl groupings. Elementary analysis and molecular weight determination spoke to a composition $C_{21}H_{34}O_5$ or $C_{21}H_{35}O_5$. They also extracted several inactive compounds A, B, C, D, E, F, G.

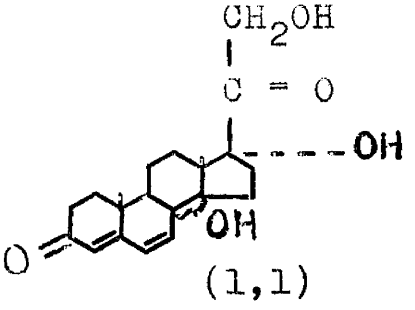
In the Mayo Clinic, starting about 1930, Mason, Myers and Kendall (1936a and b) succeeded in 1933 in extracting from adrenals a crystalline organic substance to which they assigned the letter E. This compound had a molecular formal $C_{21}H_{30}O_5$ and absorbed ultra-violet light maximally at 237 mμ. speaking to an αβ-unsaturated carbonyl group.

Using /

Using Ingle's test (1936) they found Compound E to be physiologically active though not as active as Cortin. Compound E could be oxidised with chromic acid yielding a ketone $C_{19}H_{24}O_3$ which had one sixth to one quarter the activity of androsterone in stimulating comb-growth in the capon. This finding linked Cortin with the sterol ring system. Also Compound E could be oxidised with periodic acid to yield an acid $C_{20}H_{26}O_5$ and formaldehyde. Clutterbuck and Reuter the year before had shown that these products could only come from the group:-



They were therefore able to suggest a provisional formula for Compound E. (1,1).



(1,1)

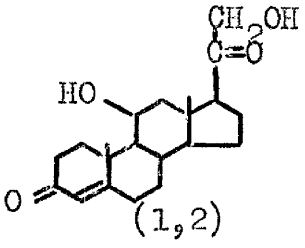
By the end of 1936 they had designated compounds A, B, C, D and E. Only Compound E had shown physiological activity.

Most spectacular, however, in results as well as in scientific content was the work of Reichstein on gland extracts provided by Organon (Holland). Publishing solely

(Reichstein /

(Reichstein 1936a, b, c, d, e,; 1937 a, b, c, d, and 1938) and with Steiger (Steiger and Reichstein 1937 a,b,c) he gav proof of having isolated compounds which he designated A to G, and came close to elucidating some of their molecular structures. The physiological activity of these compounds was assayed at the Organon Laboratory, by the muscle work test devised by Everse and de Fremery (1932). Also from the gland extracts Reichstein isolated 'androsterone' an androgen active in stimulating comb-growth in the capon.

Of all the compounds which had been crystallised by the various groups from the amorphous fraction of the adrenocor extracts only 6 had physiological activity. There were corticosterone, (1,2) 11-dehydrocortcosterone, (1,3) desoxycorticosterone (1,4) and the three corresponding 17-hydroxy derivatives of these (1,5; 1,6; 1,7). Their molecular structures were assigned thus:-



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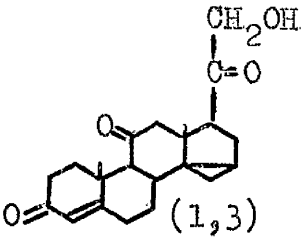

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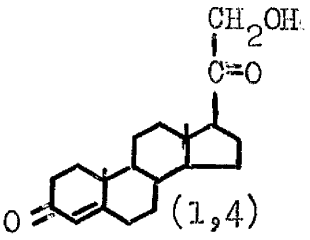

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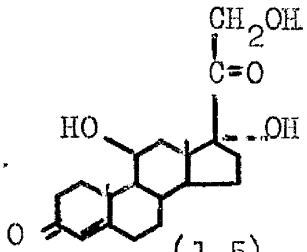
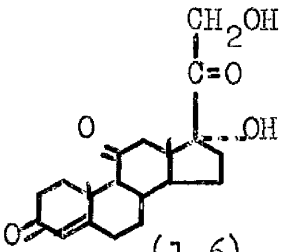


(1,5)



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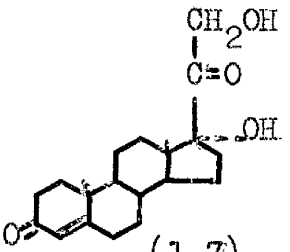


(1,7)

However, after extraction of these compounds, by crystallisation from the lipid soluble fraction there could be prepared from a second water soluble extract a principle which had a relatively low physiological activity as measured by the work test but which also had a high physiological activity measured by the growth-survival test. The significance of this finding was not to be understood until later when it was appreciated that different adrenal compounds exerted different physiological activity depending on their molecular structure.

Cartland and Kuizenga with their colleagues, (1936, and 1945), working at the Upjohn Laboratories, Kalamazoo, set out to prepare commercially and in bulk adrenocortical extracts for clinical use. They observed that hog adrenal extracts yielded much more active substance in the work test than did beef adrenals. Indeed 1,000 lbs. beef adrenals yielded 75-100 mg. of 17-hydroxycorticosterone (1,5) while 1,000 lbs. hog adrenals yielded 600 mg. in pure form. They attributed this increase to 11-hydroxylated compounds.

Tests of physiological activity.

The early workers in the field were satisfied that an adrenocortical extract had activity if when administered by injection to their totally adrenalectomised animals they remained alive. A more precise index of extract potency was obviously required and Pfiffner, Swingle and Vars (1934), defined /

defined the dog unit. This was the daily per kilogram dose of adrenocortical hormone necessary to maintain normal physiological conditions in the test dogs for a period of 7-10 days, i.e. maintenance of body weight and normal blood urea. Grollman and Frior (1933) used a rat test, the rat unit being the minimum daily dose of cortical hormone which would protect for at least 20 days, 50 per cent of a group of adrenalectomised animals at 28 days of age, the extract being given subcutaneously twice daily. Cartland and Kuizenga (1936) modified the rat unit to the minimum daily dose of hormone which administered by single subcutaneous injection daily for 20 days to 4 week old male rats weighing 50-60 g. is sufficient to protect at least 80 per cent for a 20 day period and to allow a weight gain of 20 grams for the 20 days. A comparison of the dog unit and Cartland's rat unit indicated that the rat unit was 22 times as large as the dog unit and on a per kilogram basis the growing rats required 300 times as much as the adult dogs.

Later Kuizenga, Nelson and Cartland (1940) compared by their rat method the effectiveness of oral and parenteral corticosterone (1,2), 11-dehydrocorticosterone (1,3) and desoxycorticosterone (1,4). The former two substances were isolated from the least water-soluble fraction of the gland extract, in pure form and identified by their melting points and specific rotations. Synthetic desoxycorticosterone acetate /

acetate was used. They were therefore able to administer weighed amounts of these hormones. Corticosterone and 11-dehydrocorticosterone were as effective orally as parenterally while desoxycorticosterone was 35 times more potent parenterally then when orally administered.

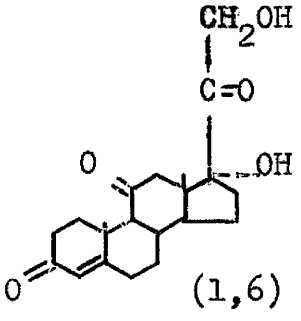
It was Steiger and Reichstein (1937) who first prepared chemically, from inactive stigmasterol, 21-oxy-progesterone, now known as desoxycorticosterone (1,4) and showed that this new substance had high cortical hormone activity.

Ingle devised a rat work test. In conjunction with Heron and Hales (1934) he had found that in contrast to the progressively increasing fatigue of skeletal muscles to repeated contractions which occurred in most animals, the rat gastrocnemius could maintain contractions at 3 per second against 100 g. weight for up to 17 days. They were unable to produce complete gastrocnemius fatigue by electrical stimulation in normal animals. After total adrenalectomy (Hales, Haslerud and Ingle, 1935) the capacity for work was reduced to a maximum of 36 hours but fatigue could be reversed or prevented by the administration of adrenocortical extracts (supplied by Hartman and colleagues). The capacity for work increased in proportion to the amount of hormone administered until an optimal dosage was reached (Ingle, 1936). Rats adrenalectomised and nephrectomised survived for 4 - 10 hours but when given cortin /

cortin they survived on average 33.7 hours (Ingle and Kendall 1936). By using such animals in the work test these authors found that they could determine the presence or absence of activity in extracts within 12 hours. This had obvious practical advantages.

Ingle's greater contribution to the understanding of steroid action was also based on his rat work test.

In 1940 (Ingle, 1940a) he found that the property for maintaining work capacity was possessed by corticosterone (1,2), 11-dehydrocorticosterone (1,3), 17-hydroxycorticosterone (1,5), 17-hydroxy-11-dehydrocorticosterone (1,6) and by desoxycorticosterone (1,4) but while the former 4 were effective when given subcutaneously at the 0.125 mg. per dose level the latter showed activity only when 2 mg. were used. Reduction of the ethylenic bond in 17-hydroxy-11-dehydrocorticosterone (1,6) rendered it inactive (1,8).



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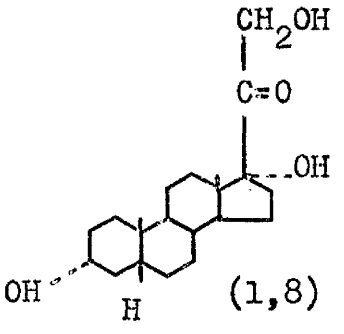


(1,8)

Desoxycorticosterone however had been recognised as extremely active in maintaining life and this at first sight seemed opposed to Ingle's observation. In subsequent work Ingle (1940b) was able to show that while desoxycorticosterone was /

was the most active known compound for life maintaining it had very little activity in maintaining work capacity. On the other hand, 17-hydroxy-11-dehydrocorticosterone (1,6) had little or no life-maintaining effects but was extremely active in the work performance test. This ability to maintain work was a capacity of the C-11 oxygenated cortical steroids.

The next link in the chain was supplied by Ingle and Leukens (1941) who showed that the work performance of adrenalectomised rats was improved by the intravenous administration of glucose in any concentration thus suggesting a link between the C-11 oxygenated cortical steroids and carbohydrate metabolism.

Almost synchronously Grattan and Jensen (1940) showed that corticosterone and 17-hydroxycorticosterone increased the deposition of liver glycogen and had a pronounced anti-insulin effect. Desoxycorticosterone even at 4 times the dosage level failed to show a similar response. Thus the adrenocortical hormones which had increased work capacity (Ingle 1940) were those which increased deposition of liver glycogen. The C-11 oxygenated steroids had therefore a significant effect on carbohydrate metabolism. Their final conclusions were that the functions of the adrenal cortex might be twofold, namely, to regulate carbohydrate metabolism and to maintain electrolyte balance and that these /

these functions were by different cortical principles.

These ideas had had earlier ventilation. Hartman and his colleagues (1939) had separated adrenal extracts into two fractions, one with little or no sodium retaining effect and one causing sodium retention. Cortin alone, they found, maintained the animal in good general condition although the plasma sodium concentration fell. The addition of their 'sodium factor' promptly raised plasma sodium to normal levels. They found too that desoxycorticosterone had a sodium retaining action and that the potency of their 'sodium factor' was approximately equal to the potency of desoxycorticosterone (Hartman and Spoor, 1940).

In 1855 Thomas Addison described cases of the lethal condition which since then has been known as Addison's disease. The suprarenal gland was from the first incriminated and empirically more than rationally sufferers were given orally, subcutaneous and rectally, large doses of epinephrin, the first humoral substance extracted from the adrenal. Dr. A.L. Muirhead (1921), himself a sufferer from Addison's disease describes his treatment with a preparation of dried whole suprarenal gland orally and compares the effects with oral, subcutaneous and rectal adrenaline.

It was not until 1932 that Loeb (1932) showed the biochemical disturbances in Addison's disease. In the serum /

serum of 3 patients there was a decrease in total base entirely at the expense of sodium. The serum potassium was either high normal or definitely increased, the blood urea was raised and the chlorides were reduced. In conjunction with Atchley, Benedict and Leland (1933) Loeb further demonstrated that in 3 adrenalectomised dogs the loss of sodium from the body was an important factor in producing the clinical features of adrenal insufficiency and that the loss was entirely through urinary excretion. A significant finding also was the fact that both the total amount of sodium and the concentration of sodium increased in the urine despite an increase in urine volume. He postulated from this work that one function of the adrenal cortex was to control the concentration of sodium in the blood and tissue fluids.

Harrop, Soffer, Ellsworth and Treschar (1933) confirmed that finding by showing in adrenalectomised dogs that haemoconcentration was due to loss of sodium and chloride plus their component of body water and that this loss was via the kidney. Administration of cortical extract reversed the changes observed and they concluded that the cortical hormone regulated sodium and chloride metabolism and consequently the balance and distribution of water. Their evidence pointed to the kidney as the site of the regulatory action of the cortical hormone.

The /

The most natural clinical development from this newly established biochemical similarity between the case of Addison's disease and the bilaterally adrenalectomised animal was the attempted treatment of Addison's disease with adrenocortical extracts. Rogoff and Stewart (1929) treated 7 cases, 4 males and 3 females with their own preparation which they had named 'interrenalin' and they reported improvement. Rowntree, Green, Swingle and Pfiffner (1931) treated 5 cases with Swingle and Pfiffner's preparation given intramuscularly and intravenously and reported good success. In this country Levy-Simpson (1930) was the first to use cortical extract - given him by Swingle and Pfiffner, to treat Addison's disease.

In 2 papers, Thorn, Garbutt, Hitchcock and Hartmann (1937a,b) demonstrated the effect of subcutaneously administere cortical extract on the electrolyte balance in Addison's disease. Not only was the negative sodium and chloride balance corrected to a positive one, but a potassium diuresis accompanied this. There was also a rise in the blood pressure and gain in weight. These features persisted during the period of cortin administration.

It will be remembered that about this time Reichstein and Steiger (1937) had synthesized chemically desoxycorticosterone. Levy-Simpson (1938) was again the first to use this substance as the acetate in the treatment of Addison's disease. He successfully treated 2 patients. In a fuller /

fuller report Thorn, Howard and Emerson (1939) confirmed Levy-Simpson's experience with desoxycorticosterone acetate, showing that its effects simulated those of cortin. Using 2 - 30 mg. in oil per day given intramuscularly in 8 patients they showed even when oral salt was withheld, that there was an increase in body weight, a rise in blood pressure, an increase in plasma volume, a restoration of serum electrolytes to normal, a positive sodium balance, a potassium diuresis and improved muscular strength. All of these features were reversed on withdrawal of desoxycorticosterone acetate. Other users of desoxycorticosterone acetate (Kuhlman, Ragan, Ferrebee, Atchley and Loeb, 1939) found that some of their patients developed cardiac insufficiency and muscular weakness even although their blood pressure remained normal. Two of them died suddenly at home. They then investigated the effects of desoxycorticosterone acetate in dogs and found that it did lower the serum potassium and produced periodic muscle weakness. There was increased water intake and excretion with raising of the blood pressure and on E.C.G. examination typical T-wave changes of hypokalaemia developed.

Desoxycorticosterone acetate then was not without its dangers, none-the-less it claimed a definite place in the treatment of Addison's disease. The following year Moehlig (1940) reported the implantation of desoxycorti-costerone acetate /

acetate pellets. 4 pellets each 150 mg. replaced treatment with adrenal extract plus salt and the general improvement, gain in weight, maintenance of blood pressure which followed all remained for 5 months. No change in the typical Addisonian pigmentation however was noted.

During the few years which followed, various investigators added to the total knowledge of the various actions of the adrenal extract. Conn and Silverman (1939) showed that cortical extracts were effective in Addison's disease when administered orally and that only half as much was required orally as parenterally to maintain sodium and chloride balance. Again, if salt were added to the diet - 7 g. sodium chloride daily - the oral dose could further be halved. Perla and colleagues (1930) found that rats and mice resisted surgical and histamine shock better after receiving cortical extract (Cortin) or desoxycorticosterone acetate and the effects were more pronounced if parenteral saline was combined. Of interest from the paediatric point of view was Wells and Kendall's (1940) findings that both corticosterone (1,2) and 17-hydroxy-11-dehydrocorticosterone (1,6; cortisone) retarded somatic growth when administered to rats.

It was not until 1946 that cortisone was synthesised chemically. Starting with desoxycholic acid Sarett (1946) prepared cortisone monoacetate and found it identical to biologically /

biologically obtained cortisone acetate supplied by Kendall. By 1949 sufficient cortisone acetate had been made by Merck and Co. Inc. to allow Hench to report on its beneficial effects on 16 cases of rheumatoid arthritis. This preliminary report (Hench, Kendall, Slocumb and Polley, 1949) also indicated that 100 mg. of cortisone acetate was only equivalent in efficiency to 89 mg. cortisone alcohol and that multiple small doses intramuscularly were better than a single larger dose. An important observation made by these workers, one to be used the following year by Wilkins in the treatment of congenital adrenal hyperplasia, was that the urinary excretion of 17-ketosteroids fell during treatment with cortisone acetate.

Pharmaceutical firms were not slow to appreciate the commercial potential in the bulk synthesis of steroid hormones. The search was on for analogues with equal or even greater pharmacological potency.

Meanwhile, Grundy, Simpson and Tait (1952) announced the isolation from beef adrenals of a substance with profound effects on mineral metabolism which was not any of the known biologically active cortical hormones. Later the same year Simpson and Tait (1952) demonstrated that a substance secreted in significant amounts was present in venous blood of the monkey and the dog which had marked effects on the urinary sodium/potassium ratio of adrenalectomised rats. It was 25 times as potent as an equivalent weight of cortisone in /

in retaining sodium. This substance was characterised as 18-oxycorticosterone or corticosterone-18-aldehyde and named aldosterone (Simpson, Tait, Wettstein, Neher, von Euw, Schindler and Reichstein, 1954).

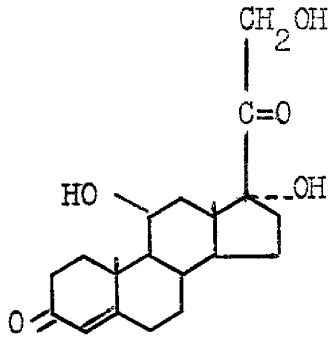
Glucocorticoid, mineralocorticoid - androgen.

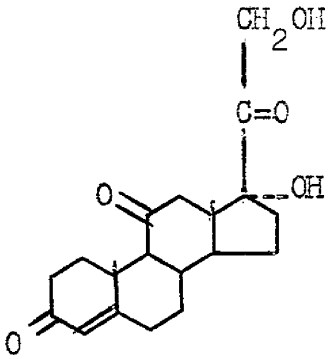
The reader will recall the earlier mention of Reichstein's (1936) finding 'adrenosterone' in adrenocortical extracts. This observation not only linked adrenocortical hormones with the sterol mucleus but indicated that the adrenal cortex also secreted hormones having biological androgenicity. Kendall, Mason and Myers (1936) oxidised their Compound E with chromic acid and the resulting diketone had one sixth to one quarter the activity of androsterone in stimulating capon comb-growth. Hodler (1936) extracted from the alkaline fraction of beef adrenals a masculinising hormone and from the benzene discard of the Swingle and Pfiffner extraction, Pottenger and Simonsen (1938) purified a white amorphous powder which when given to female rats induced functional atrophy of the uteri and uterine horns and in male rats increased testicular weight and spermatogenesis Broster and Vines (1937) extracted from the urine of virilised women, a biochemical compound which they claimed was specific to virilism of adrenal origin. This compound was active in the capon comb-growth test. After unilateral adrenalectomy quantities of the compound in the urine diminished /

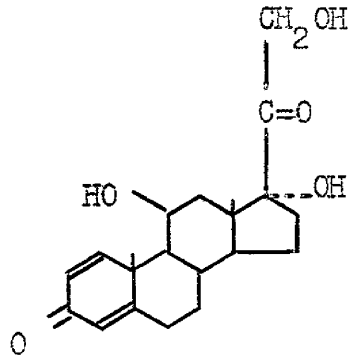
diminished and it was not found at all in the urine of normal women. That the adrenals synthesise at least 2 androgens has been shown by Short (1960) who demonstrated androstenedione and 11-hydroxyandrostenedione in adrenal venous blood.

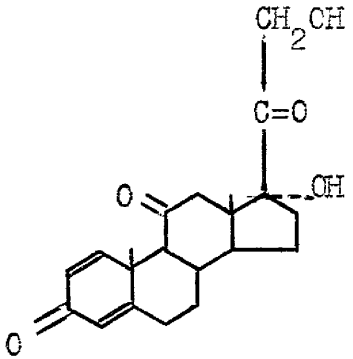
These 3 groups of steroid hormones have recently (Symington, 1962) been shown to originate from 2 histological zones of the adrenal cortex. Mineralocorticoids are synthesised in the zona glomerulosa while glucocorticoids and androgens are produced in the zona reticularis. In Symington's view zona fasciculata acts as a storage zone for steroid precursors so that in conditions of stress there is available material for immediate steroid synthesis.

1953 saw the first of the synthetic steroid analogues. Fried and Sabo (1953) working in the Squibb Institute prepared 17α-hydroxy-corticosterone and its 9α-fluoro-derivative starting with 11-epi-17-hydroxycorticosterone. This 9α-fluoro-derivative possessed strong salt retaining activity. Herzog and colleagues (1955) reported the beneficial effects of two new anti-arthritic steroids. They were prednisone and prednisolone and these were 3 to 4 times as active as cortisone or hydrocortisone. From Merck and Co. Inc., 9α-fluoro-prednisolone acetate emerged (Hirchmann, Miller, Wood and Jones, 1956) while 16α-hydroxy-lation of this compound was achieved by Bernstein and co-workers (1956). This was an important achievement for in /

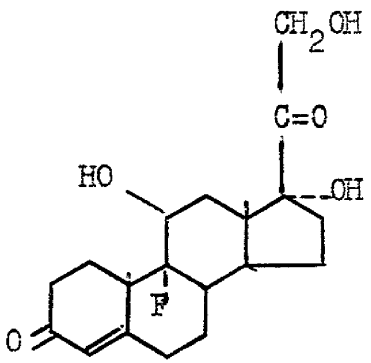


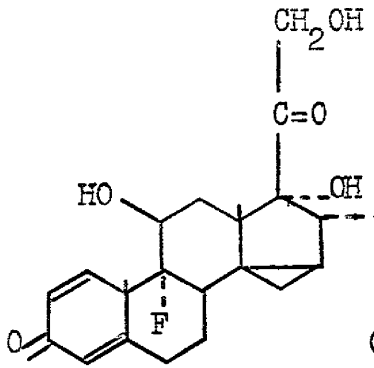


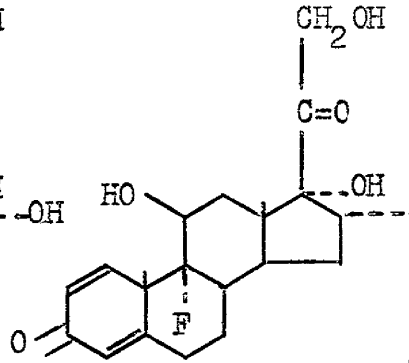


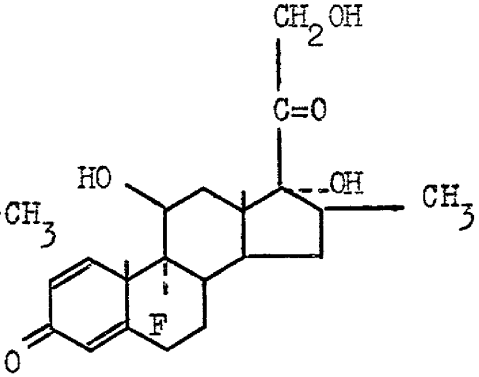


	Hydrocortisone	Cortisone	Prednisolone	Prednisone
Glucocorticoid	1	0.8	4	3.5
Mineralocorticoid	1	0.8	0.6	0.6









	9 -fluoro-hydrocortisone	Triamcinolone	Dexamethasone	Betamethasone
Glucocorticoid	10	5	30	35
Mineralocorticoid	125	0	0	0

Chart 1. The relative potencies of some hydrocortisone congeners.

in tests this new compound, triamcinolone and its diacetate were found to be 30-36 times more active than hydrocortisone in the rat liver glycogen test but they were without salt retaining properties. They concluded that 16α-hydroxylation abolishes the sodium retaining property of 9α-fluorinated steroids. This paved the way for dexamethasone (Arth and colleagues, 1958) and betamethasone (Traub, Hoffsommer, Slates and Wendler, 1958) the former with a 16α-methyl group and the latter having a 16β-methyl group. Both were claimed to exhibit an unusually high anti-inflammatory activity without having salt retaining properties. Chart I shows the molecular structures of these compounds and their accepted glucocorticoid and mineralocorticoid activity compared to hydrocortisone.

At this point I will not review the literature on the biosynthetic pathways assigned to aldosterone, hydrocortisone or the androgens for I am aware that the capacity of the average human being for sustained attention is greatly exaggerated and in every sphere there is need to practise the art of leaving off. For that matter everything is too long - lectures, speeches, sermons, books (Sir Robert Hutchison 1939).

None-the-less, the relevance of the foregoing to paediatric problems may be briefly cited. Cannata (1922) described a case of Addison's disease in an infant. In the literature /

literature he found only 2 other cases. Phillips (1887) reported 4 cases of spurious hermaphroditism in one family; this condition is now known as congenital adrenal hyperplasia. The medical treatment of this disease advanced markedly when Wilkins, Lewis, Klein and Rosemberg (1950) showed that cortisone suppressed the associated high adrenal androgen secretion. Hubble (1965) has recently claimed that dexamethasone while retarding linear growth in congenital adrenal hyperplasia, holds back skeletal maturation to a greater degree. In the text I shall present the results of treating congenital adrenal hyperplasia with prednisolone. Sikl (1948) described a case of congenital adrenal hypoplasia in an infant who died at 33 days. In 1960 Boyd and Macdonald described the condition in a family, the male members only being affected. Histologically the adrenal tissue had a characteristic morphology.

The scope then of this thesis will be as extensive as the field surveyed in the hope that having adequately reviewed the literature, I shall not only have repeated the work of others - necessarily because the diagnosis, treatment and management of patients was essential - but also that some paediatric adrenal problems if not solved will have been at least investigated.

CONGENITAL ADRENAL HYPERPLASIA

Some clinical

and

biochemical considerations.

It is appropriate to open this Chapter with some embryological, anatomical and physiological considerations.

The adrenal glands in man are formed from two separate primorida, an ectodermal portion which becomes the medulla and a mesodermal portion which becomes the cortex of the adult gland. Columnar mesothelial cells on the posterior abdominal wall, between the mesentery and the developing gonad, proliferate at about the 8 mm. stage to form a mass of cells which separate from the coelomic epithelium to become differentiated into large acidophilic cells. About the 12 mm. stage a further mesothelial proliferation of smaller cells spreads over the surface of the original condensation. These smaller cells become the definitive cortex while the initial proliferation becomes the so-called foetal cortex or X-zone.

Sympathetic cells appear on the medio-dorsal aspect of the cortex about the 11 mm. stage and invade it at the 14 mm. stage. By the 26 mm. stage they form a cell mass on the medial aspect of the more extensive cortex and are completely encapsulated later by it.

Early attempts to demonstrate steroidogenesis in foetal glands failed to reveal activity (Gersh and Grollman, 1939), but more convincing evidence that the foetal gland is capable of steroid biosynthesis qualitatively similar to /

to the adult is provided by the work of Lanman and Silverman (1957) and Solomon, Lanman, Lind and Liebermann (1958), the latter showing that the X-zone itself is active. Only the foetal glomerular zone is thought to be inactive. Ross (1962) using electron microscopy showed a lack of structural elements in foetal glemerulosa cells and this seems consistent with non-function. Some, however, believe that the foetal glomerular zone is capable of steroidogenesis (Symington, 1967). It may be that with an adequate foeto-placental exchange of sodium and water, there is no need for a foetal production of mineralocorticoid. One further interesting finding from in vitro studies with foetal zone tissue by Soloman and colleagues (1961) was the production of 16β-hydroxyprogesterone in high yield and it is of interest that the synthetic analogues of hydrocortisone with no salt retaining properties each have a C-16 substitution (Chart 1). Also, the foetal adrenal cortex has been shown to have at a very early stage of pregnancy almost the complete enzyme-histochemical characteristics of the adult tissue (Cavellero, Magrini, Dellepiane and Cizelj, 1965). The foetal adrenal gland then is capable of synthesising the cyclophentenophenanthrene nucleus, of converting the Δ5-3β-hydroxy configuration to the Δ4-3-ketone, of hydroxylation in the 17-, 21-, and 11-positions and of replacing the C-20; C21 sidechain with a 17 ketone.

Despite /

Despite this, Cathro, Birchall, Mitchell and Forsyth (1965), have isolated 3β, 21-dihydroxypregn-5-ene-20 - one from one day old infants' urine, a substance not prominent in adult urine and Villee and Loring (1965) on incubating slices of adrenal cortex from a newborn hydrocephalic child, with progesterone-4-C^{14} and pregnenolone-7-H^3 obtained hydrocortisone, androstenedione, corticosterone, progesterone, dehydroepiandrosterone, 17α-hydroxypregnenolone and 16α-hydroxypregnenolone. Very little androstenedione was obtained from progesterone. They conclude that progesterone is not necessary in the newborn for the production of hydrocortisone. This differs from the adult. Thus although the foetal adrenal cortex has normally active enzyme systems the activity of these differs from the normal adult pattern and seems to utilise biosynthetic pathways not yet completely understood.

The human foetal adrenal gland is proportionately 10 to 20 times larger than that of the adult, in the main due to hypertrophy of the central reticular zone known as the foetal zone. Shortly after birth this zone involutes by haemorrhage and necrosis and the gland remains proportionately small, gaining its birth weight again some time after puberty.

The stimulus which causes foetal adrenal hypertrophy has long been a point of controversy but since the foetal adrenal cortex is capable at least qualitatively, of /

of producing the same hormones as the adult gland there seems no reason to think that a trophic hormone other than corticotrophin should effect this highly specialised biosynthesis.

The condition which we now know as congenital adrenal hyperplasia has been recognised for more than a century. de Crechio (1865) described a patient with hypospadias, lack of external gonads, with internal genitalia of a female and with enlarged adrenal glands. Fibiger (1905) described three female pseudohermaphrodites with hyperplastic adrenals and observed that 3 siblings (2 female and 1 male) of one of his patients who died at 6½ weeks from dehydration, vomiting and diarrhoea, had similar symptoms before their deaths. Depré and Semelaigne (1925) describing a male infant, recognised the association of dehydration, vomiting and diarrhoea leading to death with congenital adrenal hyperplasia. During the late 1930's several workers (Wertheman, 1935) to mention but one, reported increased excretion of urinary 17-oxosteroids in patients with congenital adrenal hyperplasia and in 1946 Blackman published his observations from autopsy material showing that in congenital adrenal hyperplasia the reticular zone of the cortex was hyperplastic.

It will be recalled from the introductory chapter

that /

that Hench and colleagues (1949) observed the fall in urinary 17-oxosteroids in arthritic patients treated with cortisone acetate. The following year, Wilkins, Lewis, Klein and Rosenberg (1950) demonstrated that cortisone given to a female pseudohermaphrodite suppressed the secretion of adrenal hormones responsible for the high urinary excretion of 17-oxosteroids and 11-oxysteroids.

An important step in the understanding of this condition came from the work of Bartter, Albright, Forbes, Leaf, Dempsey and Carroll (1951). They administered to 3 patients with the disease both corticotrophin and cortisone. Corticotrophin caused a rise in urinary 17-oxosteroids and other reducing corticosteroids while cortisone resulted in a fall in urinary 17-oxosteroids. When administered together there was a rise in urinary 17-oxosteroids resembling the effects of administered corticotrophin alone. Their conclusions were that the high urinary excretion of 17-oxosteroids seen in the untreated case was due to excess adrenal stimulation with corticotrophin and since cortisone suppressed 17-oxosteroid secretion its action was likely to be mediated through suppression of corticotrophin. It was probably, they thought, that the adrenal glands were unable to produce cortisone sufficient to suppress corticotrophin production. How near the truth they were.

Jailer, Louchart and Cahill (1952) took the argument

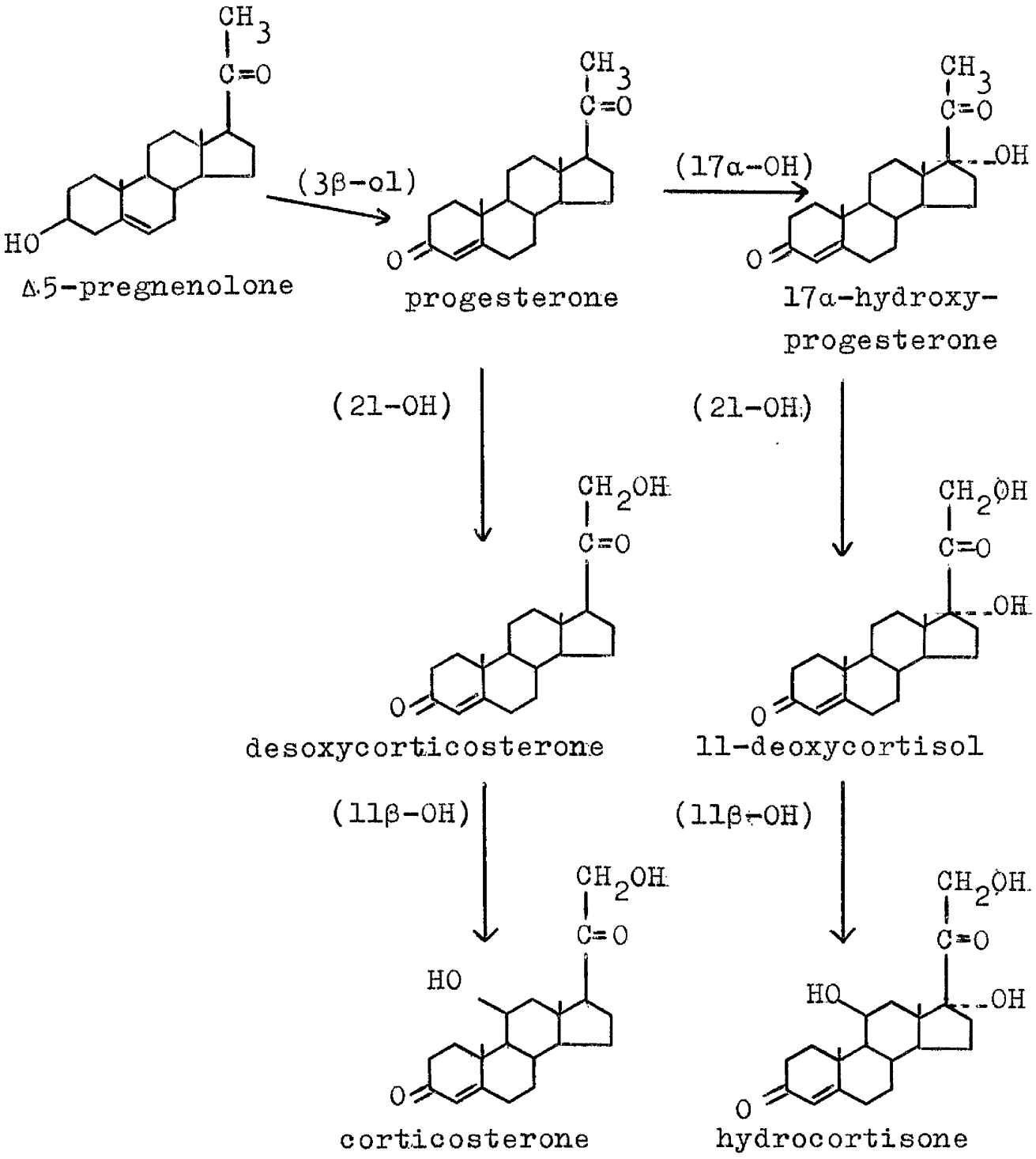


Chart 2. The biosynthetic pathways for corticosterone and hydrocortisone.

a further step forward by showing that administration of corticotrophin to 7 patients did not result in an increased adrenal production of hydrocortisone as indicated by an increase in urinary formaldehydogenic corticosteroids. It was not until 1954 that Hechter and Pincus (1954) brought together the various fragments of information, derived by several workers from adrenal gland perfusion experiments, and showed a reaction sequence for corticosteroid biosynthesis. Briefly it is as follows. Within the cortex of the adrenal, under the influence of corticotrophin, cholesterol is converted to pregnenolone (by sidechain scission from C_{27} to a C_{21} steroid) by the formation of an α,β-unsaturated ketone in Ring A, pregnenolone is converted to progesterone; C-21 hydroxylation of progesterone follows with or without C-17 hydroxylation to form either desoxycorticosterone or 17α-desoxycorticosterone (11-deoxycortisol); 11β-hydroxylation in Ring C of both these latter, forms corticosterone and 17α-hydroxycorticosterone (hydrocortisone) respectively. This is illustrated in Chart 2.

From this, the basic defect in congenital adrenal hyperplasia became more clearly understood. Butler and Marrian (1937) had isolated pregnanetriol from the urine of two women with the disease and apart from the recognition of this substance as a new triol, its significance remained obscured. When Jailor (1951) administered /

administered 17α-hydroxyprogesterone to affected patients he found an increase in urinary 17-oxosteroids and pregnanetriol. He suggested that these patients were unable to convert 17α-hydroxyprogesterone to hydrocortisone. Later Girard, Gold, vande Wiele and Liebermann (1955) administered to patients both 17α-hydroxyprogesterone and 17α-hydroxydesoxycorticosterone (11-deoxycortisol) and from the urinary findings postulated that they had a defect in C-21 - hydroxylation. The following year Eberlein and Bongiovanni (1956) demonstrated that in cases of congenital adrenal hyperplasia with hypertension there was a defect in C-11 - hydroxylation and from Bongiovanni (1961) came the finding that in some male cases with cryptorchidism and hypospadias, there is a defect in 3β-hydroxysteroid dehydrogenation. Chart 3 illustrates the sites where blocks in the biosynthesis of hydrocortisone have been detected and as a result of these, the metabolites which might be found increased in the urine.

Congenital adrenal hyperplasia is therefore a true inborn error of metabolism - the error being a defect in the biosynthesis of hydrocortisone. Genetic studies of families with the condition suggest a recessive autosomal mode of inheritance with the clinical expression in the homozyous state. Neither the incidence of the gene nor of the disease itself is known with accuracy, both /

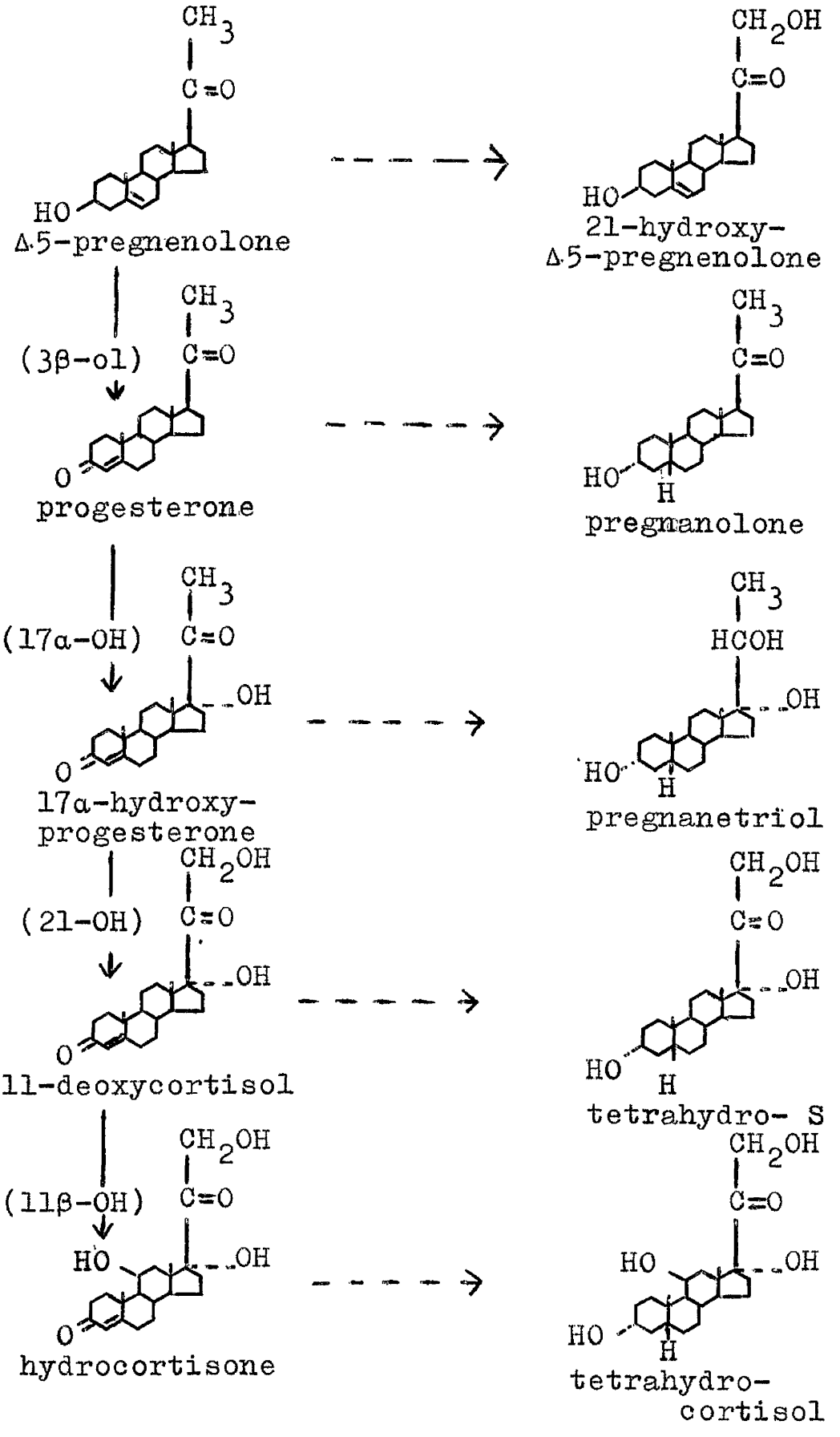


Chart 3. Urinary metabolites of hydrocortisone and its precursors in relation to the enzyme sequence.

both probably varying considerably in different populations.

Being an inborn error of metabolism, the condition is present from the beginning of steroidogenesis in the foetal cortex. As mentioned this is at a very early stage of pregnancy and the failure of hydrocortisone synthesis by a negative feedback to the foetal pituitary increases the output of corticotrophin which stimulates the adrenal cortex. Cortical hyperplasia results giving rise to an increased secretion of foetal androgens and it is to these that the abnormal genital development, particularly in the female foetus, is attributed.

Developmentally, it is about the 10 mm. stage (Table 1) when the paramesonephric ducts on each side appear as invaginations of the coelomic epithelium and as solid buds, they burrow caudally. Until about the 27 mm. stage the ducts in both male and female are identical. From then on in the female as they increase in length a lumen appears in the upper part and becomes continuous with the ostium of the invagination - thus the uterine tubes are formed. Caudally the paramesonephric ducts fuse and at the 56 mm. stage a single cavity forms within to become the utero-vaginal canal opening into the urogenital sinus. At the 63 mm. stage, on each side of the sinus the sinu-vaginal bulbs form and that part of the urogenital sinus immediately /

Table 1. Developmental processes in relation to size of foetus and gestational period.

Developmental Process	Approx. Size of Embryo.	Approx. Time Period of Gestation.
Genetic and chromosal inheritance.	Zygote.	Fertilization.
Primordia of gonads.	4-5 mm.	30-32 days.
Paramesonephric ducts develop.	10 mm.	37 days.
Primordia of adrenal cortex.	12 mm.	39 days.
Differentiation in genital duct system of male and female i.e. meso- and paramesonephric duct systems.	35-40 mm.	9-10 weeks
Sinu-vaginal bulbs in female for part of vagina and in male for prostate.	55 mm.	3 months.
Divergence of differentiation of external genitalia.	50 mm.	$2\frac{1}{2}$-3 months.
Definitive forms of external genitalia in both sexes.	100-125 mm.	4-5 months.

immediately cranial to the bulbs narrows and elongates to form the female urethra. Meanwhile the sinu-vaginal bulbs develop and fuse, later to become canalised completely so that by the 162 mm. stage the vagina is formed. By growth and elongation of the anterior vaginal wall, the vagina is carried downwards towards the perineum thus separating the urethra and vaginal orifices. In the male, after the 27 mm. stage, the paramesonephric ducts degenerate and persist only as remnants.

The external genitalia of the foetus 'in the neuter phase' appear as a central genital tubercle, flanked on either side by the genital swellings. The portion between these swellings is the shallow ectodermal closure which later is separated into a posterior portion destined to become the anus and an anterior portion which becomes the urogenital sulcus. Further sex differentiation depends on the growth of the genital tubercle. In the male it becomes the definitive phallus and from the anterior part of the urogenital sinus the urethral groove develops to extend along the ventral surface of the genital tubercle as the urethral folds and becomes the urethra. In the female, at the 25 mm. stage, the urethral tubercle is bent caudally and is not encroached upon by the urethral groove. Therefore there is no penile urethra.

The genital swellings enlarge to become the labia majora, the /

the labia minora being the unfused urethral folds.

This foetal sexual differentiation is controlled by foetal endocrine secretions and Jost (1961) has shown that foetal androgens exert a stimulating influence on mesonephric duct derivatives, a masculinising effect on the urogenital sinus and external genitalia, and a suppressing effect on the paramesonephric derivates.

Thus in congenital adrenal hyperplasia, where the foetal androgens from the adrenal cortex are increased in both sexes, the male child exhibits macrogenitosomia praecox and in the female, clitoral enlargement and fusion of the labia majora posteriorly leaving anteriorly a single perineal opening into a urogenital sinus. Frequently the urethra in the female fails to descend so that it communicates with the urogenital sinus through the anterior vaginal wall. Fortunately there is no suppression of the paramesonephric derivates and the female case has a vagina, uterus and tubes.

Clinically, affected neonates may be detected by the somatic abnormalities mentioned but it is vitally more important to recognise the possible biochemical upset resulting from complete failure of hydrocortisone biosynthesis. The features are those of adrenal insufficiency.

Before this information was available Butler, Ross and Talbot (1939) described a male infant, aged 20 months with /

with macrogenitosomia praecox who showed also low serum sodium and high serum potassium levels and who had collapsed frequently from the age of 2 weeks if fluid and salt were restricted. By giving relatively large quantities of sodium chloride and a potent adrenocortical extract the child gained weight and remained in reasonable health. The following year Wilkins, Fleischmann and Howard (1940) described a boy aged 3 years 7 months with macrogenitosomia from birth; pubarche at the age of 15 months and testicular enlargement from the age of 18 months. From the age of 1 year he had shown a marked craving for salt and highly salted food. He died suddenly and at the time of death the serum sodium was 111mEq./litre. At autopsy the adrenals were enlarged with marked hyperplasia of the androgenic foetal cortex. There were also aberrant adrenal cells in the testes.

Although the powerful mineralocorticoid, aldosterone had been isolated from beef adrenals in 1952 (Grundy, Simpson and Tait, 1952) it was not until 1959 that Blizzard, Liddle, Migeon and Wilkins (1959) were able to show that in cases of congenital adrenal hyperplasia with salt-loss there was no increase in urinary aldosterone if oral salt was withheld. Bryan, Kliman and Bartter (1962) demonstrated impaired production of aldosterone in salt-losing /

losing cases and showed that salt restriction increases aldosterone secretion minimally.

It is clear from Chart 4 that failure of aldosterone production in the context of congenital adrenal hyperplasia could be due either to a block in C-21-hydroxylation or to a failure of 3β-hydroxysteroid dehydrogenation. If C-21-hydroxylation is completely absent then in addition to the somatic abnormalities the patient has salt-loss. If the defect is only partial, then by hypertrophy the gland compensates and produces adequate amounts of glucocorticoids and mineralocorticoids. Such a patient has no salt-loss. In both instances because of the hyperplasia there in increased production of adrenal androgens resulting in early and rapid increase in stature and muscular development, advanced ossification with early epiphyseal closure, early pubarche often during the first year of life associated with macrogenitosomia praecox in the male and masculisation in the female. If 3β-hydroxysteroid dehydrogenation is defective there is impaired formation of the Δ4-3-oxo configuration in Ring A of the steroid nucleus. This is an important conversion for it is this configuration which gives to glucocorticoids, mineralocorticoids and androgens physiological activity. Thus in congenital adrenal hyperplasia due to this enzyme deficiency neither hydrocortisone, aldosterone nor testosterone /

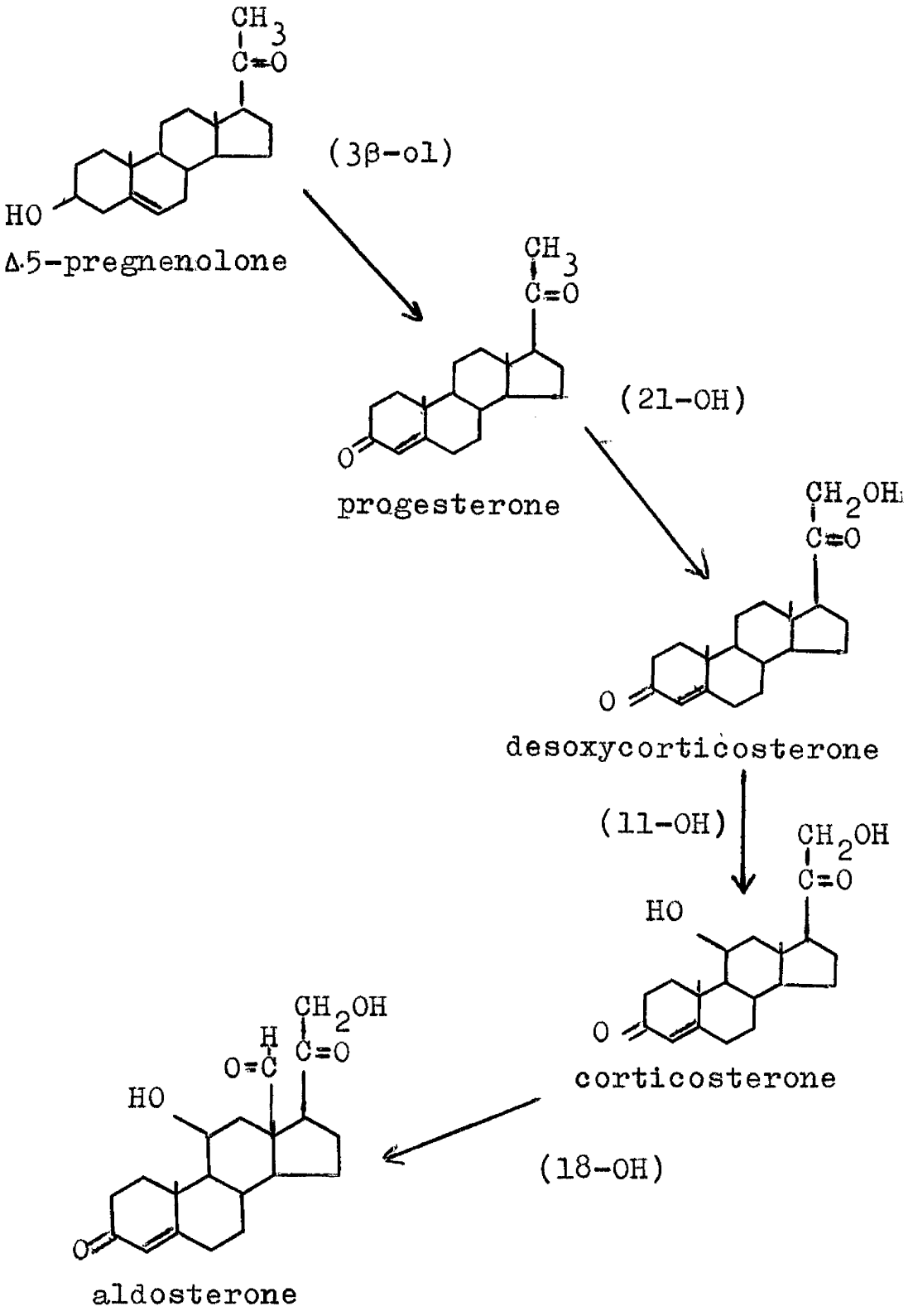


Chart 4. The biosynthetic pathway for aldosterone.

testosterone can by synthesised. The patients have salt-loss, showing early signs of adrenal insufficiency and in addition the male case is incompletely masculinized (cryptorchidism, bifid scrotum, hypospadias) for lack of potent foetal androgen. That foetal testes are capable of synthesising testosterone, albeit in small amounts, has been shown by Rice, Johanson and Sternberg (1966).

A discussion on the management of congenital adrenal hyperplasia will be more appropriate after the presentation of the clinical and scientific data concerning the 11 cases to be presented in this chapter.

CASE REPORTS.

This series of 11 cases is comprised of 8 females and 3 males (Table 2). Two males and 2 females have salt-loss, and 7 have no salt-loss. One male (Case 4) has deficiency of the enzyme 3β-hydroxysteroid (3β-ol) dehydrogenase and the other 3 with salt-loss have 21-hydroxylase deficiency. The 7 without salt-loss (6 females and 1 male) have partial 21-hydroxylase deficiency. Two cases (Cases 4 and 10) are the second affected children in their families. The sibling of Case 4, an alleged male, died aged 1 month in 1960 as a result /

Table 2. Evidence relating to diagnosis in 11 cases of congenital adrenal hyperplasia.

Case	Sex	Age at which spec. tested	Urinary steroids mg./day			Clinical Type	Enzyme Deficiency Biochemical Type
			17-OS	Pregnanetriol	Others		
1	F	8.8	10.2	40*		Simple Virilism	21-OH
2	F	7.5	7.4	12		Virilism salt-loss	21-OH
3	M	3.3	9.3	14.3		Macro-genitosomia	21-OH
4	M	2 days	4.7	0.18	Dehydroepiandrostene in excess 3 ,21-dihydroxy-pregn-5-ene-20 one (Cathro; et al.)	Incomplete or cryptorchid male	3 - ol.
5	F	0.16	3.1	1.2		Simple Virilism	21-OH
6	F	8.16	6.0	4.4		Simple Virilism	21-OH
7	F	0.24	3.4	2.5		Simple Virilism	21-OH
8	F	3.4	5.0	33		Simple Virilism	21-OH
9	F	3.16	5.2	13.6		Simple Virilism	21-OH
10	F	0.24	3.6	4.2		Virilism Salt-loss	21-OH
11	M	0.08	3.8	20.6*		Macro-genitosomia salt-loss	21-OH

*Falsely elevated by non-steroid urinary pigments.

result of diarrhoea, vomiting and dehydration. He is said to have had a normal penis, scrotum and testes. At autopsy the adrenals were hyperplastic and the diagnosis was made post-mortem. The sibling of Case 10, a registered male, died aged 1 month in 1952 from diarrhoea, vomiting and dehydration. He is said to have had a penis with hypospadias and a scrotum containing two testes. At autopsy the adrenals were hyperplastic weighing 16 g. and 17 g. There is no comment regarding internal genitalia found at autopsy. Since Case 4 has 3β-hydroxysteroid dehydrogenase deficiency, his dead sibling could not have been completely masculinized, since deficiency of this enzyme results in incomplete masculinization. It is unlikely that 2 gene mutations would occur in 1 family. For a similar reason, the dead sibling of Case 10 (21-hydroxylase deficiency) could not have been a male with hypospadias since macrogenitosomia is characteristic of the affected male with this enzyme deficiency. Dr. M. A. Ferguson-Smith was invited to determine the sexes of these two children from H and E stained sections from specimens taken at autopsy. His comments are that both are chromatin negative. If one does not doubt the pathologist's findings referable to the gonads and adrenals then there is a possibility that other clinical types of congenital adrenal hyperplasia exists.

Table 3 /

Table 3. Chronological data and development in relation to treatment.

Case	1. F	2. F	3. M	4. M	5. M
Date of birth	27.1.54	16.5.55	8.7.58	10.10.62	13.1.62
Ages at diagnosis	5 weeks	2 weeks	15 mos.	2 days	2 weeks
Salt-losing	No	Yes	No	Yes	No
Age at beginning of treatment	8 years 10 mos.	3 weeks	15 mos.	2 days	16 weeks
Bone age at beginning of treatment	Normal	Normal	4 years	Normal	Normal
Treatment	P	I.V.Saline C. DOCA	DX.P. C.	Cr.DX.P	C. PTA.
Onset of puberty	Menses 9.75 yr.	Menses 11½ yr.	Pubarche 3 yr.	Not yet	Not yet
Age at epiphyseal closure or present bone age	11 years	13.8 years	11.6 years	8.7 years	7.8 years
Actual age (Jan. 1967)	13 years	11½ years	8½ years	4 years 3 mos.	5 years
Actual height	143 cm.	1.37 cm.	121 cm.	93 cms.	105 cms.
Mother's height	152 cm.	160 cm.	156 cm.	158.75cm.	161.5cm.
Father's height	159 cm.	172 cm.	162 cm.	176.5 cm.	182.8cm.

P. Prednisolone
C. Cortisone
DX. dexamethasone
DOCA. desoxycorticosterone acetate
P.P.A. prednisolone trimethylacetate

F. Female
M. Male

Table 4. Some data of cases 6 - 11.

Case	6.Female	7.Female	8.Female	9.Female	10.Female	11.Male
Date of Birth	7.8.56	23.4.65	28.9.63	24.2.63	11.7.66	18.12.66
Age at diagnosis	8 yrs. 2 mos.	8 weeks	3 years	3½ years	4 weeks	17 days
Bone age at diagnosis	11 yrs.	Normal	7 years	6.2 yrs.	Normal	Normal
Treatment	P.	None	DX	DX	C;9 flF.	C;DOCA:9flF.

P. prednisolone
DX. dexamethesone
C. cortisone
9α-flF 9α-fluorohydrocortisone

Table 5. Biochemical findings in salt-losing cases of congenital adrenal hyperplasia.

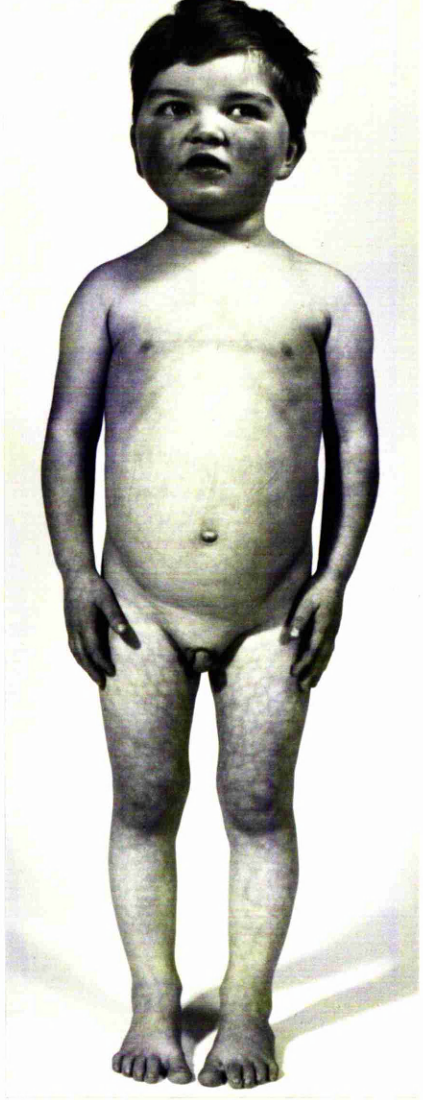
Case	Sex	Clinical Onset	Serum electrolytes mEq./litre			Birth Weight Kg.	Admission Weight Kg.	Enzyme Defect
			K^+	Na^+	Urea			
2	F	2 weeks	13.8	131	67.5	3.2	2.9	21-OH
4	M	2 days	6.5	140	93.3	3.2	3.2	3 -ol
10	F	30 days	9.5	130	200	3.5	3.2	21-OH
11	M	12 days	5.0	118	40	2.63	2.1	21-OH

Table 3 gives data of Cases 1 - 5 who have been under observation for more than 4 years. In Table 4 are the corresponding data for Cases 6 - 11 and although these patients have been under treatment for a short time only they are included because urine obtained before treatment was started in each case has provided material for research. Also some clinical aspects of their management are of importance.

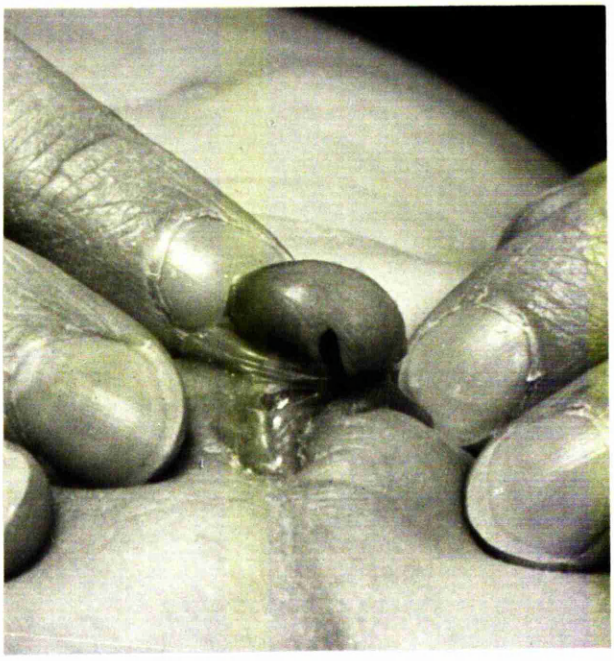
Incidence.

Four of the 11 cases are indigenous to Glasgow, the others coming from neighbouring shires - Lanarkshire 3, Ayrshire 2, Stirlingshire 1, and Renfrewshire 1.
It would be inadvisable on these few cases to calculate the incidence of heterozygotes in the general population. Further, it is a cause for concern that 2 neonates have died from the salt-losing variety of the disease, the diagnosis having been made at autopsy. The true incidence of the disease might therefore be higher than is known since diarrhoea and vomiting in an infant is all too frequently assumed to have an infective basis. It is probable that in some compensated cases, adrenal insufficiency is precipitated by an infection and the true diagnosis missed. Wilkins (1962) has put the incidence in the general population of the heterozygote between 1 in 50 to 1 in 100, while childs, Grumbach and Van Wyk (1956) estimate it at 1 per 128. On either score, if /

12 days



5 years



Case 4. 3β-hydroxysteroid dehydrogenase deficiency.

if these figures are applicable to the population in this area, more cases of the homozygotes might be expected and at least 2 are on record from postmortem diagnoses.

Salt-losing cases.

The salt-losing cases present in the early days or weeks of life with variable vomiting and diarrhoea but consistently with dehydration - (see photograph Case 4) which reflects in their admission weights being under their birth weight (Table 5). Fantus' test on the urine will indicate at this stage that excess sodium chloride, the chief inorganic constituent of normal urine, is being excreted. This information is of great value for in diarrhoea and vomiting due to infection, or with vomiting due to congenital pyloric stenosis, the urinary sodium chloride excretion is diminished. Further, infants with adrenal insufficiency will excrete large quantities of urine and the test can readily be performed.

Biochemically the serum electrolytes and blood urea are characteristic of adrenal insufficiency. The serum potassium and blood urea are raised and serum sodium low normal or low. Of these, the serum potassium shows the earliest change and is raised before the serum sodium falls. This is seen in Case 4 (Table 6), in whom at age 2 days the serum potassium was 6.5 mEq./litre.

In /

In association with genital abnormalities and a positive Fantus' Test, an early rise in serum potassium is significant. An E.C.G. at this stage may not show evidence of potassium intoxication although very shortly thereafter the typical tall, broad T-wave becomes evident.

Fantus' Test. 1 drop of 20% potassium chromate solution is added to 10 drops of urine. 2.9% silver nitrate solution is added drop-wise, the tube being shaken after each addition until a yellow to brown end point is reached. The number of drops of silver nitrate used gives the concentration of chloride in the urine expressed as sodium chloride per litre of urine. Values in excess of 3 g./litre, before salt is added to the treatment are excessive.

At this stage the true sex of the child is less important than the correction of the biochemical abnormality. All 4 cases with salt-loss (Cases 4, 10 and 11, I have attended personally), received added salt immediately on diagnosis, in Cases 2, 10 and 11 as intravenous half-strength physiological saline (200 ml./kg. body weight per day), and in Case 4 orally. During the first 24-hour period corticosteroids were withheld and this permitted a collection of urine unadulterated by exogenous steroids. These specimens were reserved for steroid analysis.

Experience from Cases 2, 10 and 11 and from 2 other patients who died as a result of congenital adrenal hypoplasia, indicates that the sudden onset of adrenal insufficiency /

insufficiency occurs from the tenth to the fourteenth day of life. Case 10 had had symptoms from the second week of life but had been receiving from then onwards added salt in the feeds, prescribed by the medical attendant for the wrong reason. Case 4 was diagnosed at age 2 days and the excessive urinary loss of sodium chloride, often as high as 10 g./litre, was matched with oral replacement. Intravenous fluid therapy therefore was not required.

Notwithstanding adequate salt replacement, intravenously or orally, those with salt-loss have required remarkably high daily steroid replacements, both glucocorticoids and mineralocorticoids, to maintain them in good clinical condition, more than is required to suppress the abnormally high urinary excretion of 17-oxosteroids. This is shown in Table 6.

Table /

Table 6. Daily requirements of salt and exogenous steroids to maintain good clinical response, in 4 cases of congenital adrenal hyperplasa with salt-loss.

Case.	17-OS before treatment mg./day.	Daily requirements to maintain good clinical response.				17-OS after treatment mg./day.
		Salt gm.	DOCA mg.	9α-fl. F.mg.	Cortisone mg.	
2 F.	3.08	3	3	-	50	0.7
4 M.	4.8	3	3	-	100	1.9
10 F.	1.8	-	1	0.15	50	0.8
11 M.	3.8	1.5	3	0.3	100	1.8

DOCA desoxycorticosterone acetate.
9α-fl.F 9α-fluorohydrocortisone.

The daily hydrocortisone secretion rate for adults is 5.0 - 27.9 mgm. (Cope and Black, 1958) and for children aged 2 months to 16 years (Loras and colleagues, 1965) 9.9 mgm. (mean value). Yet Cases 2 and 10 required 50 g. hydrocortisone daily and Cases 4 and 11, 100 mg. daily. This is in keeping with the early experimental findings (Cartland and Kuizenga, 1936) that adrenalectomised growing rats required 300 times as much cortical hormone on a per kilogram basis to maintain life, as did the adult adrenalectomised dog. The values for the urinary 17-oxosteroids after treatment are /

are slighly higher than the accepted normal for this age group (0.5 mg. + 0.2) but these values are augmented by a contribution from the administered cortisone, 1 - 2 per cent of which is excreted as a 17-oxosteroid.

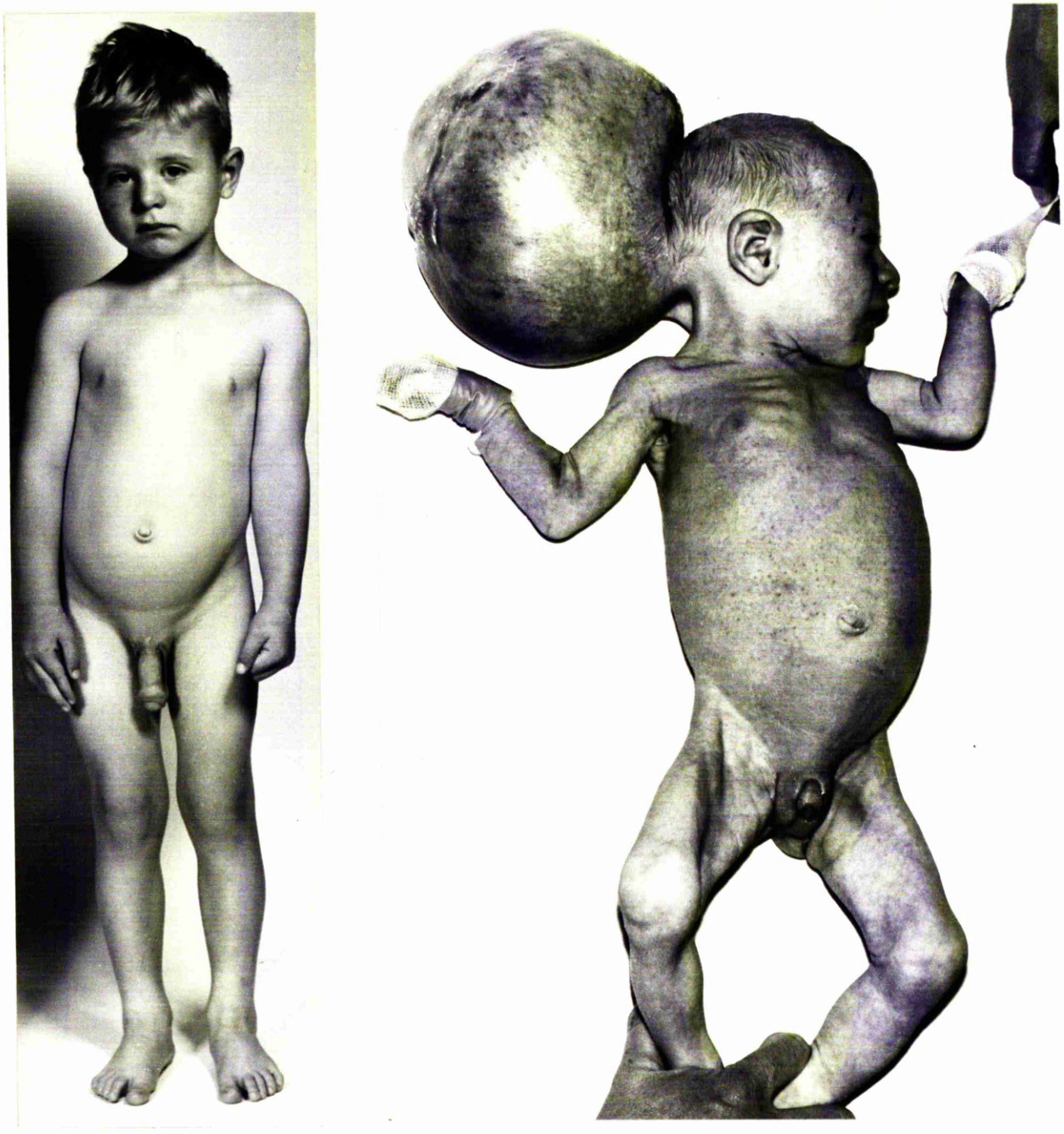
Case 2 is now 12 years. Until 6 years she was maintained on DOCA subcutaneous implants (200 mg.) every 4 to 6 weeks, prednisolone 7.5 mg. daily and added salt to satisfy a craving which had slowly developed. She was, in fact, eating 25 - 30 g. salt daily which created intense thirst with consequent polydipsia and polyuria of 3 litres per day. During her early years she had frequent adrenal crises associated with the zymotic illnesses. Under my personal supervision she was given 9α-fluorohydrocortisone 0.1 mg. daily, increasing by 0.1 mg. daily at weekly intervals. When the dosage reached 0.3 mg. daily she apruptly stopped eating salt and the polydipsia and polyuria ceased. Until the present she has remained normotensive, and has been free from adrenal crises. She menstruated for the first time on 1.1.1967.

Case 4 is now 4 years 3 months. During the first nine months of life he was maintained on cortisone 37.5 mg. daily, DOCA intramuscularly 1 mg. daily and 6 - 8 g. salt daily. He had one adrenal crisis during this period. At age 9 months DOCA was replaced by 9α-fluorohydrocortisone 0.1 mg. b.d. and on the second day of administration he refused the customary salted food /

food and has since then required no extra salt. He has remained normotensive requiring now 9α-fluorohydrocortisone 0.1 mg. daily. There have been no further adrenal crises.

Cases 10 and 11 have both received 9α-fluorohydrocortisone orally (0.15 mg. daily) when they were able to retain milk feeds. This has simplified their management in the acute phase of treatment after diagnosis, and although both require extra salt (up to 2 g. daily) this was a temporary requirement. These patients are at present 6 months and 1 month respectively and are making progress although Case 11 has required, before progress was evident, DOCA 3 mg. daily, 9α-fluorohydrocortisone 0.3 mg. daily and cortisone 100 mg. daily.

From this experience, I make the claim that 9α-fluorohydrocortisone with its powerful mineralocorticoid effect, is a drug eminently suitable for correcting the salt-losing state in neonates and can be given with safety one the infant retains oral feeds. With carefully graduated dosage, there is no need for long-continued extra dietary salt, neither is there great risk of hypertension or oedema. DOCA has an undoubted place in the treatment of adrenal crises or when vomiting is troublesome, but from this limited experience of 4 cases I have found that crises are fewer when daily 9α-fluorohydrocortisone is the mineralocorticoid used.



Case 3. Macrogenitosomia praecox aged 4 years with sister (Case 7) aged 3 months

Non-salt-losing cases.

The 7 cases in this group, with the exception of Case 7, presented with genital abnormalities. Cases 1 and 5 were investigated within the first few weeks of life, while Cases 3, 6, 8 and 9 did not come under observation until aged 15 months, 8 years, 3 years and 3½ years respectively, this delay being due largely to parental desire to conceal the abnormalities of the genitalia. Cases 1, 5, 7 and 8 had an enlarged clitoris with a single perineal opening into a urogenital sinus, Cases 6 and 9 had an enlarged clitoris but with a normal vaginal opening and Case 3 had macrogenitosomia praecox. Case 7, a registered male, but a genetic female, was the sister of Case 3 (see photograph) and had a massive encephalocele. In this group it is important to establish the genetic sex. Case 8 had been admitted to a surgical ward to have the 'hypospadias urethral opening' brought to the tip of the 'penis by plastic surgery but when her true sex was determined she was submitted to further endocrine studies and the true diagnosis made. Of the 7 without salt-loss, 6 are genetic females and one a male.

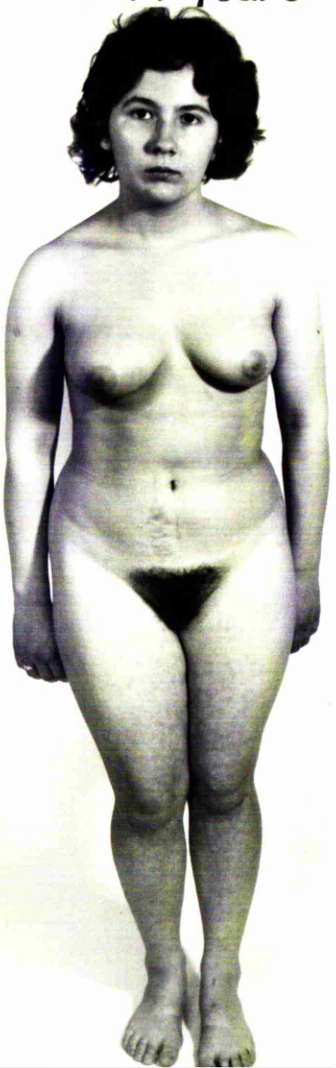
Vaginograms and cystograms were performed on all the female cases, salt-loser and non-salt-losers. Only Case 6 has a normally sited vagina and urethra, Cases 1, 2, 5, 7 and 8 having a urogenital sinus leading into a vagina /

At birth

Case 1. Virilism:no salt-loss

Vaginoplasty with amputation of clitoris.

14 years



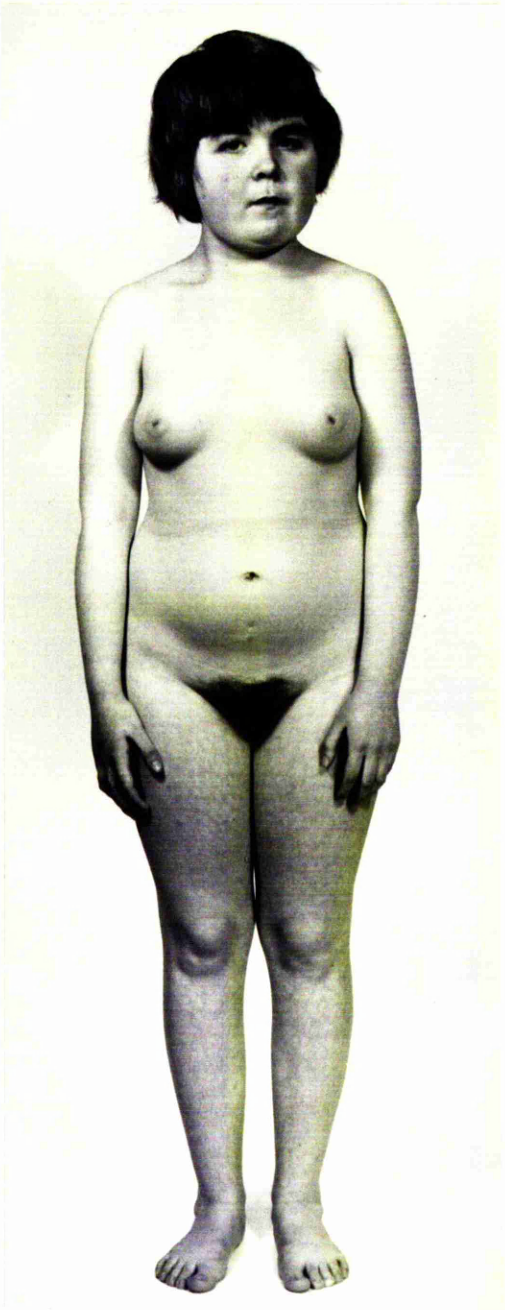
vagina, and Cases 9 and 10 have a vaginal orifice through which the bladder can be entered by a catheter from a point 0.5 cm. along the anterior vaginal wall.

Surgical treatment of the perineum.

Because of the gross cerebral abnormality in Case 7, all treatment was withheld, the parents took the infant home and she has since died in Ireland.

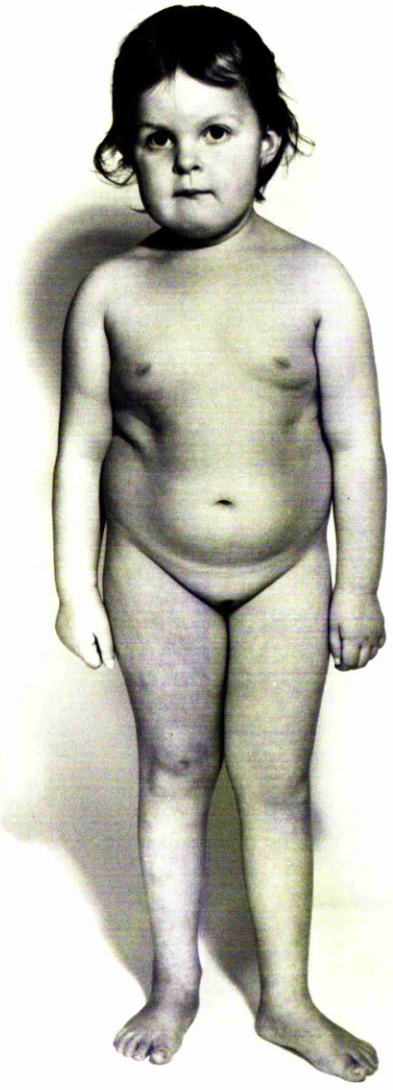
Although Case 1, (see photograph) was diagnosed early in 1954, suppressive treatment was not then started. There were long lapses between follow-up attendances. In 1960 when aged 6 years she attended a surgical clinic and was admitted for investigations. These included laparotomy to determine the nature of the internal genitalia, and when uterus, tubes and ovaries were seen amputation of the clitoris was performed. In 1962 when aged 8 years, the urogenital sinus was explored through a midline incision. A small normally sited vaginal opening was found with a urethral opening on its anterior wall immediately within it. Mucosa was sutured to skin and the wound allowed to heal. The good results are seen in the photograph. From 1962 she has been on suppressive treatment which will shortly be discussed.

Cases 2, 5 and 8 had similar anatomical abnormalities. Cases 2 and 5 were diagnosed at birth and have since then been receiving steroid therapy. Case 2 has salt-loss and /



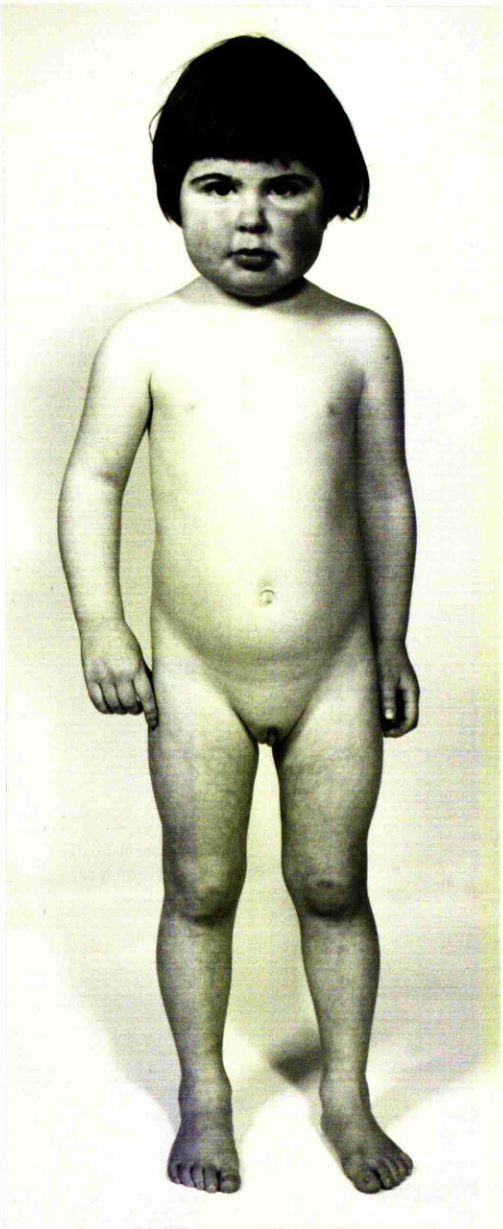
12 years

Case 2. Virilism with salt-loss. Vaginoplasty only.



5 years

Case 5. Virilism:no salt-loss. Vaginoplasty only.



3.5 years

Case 8. Virilism:no salt-loss. Vaginoplasty only.

and is well controlled with 9α-fluorohydrocortisone and prednisolone. When aged 9 years vaginoplasty was performed, (Mr. Wallace M. Dennison) but the clitoris (2.5 cm.) was not amputated on the reasoning that with adequate suppressive treatment there should be no further clitoral growth and by subsequent adnexal development it would become normally concealed. Vaginoplasty (W.M.D.) was performed on Case 5 when aged 4 years and again the clitoris 1.5 cm. was not amputated. Both cases 2 and 5 required supportive therapy during the operation, Case 5 collapsing twice during the period of anaesthesia. In 1950 when Case 8 (no salt-loss) came under observation it was decided to perform the vaginoplasty immediately and to commence suppressive treatment concurrently. The clitoris was not amputated. The clinical photographs show the post-operative results (q.v.).

Few papers comment on the surgical treatment of congenital adrenal hyperplasia. In the past the surgeon was invited to implant DOCA pellets subcutaneously and correction of the external genitalia was postponed until puberty or later by which time in the absence of steroid replacement therapy menses did not occur. Raiti and Newnes (1964) recommend subtotal clitoroidectomy and vaginoplasty at the age of 18 months to 2 years.

Under adequate suppressive therapy a large clitoris diminishes /

diminishes in size and only when treatment has been commenced late need amputation be considered. Lattimer (1960) has given details of a procedure which aims at reduction in clitoral size but with preservation of the glans. This may be important for later marital relationships. Subtotal amputation of the clitoris is therefore inadvised since some reduction in size can be expected during treatment. If at puberty the clitoral size is still unacceptable, the Lattimer operation (1961) would seem to be preferable to amputation.

Growth.

Only Cases 1 to 5 will be discussed, these having been under observation and treatment for more than 4 years. Increased statural growth in congenital adrenal hyperplasia is caused by the excessive secretion of adrenal androgens. These also cause early epiphyseal fusion, growth ceasing early and the patients becoming small adults. It is the rule in the untreated case for ossification to proceed much more rapidly than height is gained so that although patients are taller than average until epiphyseal fusion the maximum height achieved falls short of normal adult standards.

The aim of suppressive treatment with reference to growth is to suppress adrenal androgen production sufficiently to delay epiphyseal fusion but at a dosage level which will /

will allow normal linear growth. However, the ultimate height attained will in part be determined by genetic factors governing family height so that each patient has to be seen against his own genetic background. Wilkins and his colleagues (1950) suppressed the androgen secretion with cortisone and reviewed the effects after 4 years (Wilkins and Cara, 1954). The same group (Green, Cleveland, Wilkins, 1961) reported that triamcinolone suppressed androgen production, osseous development and growth, the latter two equally. Side-effects such as fever delirium and blurring of the optic discs, were too frequently encountered to justify its continued use. The use of the long-acting steroid preparations, prednisolone trimethylacetate and methylprednisolone acetate given intramuscularly at intervals up to 28 days is reported (Dingman, Maranan and Staub, 1962) to give uniformly smooth adrenal suppression although in one of their children growth was slowed but osseous development continued. In another child, both ossification and growth were slowed and in the other four patients they only claim that excessive retardation of growth was not apparent.

The ideal drug would suppress adrenal androgen secretion, retard the skeletal maturation and allow somatic growth to proceed normally. Unfortunately, the /

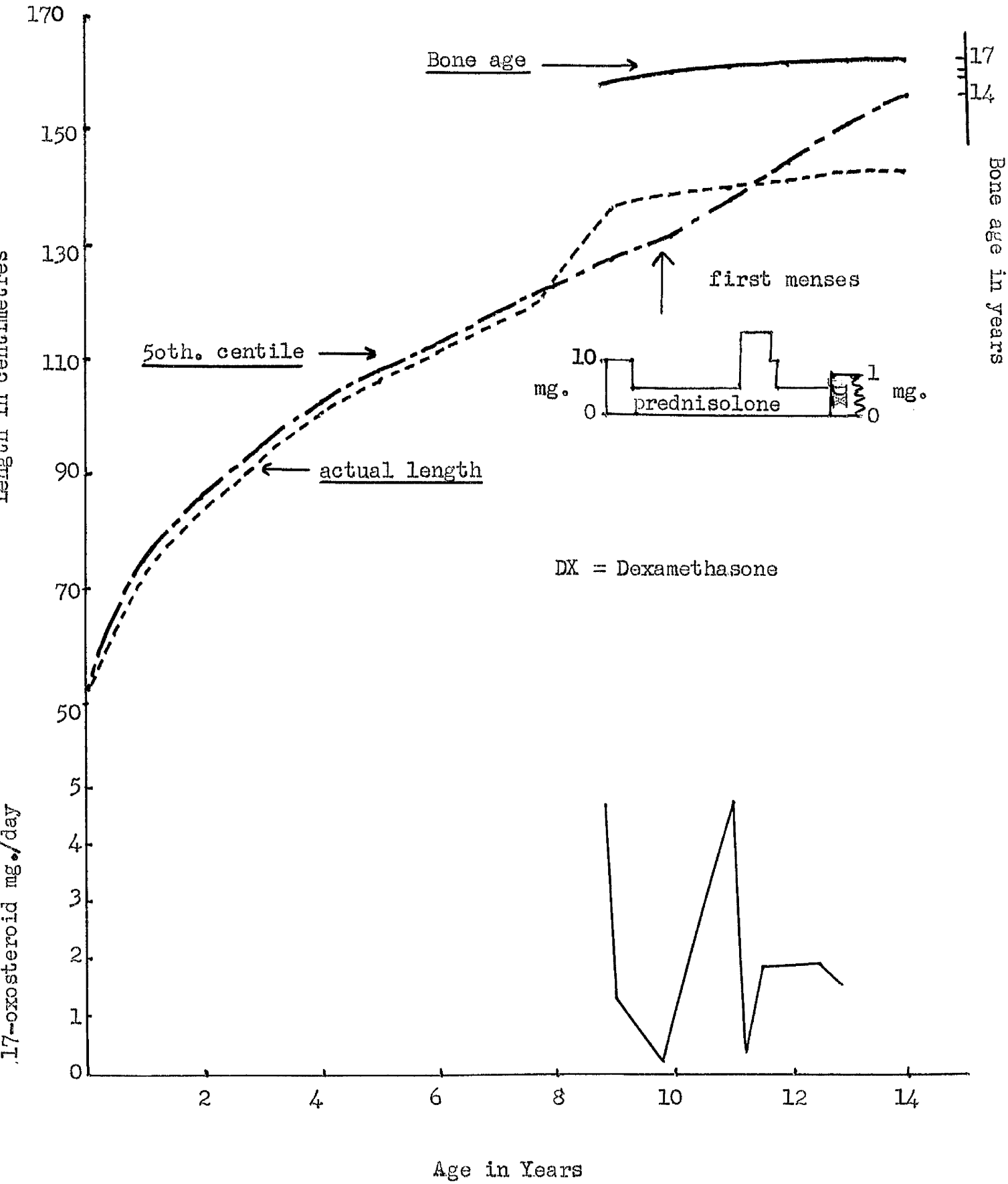


Chart 5. Case 1 - female with simple virilism. Linear growth and bone maturation in relation to suppressive treatment.

the synthetic glucocorticoid substances used in suppressive doses all retard somatic growth but Hubble (1965) has given evidence that dexamethasone, at least in the simple virilising form of congenital adrenal hyperplasia retards bone maturation more than it does linear growth.

The growth charts for the five cases are shown in Charts 5, 6, 7, 9 and 10.

The overall evidence from this group is that when the dosage of suppressive steroids is so graduated to allow linear growth, bone maturation advances more rapidly creating an increasing gap between height age and bone age. If steroid dosage is increased to delay bone maturation, the trend in the growth curve flattens more than does the curve for ossification. This is seen in Cases 1, 2, 3 and 4. Only in Case 5 are both parameters increasing in parallel, bone maturation being in advance.

Case 1 (female simple virilism: Chart 5) was not treated until aged 8 years 10 months when prednisolone 10 mg. daily was given. Linear growth till then had proceeded along the 50th percentile and from 8 years to 9 years there was a 14 cm. gain in height which might have represented the prepubertal growth spurt for menstruation occurred within a further year. At the onset of menstruation, one year after treatment, the bone age was 14 years. Epiphyseal fusion followed rapidly

despite /

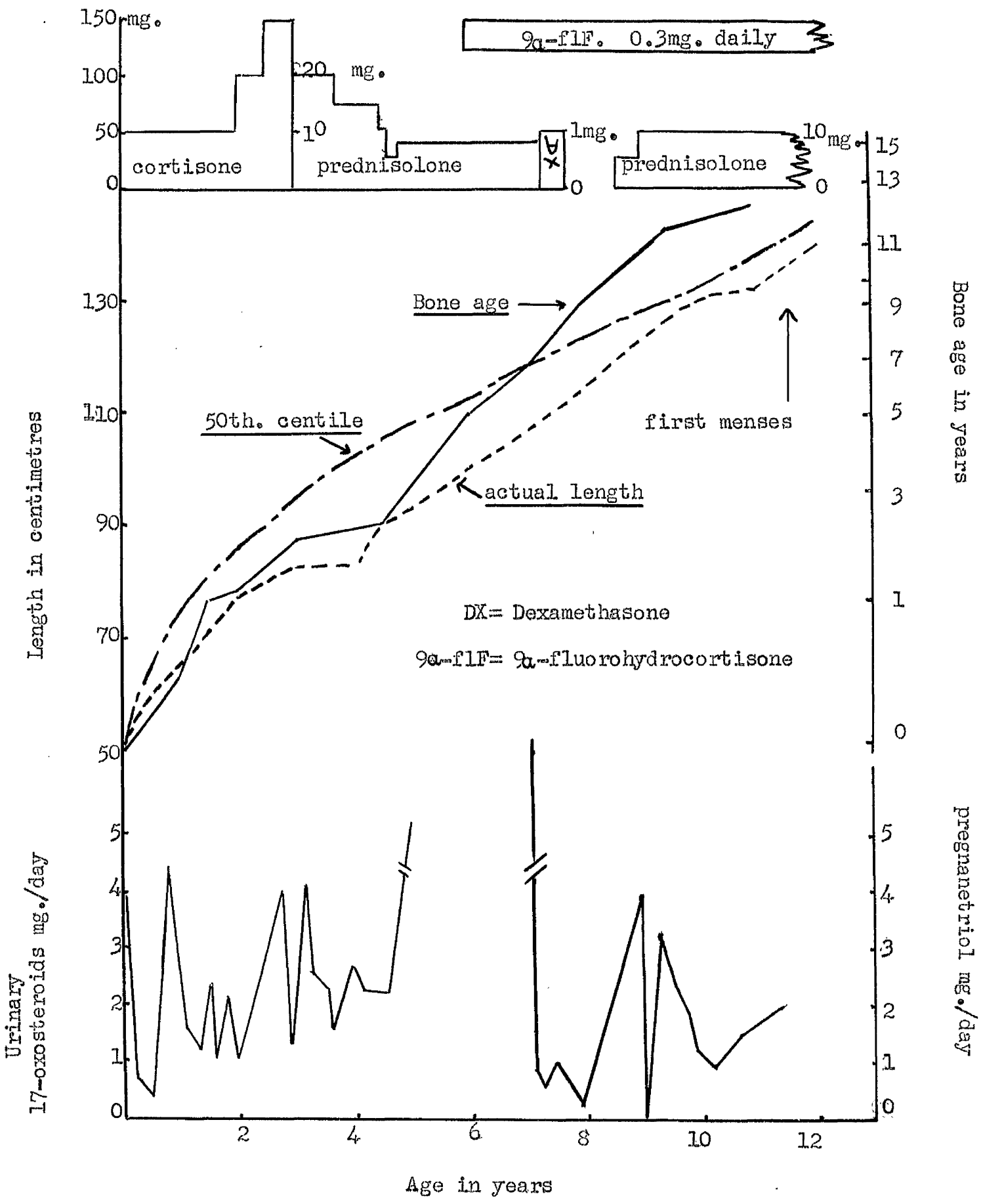


Chart 6. Case 2 - female with salt-loss. Linear growth and bone maturation in relation to suppressive treatment.

despite an increase in prednisolone to 12.5 mg. daily and presumably both factors contributed to the final short stature of 140 cm.

Case 2 (female with salt-loss: Chart 6) required high steroid dosage during the first 4½ years of life as shown. Both linear growth and bone maturation were retarded almost equally. Between 4 years 9 months and 7 years 9 months the daily glucocorticoid was reduced to 7.5 mg. prednisolone or the equivalent of dexamethasone allowing a more rapid increase in bone age than height age although the height did approach the 50th percentile. During one year, 7 year 9 months to 8 years 7 months when all glucocorticoid drugs were withdrawn there was no gross widening of the gap and the actual height came near to the 50th percentile. However, it was clear that 9α-fluorohydrocortisone, the only drug used during that period did not prevent further adrenal hyperplasia and prednisolone was restarted at 5 - 10 mg. daily. With this, growth has almost ceased while the bone age has continued to advance. When the bone age reached 13.5 years the girl menstruated. Epiphyseal fusion has not yet taken place, her height being 137 cm.

It is interesting that Cases 1 and 2 menstruated at an age more in relation to their bone age than to their chronological /

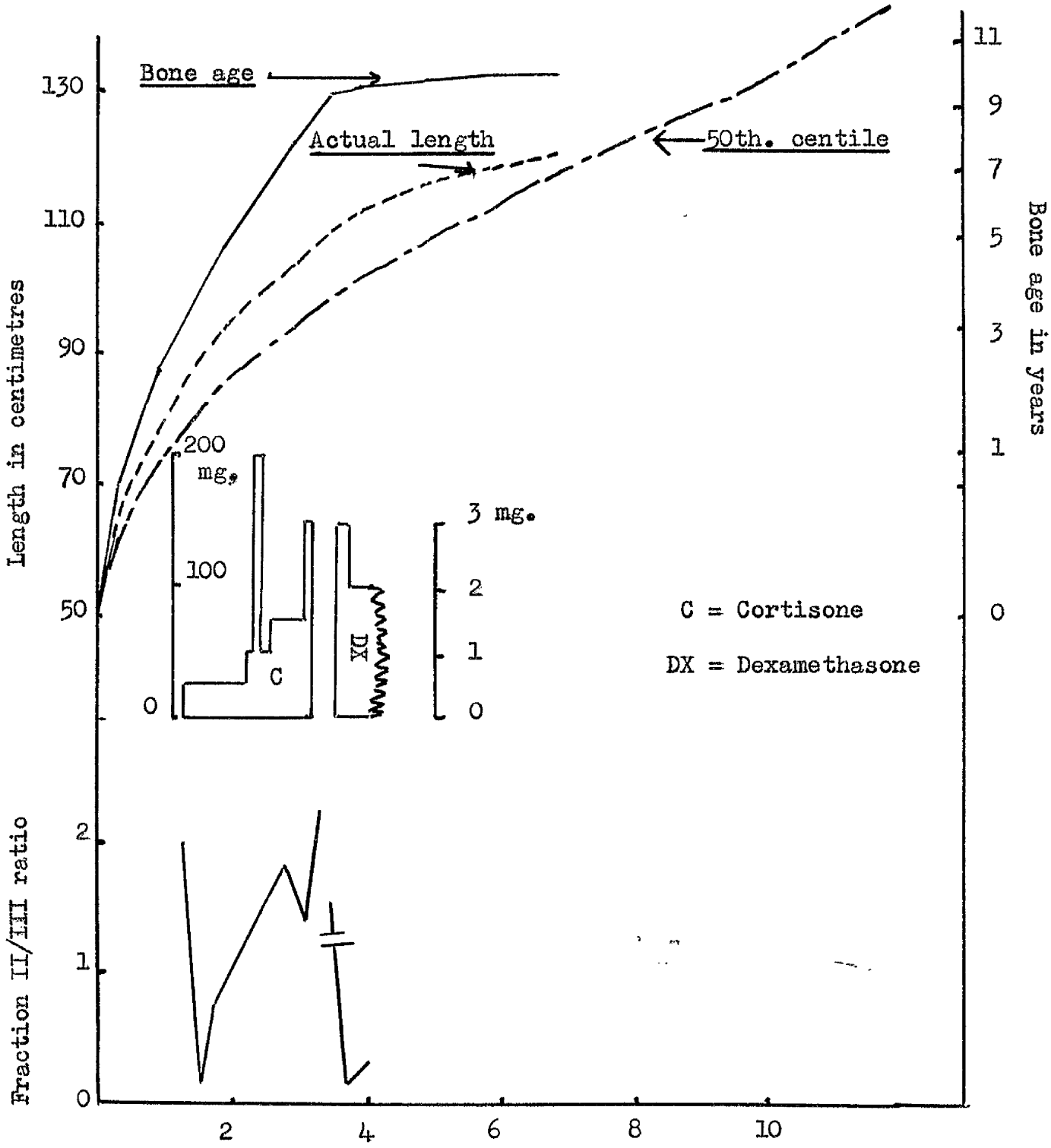


Chart 7. Case 3 - male with macrogenitosomia praecox. Linear growth and bone maturation in relation to suppressive treatment.

chronological age although to start menstruation at 11½ years is probably not abnormal. Visser (1966) has also noted the correlation of the onset of puberty with bone age.

Case 3 (male with macrogenitosomia praecox: Chart 7) showed a remarkably rapid bone maturation during the first 3½ years despite high cortisone dosage and his height then was above the 97th percentile. Since he appeared to require about 150 mg. cortisone daily, medication was changed to dexamethasone 3 mg. daily with a reduction after 3 months to 2 mg. daily. The curve of the bone age flattened and his growth curve less so. He is now on the 50th percentile for height with a bone age of 10.5 years. This one case in the series supports Professor D. Hubble's view (1965) that dexamethasone retards bone maturation more than linear growth. One point of clinical interest in Case 3 is that the testes are normally large and it is generally recognised that in congenital adrenal hyperplasia the testes remain infantile in size. The possibility of his being a case of true sexual precocity was considered but a positive dexamethasone suppression test, (Chart 8) the high urinary pregnanetriol values (Table 2) and later an affected female sibling all supported the diagnosis of congenital adrenal hyperplasia. There is a possibility that the enlarged testes contain adrenal /

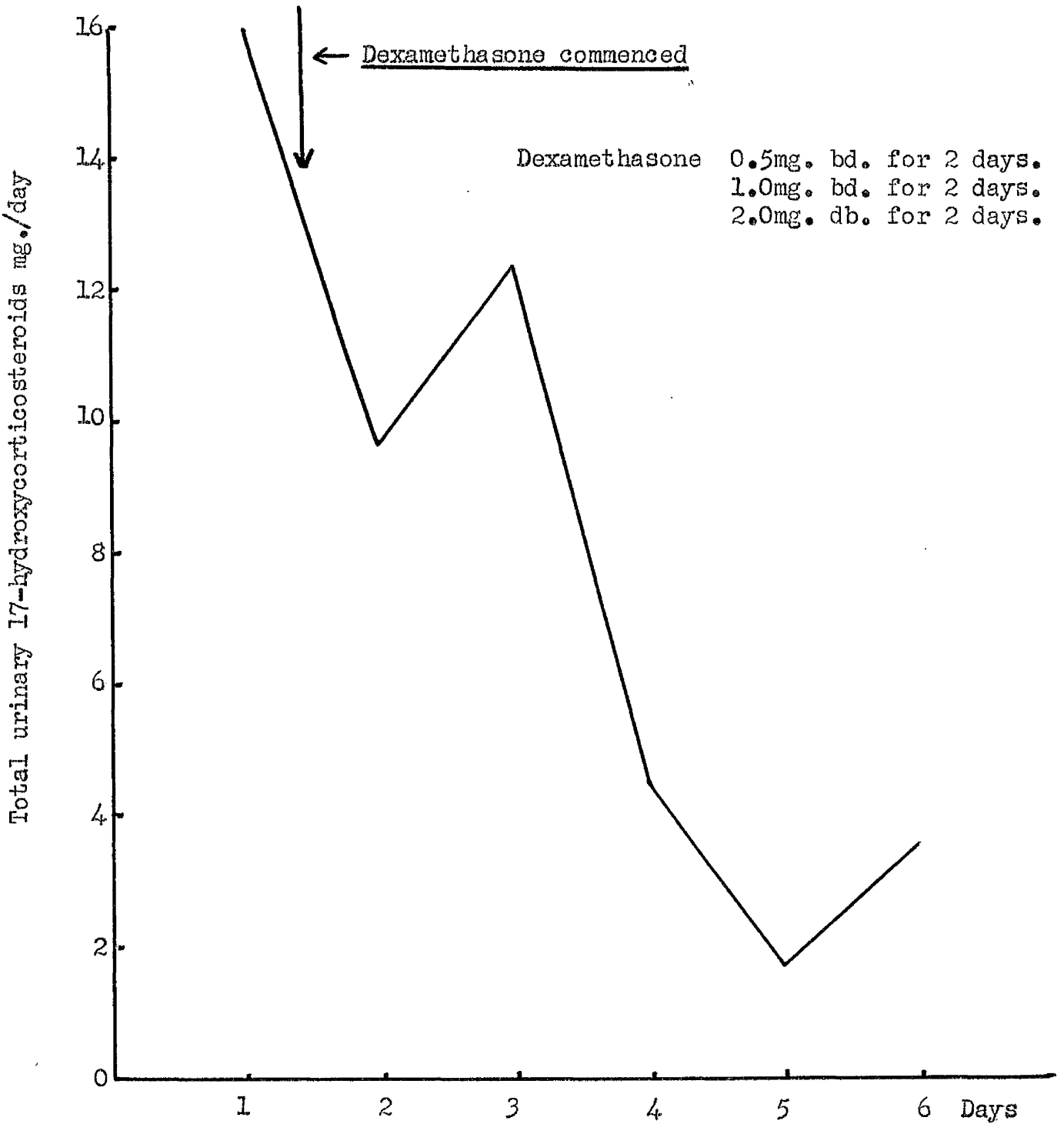


Chart 8. Suppression of urinary 17-hydroxycorticosteroids with dexamethasone in Case 3.

adrenal rest tissue. His plasma corticotrophin level was elevated (5 milli-units/100 ml.) and Hamwi, Gwinup, Mostow and Besch (1963) have shown that prolonged excessive corticotrophin production does activate testicular rest tissue.

Case 4 (male with salt-loss: 3β-ol deficiency) is of considerable clinical and biochemical interest. He i s a genetic male with cryptorchidism, hypospadias, bifid scrotum and has salt-loss typical of the syndrome (Bongiovanni, 1961). It is generally accepted that testosterone is the most potent androgen and in congenital adrenal hyperplasia its production rate is increased (Visser, 1966). In the absence of 3β-hydroxysteroid dehydrogenase, the Δ4-3-oxo configuration in Ring A cannot be achieved and this configuration is necessary for biologically active testosterone. Case 4 cannot synthesize testosterone or androstenedione an adrenal C19-precursor of testosterone of lesser biological activity. The adrenal C19-precursor, dehydroepiandrosterone is therefore his most potent androgen. Dehydroepiandrosterone has a potency of 1/13th to 1/14th (Fieser & Fieser, 1959) that of testosterone.

It might have been expected that bone maturation in this case would have been retarded or at least readily controlled. With this reasoning and in the hope that increased adrenal production of dehydroepiandrosterone might stimulate /

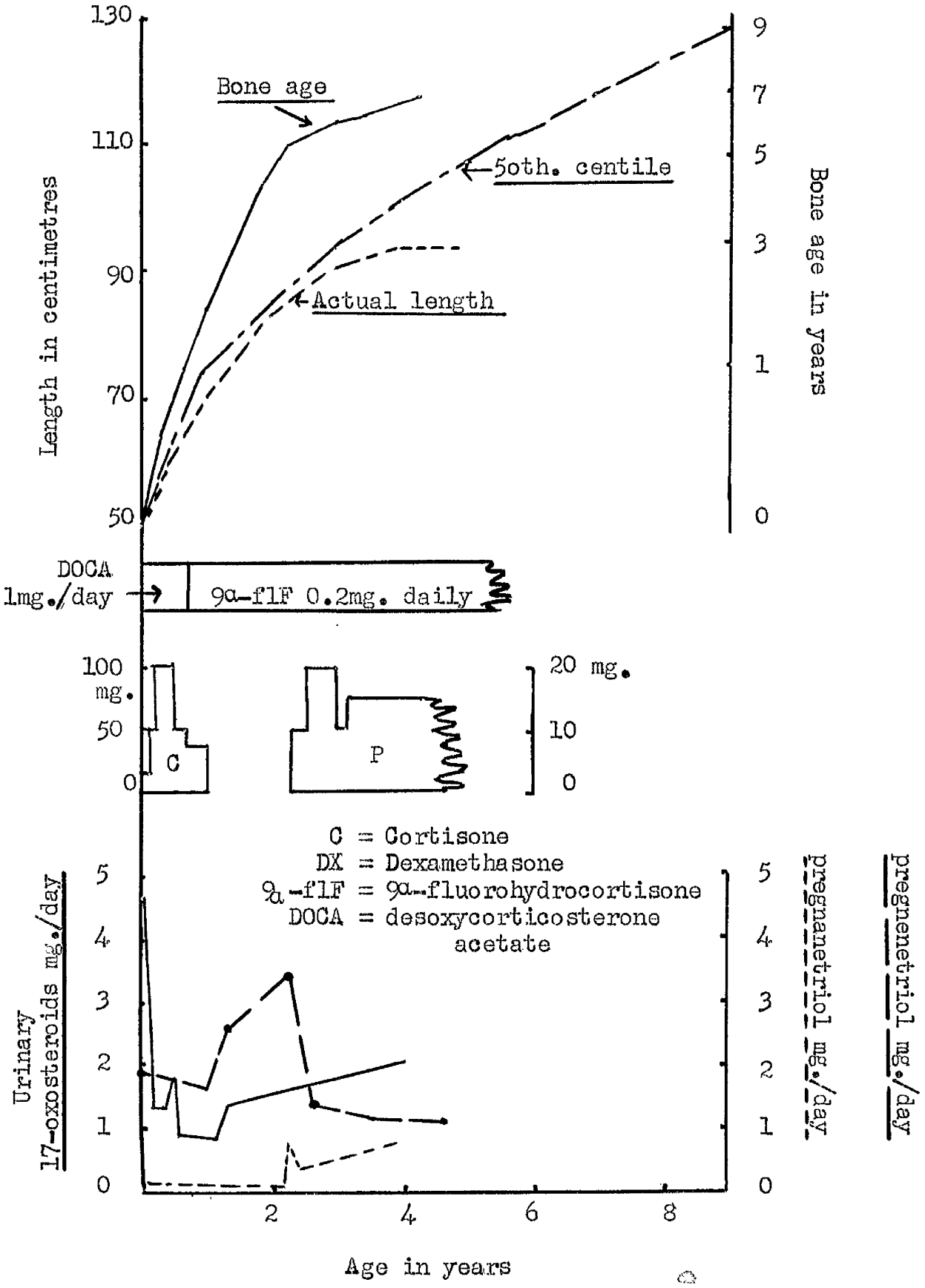


Chart 9. Case 4 - male, 3β-ol deficiency with salt-loss. Linear growth and bone maturation in relation to suppressive treatment.

stimulate testicular descent, glucocorticoid was withdrawn from age 1 year to 2 years 3 months and he was controlled on 9α-fluorohydrocortisone alone, (Chart 9). During this period the bone age increased rapidly to 5.7 years while his height only approached the 50th percentile. Since then prednisolone has been introduced and although he received up to 20 mg. daily there has been no marked arrest in the onward progress of bone maturation. This raises the possibility that the adrenal gland may secrete an androgen at least as potent as testosterone in these cases of congenital adrenal hyperplasia. One further point is of interest. During the period when glucocorticoids were withdrawn, what was the nature of the endogenous adrenal androgens secreted? An attempt will be made to answer this from urinary studies to be reported presently.

Case 5 (female, simple virilism: Chart 10). Until aged 2½ years she has been very responsive to cortisone 50 mg. daily, or prednisolone trimethylacetate 25 to 37.5 mg. at 4 weekly intervals, both bone age and height being under the 50th percentile. At age 1 year 9 months cortisone was reduced to 25 mg. daily which allowed an escape of bone maturation with a much slower increase in height. From age 4 years cortisone 50 mg. daily allowed linear growth to continue at the same time restricting ossification perhaps more so.

From /

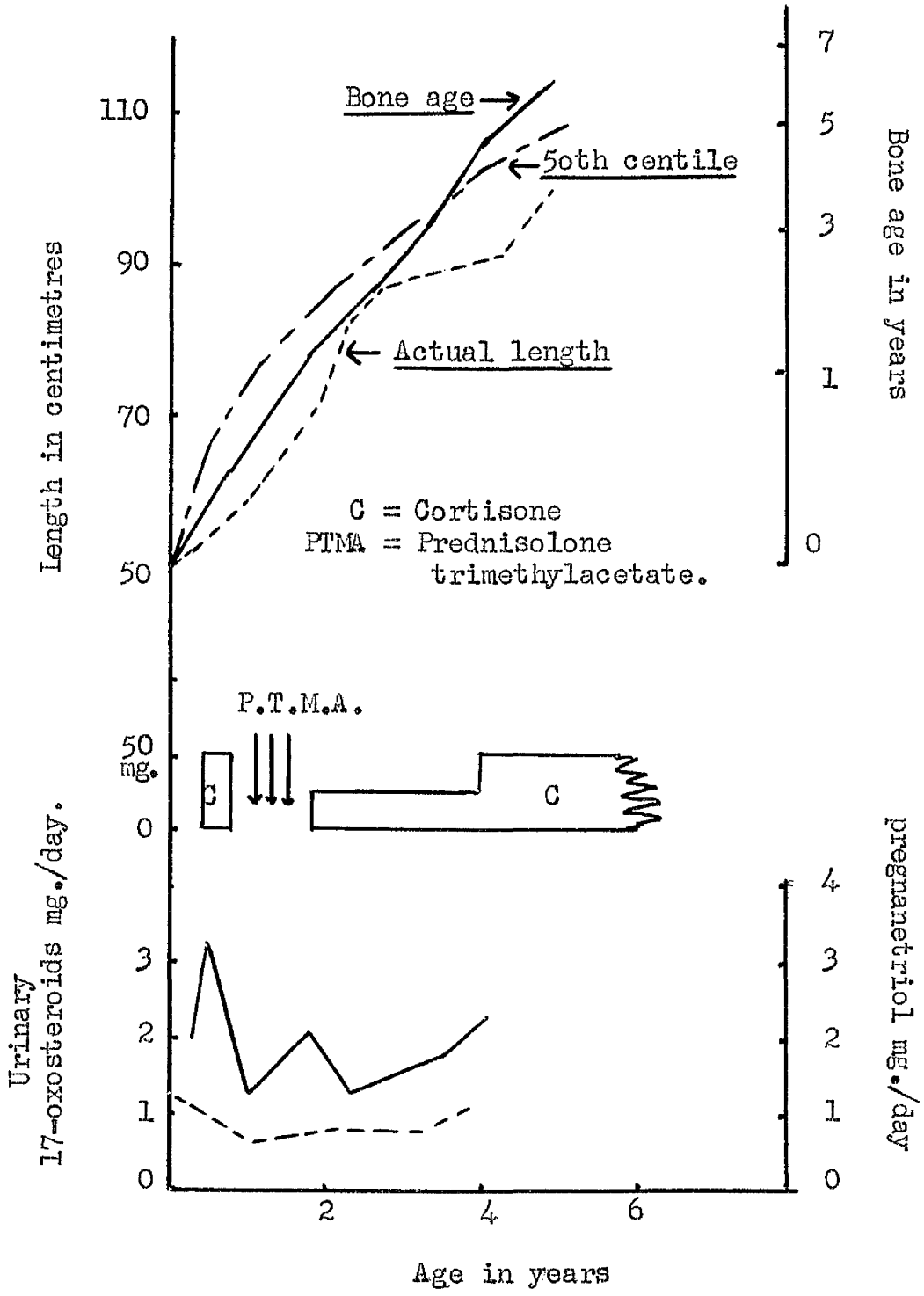


Chart 10. Case 5 - female with simple virilism. Linear growth and bone maturation in relation to suppressive treatment.

From this series of 5 cases, it would appear that each patient presents a different growth response to treatment. While the aim is to achieve maximum height by delaying fusion of epiphysis, the fact that steroids themselves inhibit linear growth, almost dooms the exercise to failure except in the mildest cases. Genetic factors may play a part in the ultimate height attained and in this series all the parents might be regarded as the smaller members of the community. (Tallest in the series 182.8 cm.).

Clearly the present synthetic steroid analogues have been of undoubted value, particularly in the virilized female, to allow normal sexual development. Successful pregnancies in cases of congenital adrenal hyperplasia have been reported. (Laron 1959; Mason 1961; Swyer and Bonham, 1961). The powerful salt-retaining substance 9α-fluorohydrocortisone is of proven value in the salt-losing case particularly for maintenance and I believe that this drug can be increased to 0.3 to 0.4 mg. daily without producing unwanted side-effects. This, of course is only so if no extra salt other than that added while cooking is allowed. Indeed 3 older children with salt-loss in this series voluntarily refused extra salt when the dosage of 9α-fluorohydrocortisone reached optimum levels.

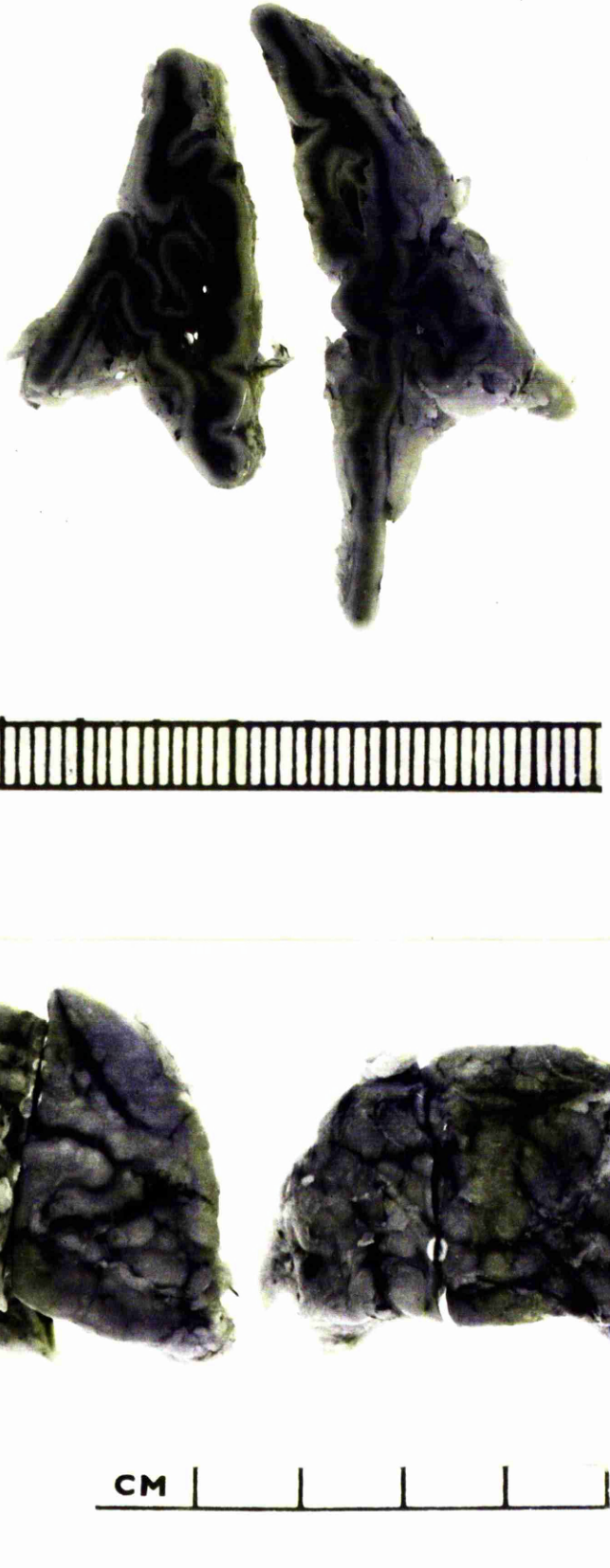
We still await the introduction of a drug which will specifically /

specifically control bone maturation thus necessitating glucocorticoid for replacement only rather than for complete adrenal suppression. Such a drug may have been discovered by Schering A.G. Berlin. Cyproterone (1,2-methylen-6-chlor-Δ4,6-pregnadien-17-ol-3,20-dione) a powerful antiandrogenic drug has been shown capable of inhibiting normal masculine development in rat foetuses so that when the drug is fed to pregnant rats, the whole litter consists of somatic females even though some are genetic males (Newmann and Elgar, 1966). Cyproterone is very much at the experimental stage but it holds out possibilities as valuable as the present animal experiments are fascinating.

Deaths.

In the series of 11 cases, one, case 9 died. This child, a female, came under observation when 3 years 5 months. A vaginal opening was present through which a catheter could be passed into the bladder from a meatus ½ inch inside the anterior vaginal wall. The clitoris was 1 cm. long. She was adquately controlled on dexamethasone 0.75 mg. daily and 2 g. extra salt daily. After 4 months she developed a viral bronchitis during which the family practitioner discontinued steroid treatment. She collapsed and died suddenly on the 2nd day of the illness.

The /

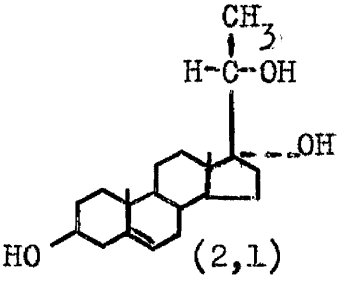


Adrenal glands from Case 9. Postmortem specimens.

The adrenal glands are shown but because of autolysis the histological appearance of the tissue is unrepresentative and is not shown.

Steroid analyses in diagnosis, management and research.

The simplest and most rapidly performed quantitative estimation of urinary steroids is that of 17-oxosteroids. As a diagnostic procedure, the test is extremely valuable since in congenital adrenal hyperplasia the daily excretion of 17-oxosteroids is invariably increased. On the result of this test, I have made the diagnosis in the case of this series (Table 2) although clearly the clinical and biochemical features were supportive. The estimation of pregnanetriol is more time-consuming and although it was performed (Table 2) the higher results obtained served generally to confirm the diagnosis. It will be remembered however that in congenital adrenal hyperplasia due to a deficiency of 3β-hydroxysteriod dehydrogenase, urinary pregnenetriol (2,1) and not pregnanetriol (2,2) would be excreted in increased amounts.



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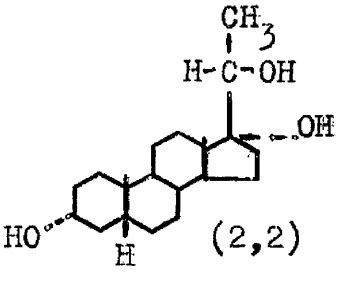


(2,2)

Also with such a defect, the adrenal androgen would also have the Δ5-configuration in Ring B but would nonetheless react positively with the Zimmermann reagent - assuming that the androgen under these circumstances retains a 17-ketone. The urinary 17-oxosteroids in Case 4 (3β-hydroxysteroid dehydrogenase deficiency) were found to be increased (4.7 mg.) before treatment commenced.

Once treatment is started, part of the administered steroid will be excreted in the urine as a 17-oxosteroid and for this reason, the estimation of urinary pregnanetriol might afford a truer index of adrenal suppression. Gallagher (1954) found that about 2 per cent of hydrocortisone (800 mg.) administered to a subject could be recovered from the urine as 17-oxosteroids. If this finding is applicable to infancy and childhood, the contribution to the urinary 17-oxosteroids by orally administered steroids, cortisone, prednisolone or prednisone, will be micrograms only and this will not significantly increase the daily 17-oxosteroid excretion. The metabolites of the C-16 substituted (Chart 1) synthetic analogues (dexamethasone, betamethasone and triameinolone) retain this configuration and therefore do not yield Zimmermann chromogens. Such drugs do not contribute to the total urinary 17-oxosteroids estimated by this reaction.

It will be seen from the composite charts (Charts 5-10) that the level of urinary 17-oxosteroids reflects the progress in bone maturation more than it does that of the linear growth. In the 5 cases presented the excretion of 17-oxosteroids was rapidly suppressed but the difficulty in maintaining continuous even suppression is clear in retrospect.

Despite ostensibly good control achieved in Case 1 there was escape after puberty from age 10 to 11 years, when bone maturation progressed to epiphyseal fusion while the height remained static. She is now biochemically controlled, with a daily urinary 17-oxosteroid level just under 2 mg./day. She reports, however, that when the daily dosage of prednisolone is 10 - 12.5 mg. the menstrual flow lasts 4-5 days with an average loss but lasts only 1 day and is scant when prednisolone is reduced to 5 mg. daily. It may be that after puberty when epiphyseal fusion is complete the correct dosage schedule should be geared to achieving a normal menstrual cycle and flow rather than to a biochemical test. Three women who each had a successful pregnancy Laron, 1959; Mason, 1961 and Swyer and Bonham, 1961) were receiving hydrocortisone 100 mg. intramuscularly every third day, cortisone 25 mg. thrice daily and prednisolone 5 mg. thrice daily respectively.

Cases 2 and 4 (salt-losers) and Case 5 (non-salt-losing) /

losing) required high dosage of cortisone during their early life to maintain clinical progress and although the levels of urinary 17-oxosteroids were about 2 mg./day the retardation of growth was greater than might have been expected with urinary 17-oxosteroid levels of this order. Presumably the high doses of glucocorticoids inhibited protein anabolism and retarded growth - well recognised effects of steroid administration.

Case 3 is of considerable interest. From age 1 year 3 months when he first presented, until age 3 years 1 month, serial X-rays at short intervals showed an unrestrained advance in bone age from 2.8 years to 8 years despite large daily doses of cortisone. During this period biochemical control was assessed by the Fraction II/III index (Hill, 1960). This index is the ratio of the 11-deoxy-17-oxosteroids to the 11-oxy-17-oxosteroids derived from the 17-ketogenic steroids. Being a ratio, complete 24-hour collections of urine are not necessary. While of some value the test falls on two scores, firstly that absolute values are not obtained and secondly Cox (1962) has pointed out that pregnanetriolone is slower to be removed under treatment than is pregnanetriol, both being present in the urine of the compensated and salt-losing patient with 21-hydroxylase deficiency. The nett result would be a more /

more rapid fall in the 11-deoxy-17-oxosteroids than in the 11-oxy-17-oxosteroids.

Despite these considerations the Factor II/III ratio in Case 3 remained high at 1-2.5 (normal 0.5) while on cortisone. The correctness of the diagnosis was questioned. Cortisone was abruptly stopped without any apparent untoward effects. After a suitable period of treatment, plasma corticotrophin was estimated and reported by Dr. Beryl Connor as 5 milli-units/100 ml. Adrenal suppression tests were performed using dexamethasone and when adequate suppression (Chart 8) was observed, treatment with dexamethason was commenced. Later the adrenal glands were shown to be unresponsive to corticotrophin stimulation during dexamethasone treatment. About this time the patient removed to Ireland and is now in the care of Professor I.J. Carre.

Aldosterone excretion in congenital adrenal hyperplasia.

It might be expected that aldosterone production in the salt-losing syndrome and in the compensated case would differ. If 21-hydroxylation is completely absent it would be reasonable to expect a complete failure in aldosterone production while if the defect is partial, near normal production might be expected.

At present there is no agreement on the level of aldosterone production particularly in the salt-losing case. /

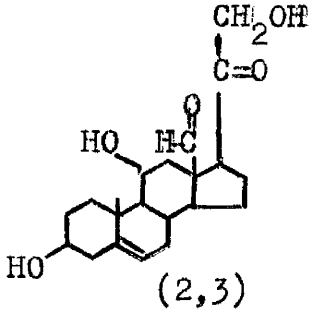
case. Pradar, Spahr and Neher (1955) have reported high urinary aldosterone values while Blizzard, Liddle, Migeon and Wilkins (1959) found that urinary aldosterone, by a bio-assay technique was abnormally low. Recently two groups have demonstrated depressed aldosterone production and excretion in the salt-losing form (Bryan, Kliman and Bartter, 1965; New, Miller and Peterson, 1966) while the latter group has shown that in compensated cases the urinary excretion of aldosterone is normal.

To explain why some cases with a 21-hydroxylase defect synthesize neither hydrocortisone nor aldosterone while others syntheseize aldosterone but not hydrocortisone, at least in adequate amounts, one might postulate that in all cases there is a defect in 21-hydroxylation of 17α-hydroxyprogesterone - therefore impaired hydrocortisone production without salt loss - and in addition in the salt-losing form there is a defect in the 21-hydroxylation of progesterone. This postulate will be proven when it is shown from incubation studies that adrenal tissue from the salt-losing case can 21-hydroxylate progesterone while that from the salt-losing case cannot.

Secretion rates and incubation studies are outside the scope of this work, but I have estimated the urinary excretion of aldosterone (free and 3-oxo-conjugated) and tetrahydroaldosterone in the 11 cases of this series. Brooks' /

Brooks' (1960) method was employed for the free aldosterone and that of Pasqualini, Legrand and Jayle (1963) for tetrahydroaldosterone.

Of the total aldosterone secreted, 5 - 15% appears as the 3-oxo-conjugate in the urine and a further 10% as tetrahydroaldosterone conjugated with glucuronic acid (Ross, 1959). Table 7 shows the daily excretion of aldosterone and tetrahydroaldosterone in the present series. Free aldosterone was not detected in the urine of the salt-losing cases. To observe the ability to increase aldosterone production in response to salt deprivation, salt was withheld from Case 4, but she developed hypotension and lethargy within six hours and the test was abandoned. Salt restriction therefore was not attempted in the other 3 salt-losing cases.
In the same group tetrahydroaldosterone was detected in Case 4 only (8 mg. per day). This was an unexpected finding since Case 4 has a defect in 3β-hydroxysteroid dehydrogenase. If satisfactory hydroxylation were achieved at positions C-18 and C-21, Case 4 might produce the hypothetical compound (2,3) not dissimilar structurally to aldosterone (2,4).



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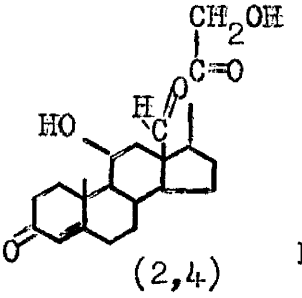

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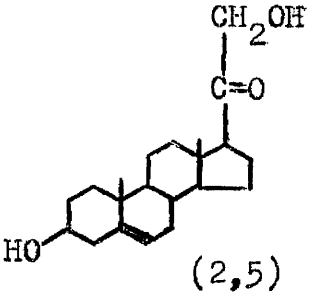
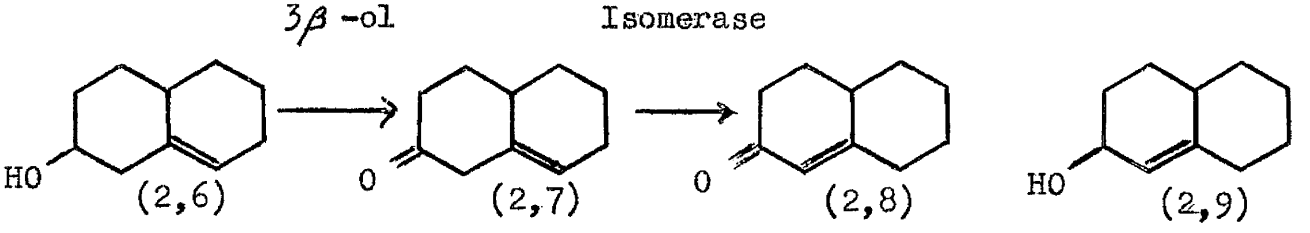


(2,5)

Table 7. The daily urinary excretion of free aldosterone and tetrahydroaldosterone in salt-losing and non salt-losing congenital adrenal hyperplasia.

Case	Sex	Enzyme Block	Clinical Type	Age	µg. per day	
					Aldosterone	Tetrahydro-aldosterone
1	F	21-OH	No salt-loss	8yr.10mo.	3.5	39.0
2	F	21-OH	Salt-loss	7yr.2mo.	0	2.2
3	M	21-OH	No salt-loss	3yr.2mo.	2.5	26.0
4	M	3 -ol	Salt-loss	4 - 7dys.	0	0
				3.5 yr.	0	1.0
5	F	21-OH	No salt-loss	2yr.3mo.	1.2	1.0
6	F	21-OH	No salt-loss	8yr.	6.35	45.2
7	F	21-OH	No salt-loss	9 wks.	0	4.6
8	F	21-OH	No salt-loss	2yr.10mo.	3.75	15.8
9	F	21-OH	No salt-loss	3yr.5mo.	2.0	12.5
10	F	21-OH	Salt-loss	6 wks.	0	0
11	M	21-OH	Salt-loss	3 wks.	0	0

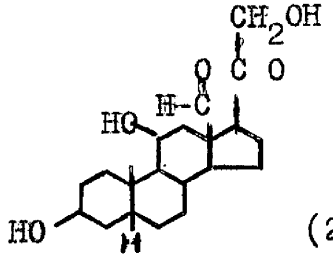
While unlikely to be physiologically active as a mineralocorticoid, this compound (2,3) would be excreted as a disulphate, having a 3β, 21-dihydroxy structure (Pasqualini and Jayle, 1962). The compound (2,5) has in fact been isolated by these authors from human urine, and Cathro, Birchall, Mitchell and Forsyth (1965) have identified this same compound in the urine of Case 4 in excess amounts. It would not, however, be hydrolysed by β-glucuronidase although the British Limpet preparation of β-glucuronidase powder may also contain steroid sulphatases. These sulphateses are only active for the 3β-sulphates of 5α and Δ5-compounds and are not active for 21-sulphates (Roy, 1956). According to the modern genetic concept of 1 gene 1 enzyme, it is probable that the conversion of a Δ5-3β-ol compound to a Δ4-3-oxo compound is a 2-step reaction involving a 3β-hydroxydehydrogenase and an isomerase. This reaction is indicated thus:-



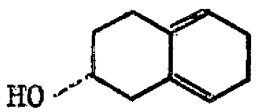
While Δ5-3β-OH compounds (2,6) have been detected in the urine of healthy infants and children with 3β-hydroxysteroid dehydrogenase deficiency (Bongiovanni 1962; Reynolds 1965), so far compounds having Δ5β-3-oxo (2,7) and /

and Δ4-3β-ol (2,9) configurations in Rings A and B have not been isolated from the urine of these cases. Ewalt, Werbin and Chaikoff (1964) and Kruskemper, Forchielli and Ringold (1964) have presented evidence for 2 adrenal isomerases specific for C-19 and C-21 Δ5-3-oxosteroids and these could be separated from the 3β-hydroxysteroid dehydrogenase.

The substance which I have estimated in Case 4 as tetrahydroaldosterone gives a blue tetrazolium reduction in the loci of tetrahydroaldosterone in the 3 chromatograms used in the method. The possibility that



(2,10)



(2,11)

this compound has other than a 3α,ol,5β configuration (2,10) exists and the further possibility that it is a metabolite of a Δ5 -3-oxo compound having the configuration Δ5 -3-α -ol (2,11) is being investigated. The difficulty of obtaining standard steroids with this configuration is at the moment an impediment.

Free aldosterone was detected in the urine of all the non salt-losing cases. These figures are within the normal range for age (Minick and Conn, 1964) and are in agreement with those found by others in the simple virilising form (New, Miller and Peterson, 1966).

The same authors (New, Miller and Peterson, 1966)

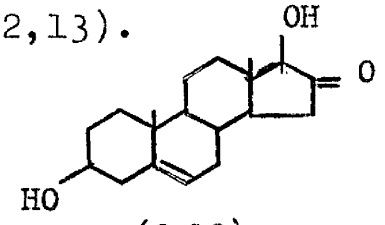
using different chromatographic solvent systems report urinary tetrahydroaldosterone for simple virilising cases aged 7 months to 10 years as 10-57 µg. /day. These agree with the present findings and indicate in conjugation with the normal excretion of free aldosterone that there is no disturbance in the production of aldosterone in the non salt-losing form of congenital adrenal hyperplasia. This investigation of aldosterone excretion in the present series was not designed to answer the basic problem of the preferences of 21-hydroxylase in both forms of the disease but further work on the urinary steroid metabolites in Case 4 (3β-hydroxysteroid dehydrogenase deficiency) might uncover pathways of steroid metabolism still obscure.

The nature of the urinary 17-oxosteroid in congenital adrenal hyperplasia.

At birth the infant with congenital adrenal hyperplasia does not show advanced ossification yet abundant evidence was quoted in the introduction to this chapter indicating that foetal androgens in excess amounts are being produced from an early stage of pregnancy. The increased urinary 17-oxosteroids have been shown to be due to dehydroepiandrosterone and androstenedione (Fukushima, Bradlow, Hellman, Zumoff and Gallagher, 1961) and Degenhart, Visser, Wilmink and Frankena, 1965,

report /

report high excretion and secretion rates of testosterone in congenital adrenal hyperplasia. Testosterone is the most potent endogenous androgen so far known and it is held responsible for the virilism, macrogenitosomia praecox, rapid growth and accelerated bone maturation characteristic of the syndrome. In the comb-growth test, dehydroepiandrosterone and androstenedione have only one-sixth and one-eighth the androgenic activity of testosterone. Recently Reynolds (1965) has isolated from the urine of cases with 3-β-hydroxysteroid dehydrogenase deficiency, 16-oxo-androstenediol (2,12) and 16α-hydroxydehydroepiandrosterone (2,13).



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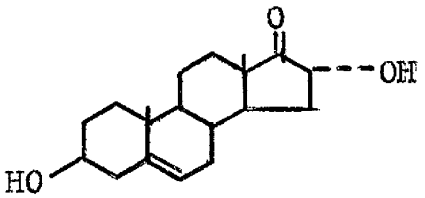


(2,13)

Both compounds, however, have been found in the urine of normal infants (Reynolds, 1964; Bongiovanni, 1962) and would therefore seem unlikely to be more potent androgens than testosterone. It is equally unlikely that they have any function after birth since they are reduced in quantity with age and were found in only one of Reynolds (1965) cases after the age of 5 months.

Case 4 of this series, with 3β-hydroxysteroid dehydrogenase deficiency showed, during the period of glucocorticoid withdrawal /

withdrawal, a rapid advance in bone maturation. In theory he cannot synthesize testosterone so that an androgen or androgens as potent as testosterone would seem to be available. If such a compound exists, is it peculiar to all forms of congenital adrenal hyperplasia or only to the 3β-hydroxysteroid dehydrogenase deficiency type?

To examine this question it was decided to carry out a qualitative and where possible a quantitative analysis of the urinary 11-deoxy-17-oxosteroids and 17-hydroxy-C-19-compounds, excreted by the 11 cases of the series. Pre-treatment urine was used where possible but in two instances urine obtained while the glucocorticoids were withdrawn formed the basis of the analysis.

The total conjugates of the C-19 compounds were extracted by the method of DePaoli, Nishizawa and Eik-Nes (1963) and the glucuronides and sulphates hydrolysed as indicated by these authors. The combined steroids were purified on silica gel and the aliquots chromatographed on the Bush 3 system, before and after oxidation.

In Table 8 is shown the age of each patient at the time of the analysis, the total 17-oxosteroids in the sample of urine assayed, and a breakdown of the 17-oxosteroids into the various fractions as indicated.

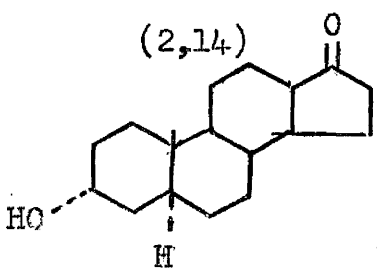
Dehydroepiandrosterone /

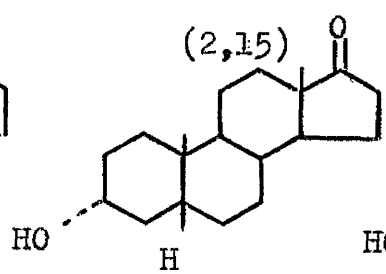
Dehydroepiandrosterone formed about 10% (mean 9.4 for 10 cases) of the total 17-oxosteroids with the mean androsterone/etiocholanolone ratio greater than unity. Case 4 had the greatest output of dehydroepiandosterone (18.96% of the daily total). Androsterone and etiocholanolone were also detected in this case and the immediate precursor of these must have had the Δ4-3-oxo-configuration. These findings might suggest that here less dehydroepiandrosterone is converted to androstenedione than in the others of the series and this would be in keeping with a block in 3β-hydroxysteroid dehydrogenase activity. It is generally accepted that only the Δ4-3-oxo compounds are metabolised to form the 3α-ol metabolites such as are androsterone (2,14) and etiocholanolone (2,15). Cathro and colleagues (1965) have demonstrated excess amounts of 3β-hydroxy-compounds in third day urine from this case (21-hydroxypregnenolone, 132 µg./day and dehydroepiandrosterone, 30.3 µg./day) but have also shown that in the newborn during the early days of life 3β-hydroxysteroid dehydrogenase is temporarily inefficient. On theoretical grounds, Case 4 may have in part recovered some of this enzyme activity and at present the deficiency is less than at birth. This is suggested also on clinical grounds for he now requires only 0.1 mg. 9α-fluorohydrocortisone as mineralocorticoid substitution. It will be remembered

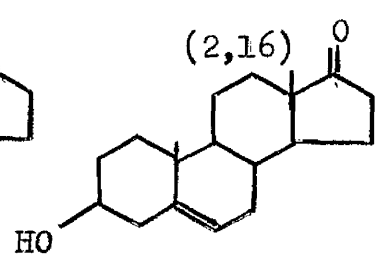
lso /

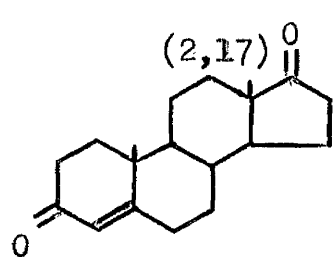
also that traces of tetrahydroaldosterone were detected.

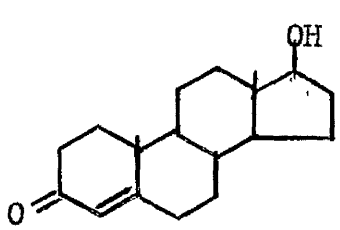
Few reports of this type of analysis are available for comparison. Masuda (1957) has studied the pattern of urinary 17-oxo-steroids in 5 untreated cases of congenital adrenal hyperplasia. Only 2 were in the paediatric age group - boys with macrogenitosomia praecox, aged $4\frac{1}{2}$ and $7\frac{1}{2}$ years. He found that dehydroepiandrosterone, androsterone and etiocholanolone were all increased and the ratio androsterone/etiocholanolone was greater than unity. Rubin, Dorfman and Pincus (1954) and Jailer, Gold, vande Wiele and Lieberman (1955) had earlier drawn attention to the high ratio of androsterone/etiocholanolone in congenital adrenal hyperplasia. The real significance of these findings is that they reflect increased /











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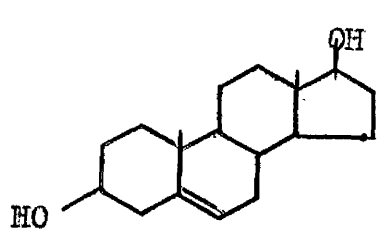

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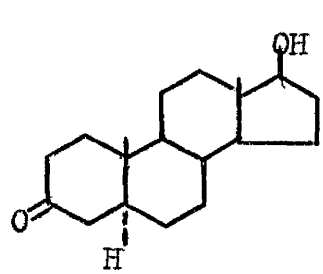


(2,20)

increased secretion by the hyperplastic adrenal cortex. Dehydroepiandrosterone (2,16) is secreted by the adrenal as the sulphate (Baulieu, 1962) and is a precursor of testosterone (2,18) via androstenedione (2,17) (Mahesh & Greenblatt, 1962). However, Klempien, Voigt and Tamm (1961) have given evidence that the major pathway from dehydroepiandrosterone to testosterone is via Δ5-androstene-3β,17βdiol (2,19) and not Δ4-androstenedione (2,17). If this is so, it is an important step, for in congenital adrenal hyperplasia the high levels of circulating dehydroepiandrosterone might push the reaction in favour of testosterone (2,18) production. An earlier quoted, increased secretion and excretion of testosterone in congenital adrenal hyperplasia has been reported by Visser (1966).

From Klempien's finding, Δ5-androstene-3β,17βdiol might reasonably be expected to undergo dehydrogenation at C3 with isomerisation of the Δ5-structure to a Δ4, to form testosterone. However, dehydrogenation followed by reduction of ring B to the 5α compound would result in androstane-17β-ol-3-one (2,20). This compound has been prepared in vitro by Butenandt, Tschering and Hanish (1935), and it is almost as active as testosterone in the Comb-growth test. Its synthesis in congenital hyperplasia has not heretofore been reported but I am of the opinion /

opinion that I have detected a substance in Cases 4 and 11 which behaves chromatographically before and after oxidation with chromic acid similar to androstane-17β-ol-3-one. In extracts previously oxidised with chromic acid, a Zimmermann positive spot was present in the Bush C system at $R_{S(DOC)}$ 3.2. This spot was not found in unoxidised extracts. It seemed possible that this unknown 17-ketone might have derived from a compound having a 17-hydroxyl group. To test this hypothesis chromatographic strips of the unoxidised extracts were oxidised with copper acetate in methanol according to Neher (1964) and thereafter a faint Zimmermann reaction was observed at $R_{S(DOC)}$ 1.28 where none had occurred before. The chromatic behaviour of several likely isomers was studied and androstane-17β-ol-3-one compared most nearly to the unknown. Complete identification of this compound awaits further analysis by gas/liquid chromatographic techniques. If, however, the unknown substance is androstane-17β-ol-3-one, the continuing bone maturation observed in Case 4 when glucocorticoid suppressive drugs were withdrawn, is more readily understandable, unless he also was synthesising testosterone, which is theoretically unlikely, by the pathway suggested by Klempien, Voigt and Tamm (1961).

The increased excretion of androsterone and etiocholanolone (Table 8) /

Table 8. Urinary 17-oxosteroids detected in 11 cases of congenital adrenal hyperplasia.

Case	Age	Sex	Enzyme defect	17-OS mg./day	mg. per day				*Andro / Etio:	% Andro	% Etio	* Δ4 Andro	Testosterone
					DHEA	DHEA % of Total	Androsterone	Etiocholanolone					
1	8.8	F	21-OH	10.2	800	7.84	1020	820	1.2	10	8	+	±
2	7.5	F	21-OH	7.4	760	10.27	652	602	1.08	8.8	8.1	±	±
3	4.3	M	21-OH	9.3	870	9.4	910	744	1.2	9.7	8	±	±
4	3.5	M	3 -ol	5.6	1062	18.96	454	468	0.97	8.1	8.4	-	±
5	0.16	F	21-OH	3.1	280	9.0	140	128	1.09	4.5	4.1	N	±
6	8.16	F	21-OH	6.0	545	9.0	620	500	1.2	10.3	8.3	±	±
7	0.25	F	21-OH	3.4	352	10.3	325	230	1.4	9.5	6.7	-	±
8	3.4	F	21-OH	5.0	486	9.7	460	385	1.2	9.2	7.7	-	±
9	3.16	F	21-OH	5.2	495	9.5	425	380	1.1	8.1	7.3	-	±
10	0.25	F	21-OH	3.6	354	9.8	308	250	1.2	8.5	6.9	-	-
11	0.08	M	21-OH	3.8	365	9.6	304	314	0.96	8.0	8.2	-	-
				Mean		9.4			1.14				

(-) = None detected *Δ4-androstenedione * androsterone etiocholanolone

(Table 8) observed in the series reflects increased adrenal production of their precursors. Androsterone (3α,5α) derives from dehydroepiandrosterone, Δ4-androstenedione and testosterone. Etiocholanolone (3α,5β) is preferentially formed from pregnanetriol, 17α-hydroxypregnanolone and 17α-hydroxyprogesterone. Thus both C-19 and C-21 steroids are being produced in excess amounts. In both Masuda's (1957) and Birke and colleagues' (1958) reports, 11β-hydroxylated-5β-17-oxosteroids were not found and it has been suggested that this finding might indicate different metabolic pathways for 17-oxosteroids and corticosteroids in congenital adrenal hyperplasia (Bergstrand, Birke and Plantic, 1959).

My finding of an increased excretion of both androsterone and etiocholanolone, with the ratio androsterone/etiocholanolone greater than 1, is in keeping with these authors' findings. Only in Case 4 was this not so although the differences were minimal (androsterone 454 μg./d., etiocholanolone 468 μg./d.). It would be unwise to claim that this isolated finding is significant but further investigation along the lines suggested by Bergstrand, Birke and Plantin (1959) might elucidate its meaning.

Δ4-androstenedione was detected in the urine of Cases /

Cases 1, 2, 3 and 6 but in each the amount was less than 5 µg./day (visual comparison with 5 µg. standards on sidelanes of chromatogram). Testosterone, estimated as Δ4-androstenedione after oxidation with chromic acid was detected only in trace amount in Cases 1 - 9 (excepting Case 4). There is very little published data on testosterone excretion rates for normal children but excretion is thought to be less than 5 µg./day.

In 11 cases of congenital adrenal hyperplasia, Degenhart, Visser, Wilmink and Frankena (1965) found excretion rates ranging from 11 - 261 µg./day. The values found in this study are much lower and are related to differences in the techniques used.

It is clear from this work, and the literature that the study of urinary steroid patterns in congenital adrenal hyperplasia is not exhausted and cases with 3β-hydroxysteroid dehydrogenase deficiency hold a source of material yet to be investigated.

For instance, little is known of the steroidogenic capacity of the testes in these patients. Should their testes be placed in the scrotum at an early age? Has testicular descent failed due to the adrenal or a concocomitant testicular biosynthetic defect? Is there in fact a clinical syndrome associated with a 3β-hydroxysteroid dehydrogenase deficiency without an associated /

associated Δ5-isomerase deficiency or does a deficiency of Δ5-isomerase alone present as a clinical entity?

This chapter, far from answering any specific question has, as far as the author is concerned, opened the door to a field of research based on methods outside the scope of this present work.

S T U D I E S O F C O R T I S O L

Metabolism

in

children of short stature.

Frequently children of short stature are brought to the paediatrician in the hope that the particular cause of the retardation will be discovered and removed, to allow normal growth to proceed. When organic disease such as coeliac disease or hypothyroidism is found dietary measures or substitutionary treatment result in resumption of normal growth. Gross and irremediable congenital abnormalities in the cardiovascular and renal systems frequently result in stunting of growth and the true facts must then be given to the parents. When these and other organic conditions known to result in dwarfism have been excluded there remains a group of children whose small stature is less easily explained. In some, hereditary factors may play a part and it is often helpful to know the family growth pattern. Parents and siblings may however be normally tall and this serves rather to emphasise the smallness of the patient.

In assessing the small child it is clinically convenient to correlate height and weight with age so that the terms height age and weight age when applied to a particular patient may give the impression of a small, fat child or a tall thin one, and while this is of immediate value it does not serve as a good standard by which to compare children of different chronological ages.

Growth /

Growth charts prepared by Tanner and Whitehouse in part fill this gap. On these charts heights and weights for age are plotted in relation to average limits of normality between the 3rd and 97th percentile so that trends in the development (height or weight) of a child may be followed over several years, particularly if therapeutic measures are instituted. A good example is seen in the case of a child with coeliac disease who at the time of diagnosis may be below the 3rd percentile for both height and weight and when treatment with gluten-free diet is instituted the growth curves move towards the 50th percentile.

The subjects of the present investigation are a group of small children who have been placed in 3 categories according to clinical considerations.

1. Malnutrition.

While inadequate growth due to inadequate calorie intake is rare in the general child population of the United Kingdom environmental factors such as neglect and poverty sometimes militate against adequate nutrition. Children from such environments, accustomed to little food, readily develop small appetites, the anorexia resulting only in less food being made available to the children. Group 1 consists of 16 children (Table 1) who were inmates of an orphanage (Quarrier's Homes, Bridge /

Bridge of Weir). All had been subjected to varying degrees of malnutrition and this with other social factors had resulted in their admission to the orphanage. All had retarded bone age and all were below the 3rd percentile for height. Only 2 were above the 50th percentile for weight, 5 were on the 10th percentile, 4 were on the 3rd and 5 were beneath the 3rd percentile. There was no evidence of organic or endocrine disease in any of this group. There was no available data on the family growth pattern although Cases 1 - 4 were siblings as were also Cases 5 and 6.

Table 1. Data concerning the Group I children of small stature.

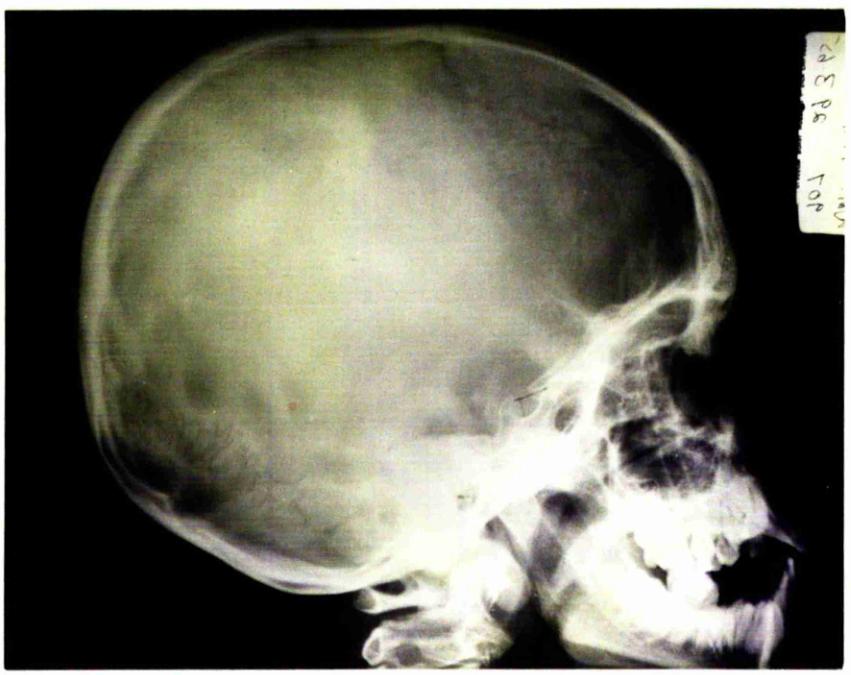
	Case	Sex	Age Years	Bone Age Years	Percentile Height	Percentile Weight
1	L.I.	F	4.5	4	-3	-3
2	E.I.	F	6.5	6	-3	-3
3	G.I.	M	10	8	-3	-3
4	M.I.	F	11.5	9.5	-3	3
5	A.M.	F	6.5	5.5	-3	3
6	J.M.	M	13.3	7	-3	10
7	M.McL.	F	7.25	4.5	-3	10
8	T.C.	M	7.5	6.5	-3	10
9	C.R.	M	8	4	-3	-3
10	W.M.	M	8.75	6.5	-3	-3
11	G.S.	M	10	8	-3	95
12	S.D.	F	10	9	-3	10
13	C.T.	F	2.5	1.5	-3	75
14	G.T.	M	5.25	3.5	-3	-3
15	G.B.	M	6	3	-3	10
16	J.M.	F	6.5	6	-3	3

2. Congenital growth retardation.

Primordial dwarfism has been applied to this group in whom smallness has been apparent since birth. Warkany, Monroe and Sutherland (1961) used the term 'intra-uterine growth retardation' to indicate that in these children growth inhibiting factors seem to be operative from a very early stage. They are usually small at birth although born at term and their growth proceeds slowly. Ossification at different centres may vary but there is usually no gross specific retardation. Their mental age is normal and sexual maturation is not delayed. When epiphyseal fusion takes place they are small, normally proportioned adults. Group 2 of this series consists of 4 children classified as primordial dwarfs, (Table 2.). Bone age was minimally retarded in all 4 and all were below the 3rd percentile for height. Organic disease was excluded.

Table 2. Data concerning the primordial and hypopituitary dwarfs in the series.

Case	Sex	Age Years	Bone Age Years	Percentile Height	Diagnosis
J.G.	M	3	2.3	-3	Primordial
R.H.	M	3	0.6	-3	Primordial
J.A.	M	3.8	0.75	-3	Primordial
D.P.	M	4.5	1.5	-3	Primordial
E.O.	F	13	6.0	-3	Hypopituitar
W.McI.	M	13	5.6	-3	Hypopituitar



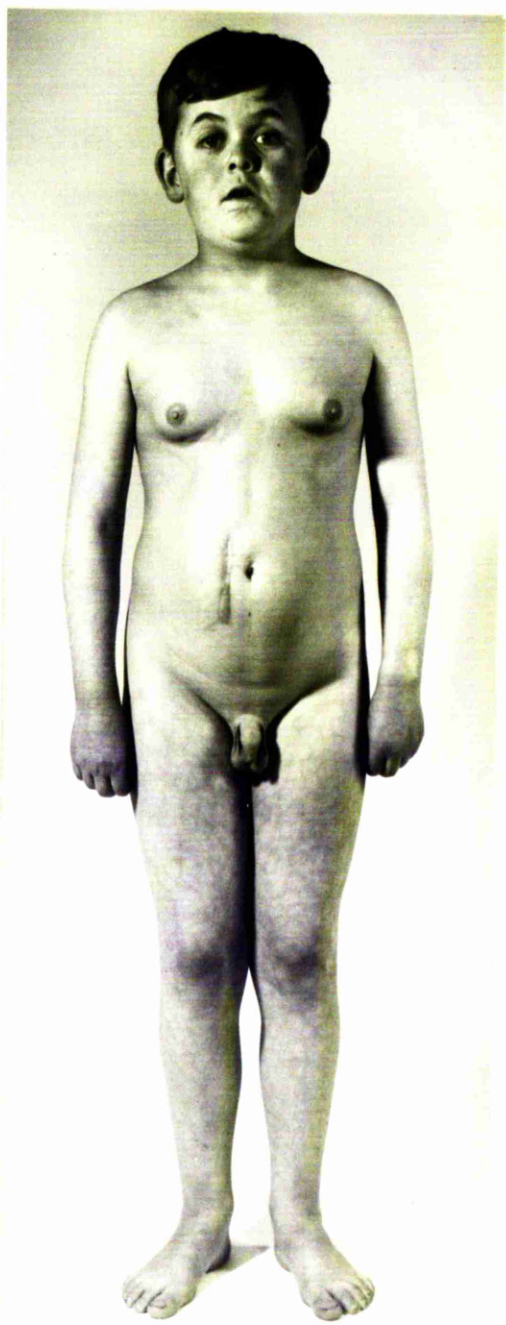


Fig. 1. W.Mcl. Male case of hypopituitary dwarfism. Gynaecomastia due to treatment with ethyl estrenol.

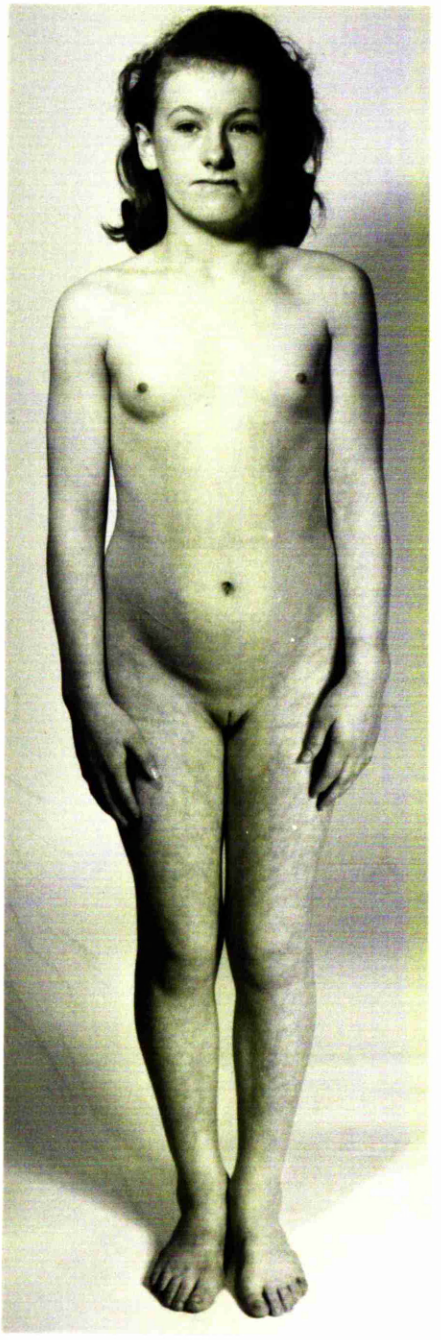


Fig. 2. E.O. Female case of hypopituitary dwarfism. Breast enlargement due to treatment with ethylestrenol.

3. Congenital hypopituitary dwarfism.

This condition is generally regarded as the gradual onset of growth retardation in the 2nd or 3rd year of life associated with an immature facies, a height-age more retarded than skeleteal age, with a height well below the 3rd percentile after the age of 4 years. These patients usually show increased insulin sensitivity and some degree of impaired sexual development. Evidence of subthyroidism and hypofunction of the adrenal cortex are usually considered to be present although in variable degrees. Group 3 of this series consists of a boy and a girl, both aged 13 years whose parents gave a history of the onset of growth retardation during the second year of life. Both showed immature facies (see photographs), and their height and bone ages were grossly retarded (Table 2). At present neither are showing evidence of sexual development although both have developed breasts when treated with ethyl estrenol. In a group of 20 children being treated with this anabolic steroid, they are the only 2 who have shown breast enlargement. Since the treatment of small stature is outside the scope of this investigation the above findings must remain sub judice. The boy showed increased insulin sensitivity and no increase in growth hormone secretion during /

during hypoglycaemia (Table 3). These two children are classified as examples of congenital hypopituitary dwarfism.

Table 3. Growth hormone and blood sugar levels after intravenous insulin (o.1 unit/Kg. body weight) in 2 cases of hypopituitary dwarfism.

	Plasma HGH mμg./ml.		Blood Sugar mg./100 ml.	
Minutes	Male W.McM.	Female E.O.	W.McM.	E.O.
0	3	0	102	79
10	3	14	51	29
20	4	25	-	16
30	3	27	67	42
40	3	16	-	42
50	2	-	-	42
60	-	20	51	53
70	-	-	-	-
80	-	-	43	-
100	1	7	51	74

Urinary steroid metabolites

The object of the investigation was to study the adrenal function in these 3 groups of children by an analysis of the urinary steroid metabolites. It might have been expected that in the primordial and hypopituitary /

hypopituitary group, adrenal function would be impaired and that resting values might afford little information, especially if the analyses were to include estimation of the free unmetabolised corticosteroids, hydrocortisone and cortisone. Cope and Hurlock (1954) have pointed out that under basal conditions urinary hydrocortisone levels are unsuitable for detecting adrenal hypofunction. Thus it was decided that each patient would receive a standard adrenal stimulation with corticotrophin and the adrenal response to this as reflected in the urinary steroid metabolites would form the basis of the comparison. A group of 14 other children, whose ages ranged from 3 months to 10 years, who were recovering from non-endocrine disease, were included in the study as normals.

Each child received corticotrophin (Acthar gel-H.P. Armour) 20 mg. at 12-hourly intervals for 2 days and on the second day, a 24-hour collection of urine was made using a small amount of chloroform as the preservative. This urine was analysed for hydrocortisone, cortisone and their tetrahydrometabolites by the methods set out in Chapter 5. The results obtained from this investigation are shown in Table 4 (normal children), Table 5 (malnutrition group) and Table 6 (primordial and hypopituitary groups).

The body handling of corticosteroid is contained in the /

the processes, secretion, plasma binding, degradation and excretion. Under normal basal conditions only that fraction of circulating corticosteroid which is not plasma bound, is available for excretion in the free state or for degradation. Thus binding inhibits both degradation and excretion of the free unmetabolised substance. To estimate one fraction of a corticosteroid gives little information regarding the processes between secretion and excretion. Further in certain diseases the total process of steroid metabolism is altered. Thus Doe, Zimmermann, Flink and Ulstrom (1960) found that in Cushing's Syndrome, the elevated levels of total plasma 17-hydroxycorticosteroids were due to an increase in the non-protein-bound fraction and Cope and Black (1959) showed that the rise in urinary hydrocortisone was twice as great as the increase in 17-oxogenic steroids, this being due to a greater proportion of free cortisol.

Degradation of corticosteroids is effected mainly in the liver by reductases and it would appear that these have marked specificity. A Δ4-steroid reductase 5β is specific for reducing the double bond of cortisone and hydrocortisone while a Δ4-steroid reductase 5α produces the 5α-metabolites. Both these reductase systems are irreversible and cannot re-introduce a double bond into the ring A saturated steroid. 5β-reductase has been found /

found in the soluble fraction of mammalian liver while the 5α-reductase is a microsomal enzyme.

To estimate total urinary 17-hydroxycortisteroids only would therefore give no information regarding these processes and if a greater understanding of adrenal function in these forms of dwarfism selected for the study is to be obtained an analysis of the several urinary metabolites of hydrocortisone might prove of value. Thus hydrocortisone, cortisone, tetrahydrocortisol (3α, 5β) allo-tetrahydrocortisol (3α, 5α) and tetrahydrocortisone were estimated and compared with the values found in the normal children.

Normals.

From Table 4 (normal group) it will be seen that free hydrocortisone appears in the urine at levels ranging from 125 to 482μg.per day with a mean for the group of 335μg.per day. Free cortisone is also present (18-120μg. per day) with a mean of 54μg. per day. The hydrocortisone/cortisone ratio was 4 to 9.1 (mean 6.4). Hydrocortisone is the principal adrenocortical glucocorticosteroid. Cortisone is not itself secreted by the adrenal cortex but it is formed peripherally by the 11-dehydrogenation of hydrocortisone. Little is known /

Table 4. Normal Group. Daily urinary hydrocortisone, cortisone and metabolites in the normal children of the series.

			Microgm./day		mg. per day				ratios			
Case	Sex	Age Years	F	E	THF	Allo-THF	THE	Total Metabolites	F/E	% allo-THF	Allo-THF / THF	Allo-THF and THF / THE
1	M	0.25	125	18	2.14	0.32	2.7	5.16	6.9	6.2	0.14	0.91
2	M	0.25	291	45	2.26	0.36	3.13	6.76	6.4	5.4	0.16	0.83
3	M	0.4	394	65	2.80	0.4	5.89	0.09	6.0	6.6	0.14	0.54
4	M	0.4	482	120	3.70	0.52	6.18	10.4	4.0	5.0	0.14	0.68
5	F	3.1	335	70	2.82	0.48	5.59	8.89	5.2	5.4	0.17	0.57
6	F	3.0	252	45	1.83	0.5	3.84	6.17	5.6	8.1	0.27	0.60
7	F	4.0	457	50	2.69	0.6	4.6	7.89	9.1	7.6	0.22	0.71
8	F	4.1	480	56	5.12	0.92	8.8	14.84	8.4	6.2	0.17	0.68
9	F	7.4	289	54	0.86	0.24	3.6	4.8	5.3	5.0	0.27	0.30
10	F	8.8	309	44	1.59	0.78	6.7	9.07	7.0	8.6	0.49	0.35
11	F	9.3	294	50	3.58	0.69	8.5	12.77	5.8	5.4	0.19	0.33
12	F	9.8	268	35	3.59	0.7	6.5	9.59	7.6	7.3	0.19	0.66
13	F	10.0	327	50	4.83	1.2	8.6	14.63	6.5	8.2	0.24	0.70
14	M	10.0	387	55	7.38	1.5	10.1	18.98	7.0	7.9	0.20	0.87
	Mean		335	54	3.22	0.65	6.05	9.93	6.4	6.6	0.21	0.52
	Range			18-420	0.86-7.38	0.32-1.5	2.7-10.1	4.8-18.98	4-9.1	5-8.6	0.1400.49	0.3-0.91

known of the extent of cortisone formation except that a substantial fraction of the urinary metabolites of hydrocortisone is tetrahydrocortisone (3α, 5β, 11-oxo). Thus before reduction is complete 11-dehydrogenation of hydrocortisone would appear to play an important part. An examination of the values obtained for the 3 metabolites, tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF) and tetrahydrocortisone (THE) immediately reveals that there is a wide range for each child in the group. What does however emerge is the relative constancy of the proportion of allo-tetrahydrocortisol in the total metabolites of the individual case. This finding probably indicates a functional constancy of reductase 5α whose function might also be hormone controlled. For instance Guignard - De Maeyer, Grigler and Gold (1963) noted that in 12 cases of Cushing's syndrome there were relatively small amounts of the 5α reduced metabolite of hydrocortisone while in congenital adrenal hyperplasia (2 cases) the percentage of allo-tetrahydrocortisol was minimally above normal.

The ratio allo-tetrahydrocortisol (3α, 5α) to tetrahydrocortisol (3α, 5β) gives an estimate of hydrocortisone metabolism with reference to hepatic 5α- and 5β-reductases. Again in the normal group this ratio is remarkably constant. The above-mentioned authors have /

have observed a fall in the ratio allo-tetrahydrocortisol/ tetrahydrocortisol (5α/5β) in children given corticotrophin and they suggest that the fall might indicate a limitation of 5α-reductase capacity or perhaps an increase in 5β-reductase. They found the 5α/5β ratio in 4 instances to range from 0.12 to 0.30 with a mean of 0.17. In the present series of 14 normal children the range was from 0.14 to 0.49 with a mean of 0.21. Visser and Cost (1964) when reporting on a family with a defect in the biosynthesis of aldosterone give some data on hydrocortisone metabolism in 3 normal infants aged 3, 5 and 8 months. In the resting state they found the 5α/5β ratio to be 8.2 and 5.3 while after corticotrophin stimulation (6.6, 5, 3, 4, 6 and 3.4) no gross change was observed.

The ratio allo-tetrahydrocortisol plus tetrahydrocortisol/tetrahydrocortisone (allo-THF+THF/THE) gives an indication of 11-oxidation of hydrocortisone. In terms of actual quantities of tetrahydrocortisone (THE) the normal infants and children in this series excreted more of this metabolite than the combined 5α and 5β metabolites of hydrocortisone. Further there tends to be a slight increase with age and although this does not corroborate Visser and Cost's (1964) figures it agrees with those found by Gupta (1966). The 11-oxidation index found in this group ranged from 0.3 - 0.91 with a mean of 0.52.

Because /

Table 5. Malnutrition Group. Daily urinary hydrocortisone, cortisone and metabolites in the children of small stature due to malnutrition.

Sex	Age	ug./day F	E	mg./day Allo-THF	Allo-THF	THE	Total Metabolites	F/E	% Allo-THF	Allo-THF / THF	Allo-THF+THF / THE
F	6.1	505	72	1.4	0.21	2.6	4.21	7.01	5.0	0.15	0.61
F	6.1	103	18	0.8	0.2	0.9	1.9	5.7	10.5	0.25	1.1
M	10.0	607	75	6.0	1.49	5.5	12.9	8.09	11.5	0.24	1.3
F	11.1	469	61	8.0	0.86	9.0	17.86	7.68	4.8	0.17	0.98
M	13.3	198	28	2.0	0.80	3.4	6.2	7.07	12.9	0.4	0.82
F	7.25	169	25	2.4	0.46	3.2	6.06	6.76	7.5	0.19	0.89
M	7.5	480	54	3.7	0.7	6.5	6.9	8.8	6.4	0.18	0.67
M	8.0	352	60	5.9	0.9	4.0	10.8	5.86	8.3	0.15	1.7
M	8.75	332	50	6.0	0.7	4.2	10.9	6.64	6.4	0.11	1.5
M	10.00	286	47	3.2	0.92	5.0	9.12	6.08	10.0	0.28	0.82
F	10.0	339	40	4.02	0.96	6.0	10.98	8.47	8.7	0.27	0.83
F	2.5	154	17	1.8	0.24	2.7	4.74	9.05	5.0	0.13	0.75
F	5.25	507	56	1.8	0.24	1.2	3.24	9.05	7.4	0.13	1.7
M	6.0	396	58	0.79	0.3	2.3	3.39	6.8	8.8	0.37	0.47
F	6.5	154	26	3.8	0.325	6.2	10.32	5.9	3.1	0.08	0.66
Mean		322	44					7.82	7.0	0.20	0.95
Range								5.7-9.05	3.1-12.9	0.08-0.37	0.47-1.7

Because of the very few studies of this kind reported in paediatric literature further comment on the significance of these findings must be withheld meantime.

Malnutrition.

In Table 5 are the results of a similar analysis of the same urinary steroids found in the 16 children whose small stature is attributed to deficient calorie intake. Once again it will be seen that there is a very wide scatter of the various metabolites for each individual although there seems to be a pattern emerging not dissimilar to that found in the normal group.

Observe that the mean daily excretion of free hydrocortisone is 322 µg. per day compared with 335µg. per day in the normal group. This difference is not significant. Cortisone is excreted in somewhat less amounts the mean being 44µg. per day with the range of 17 - 75 µg. per day (normals, mean 54µg. per day; range 18 - 120µg. per day). The range of the ratio of hydrocortisone to cortisone was not dissimilar in both groups although the mean was slightly higher in the children of small stature. This suggests that although they did not excrete as much free hydrocortisone as the normals there was a reduction in the conversion of hydrocortisone to cortisone. Because of the over-all differences in the total quantity of urinary steroids estimated in each case a correlation

of /

of values found for the 3 metabolites would serve little purpose. Table 5 however does show that of the total metabolites estimated allo-tetrahydrocortisol still forms a proportion (7 per cent) similar to that of the normal group (6.6 per cent). Also the activity of hepatic reductase 5α is similar to that of the normal group (0.2 and 0.21). The 11-oxidation index however shows a slight rise from the normal mean of 0.52 to 0.95 which bears out the finding of a higher hydrocortisone/cortisone ratio in the group. If an interpretation is permissible at this stage, it might be that these children of small stature retain more hydrocortisone in their circulating hormone pool, perhaps primarily for its more active glucocorticoid effects as well as for its anti-stress activity. This statement assumes that the nutritional deprivation may result in a lowering of the blood sugar level which is restored to normal by increased circulation of active glucocorticoid.

Primordial Dwarfs.

In Table 6 are the corresponding values found in the 4 cases of primordial dwarfism. Immediately it is clear that these children excrete less of each fraction than do those of the first two groups the mean ratio of hydrocortisone/cortisone remains similar to the normal group although the total quantities of both hydrocortisone and cortisone are markedly reduced (mean 139μg./day).

Allo- /

Table 6. Daily urinary hydrocortisone, cortisone and their metabolites in primordial and hypopituitary dwarfs.

Primordial Group.

Case	Sex	Age Years	Microgram per 24 hourly F	E	mg. per 24 hours THF	ALLO THF	THE	Total Metabolites	ratios F/E	% Allo- THF	Allo THF / THF	Allo THF + THF / THF
J.G.	M	3	135	21	1.96	0.297	2.543	4.8	6.4	6.2	0.15	0.88
R.H.	M	3	177	28	0.658	0.112	1.13	1.9	5.9	5.9	0.17	0.68
J.A.	M	3.8	204	30	1.863	0.261	2.616	4.74	6.8	5.5	0.14	0.81
D.P.	M	4	50	9	2.124	0.22	2.596	3.24	5.5	6.8	0.13	0.81
Hypopituitary Group.												
E.O.	F	13	870	75	1.728	0.382	1.05	3.16	11.5	12.1	.22	2
W.McI	M	13	800	56	1.848	0.442	1.110	3.40	14.2	13.0	.23	2.06

Allo-tetrahydrocortisol holds a normal percentage of the total metabolites although there might be a slight restriction or limitation of 5α-reductase as seen in a somewhat lower ratio of allo-tetrahydrocortisol/tetrahydrocortisol (0.147). It might, however, be argued that 5β-reductase is increased but information on minor alterations in this ratio is scant. The mean 11-oxidation index in the group is 0.795 which is higher than that of the normal group. The range however of the individual values is well within the normal range, and the higher mean value is probably not significant especially since the hydrocortisone/cortisone ratio is judged normal.

Pituitary Dwarfs.

In Table 6 is also shown the comparable figures for the 2 cases of hypopituitary dwarfism. It is regrettable that only 2 cases had been sufficiently thoroughly investigated to justify inclusion for it is in these 2 children that the most striking changes are observed. Although the total urinary metabolites remain low, more comparable to the values found in the primordial dwarfs the daily excretion of free hydrocortisone is remarkably high (800 and 870µg. per day). While the excretion of cortisone is also high (56 and 75µg. per day) the very high ratio hydrocortisone/cortisone (12.1 and 13) indicates a body handling of hydrocortisone in favour of maintaining /

maintaining increased circulation of free glucocorticoid. The percentage of allo-tetrahydrocortisol has increased to 12.1 and 13 per cent of the total metabolites while the 5α/5β ratio remains reasonably normal at 0.22 and 0.23 (normal group mean 0.21). The significance of this high percentage of allo-tetrahydrocortisol is not clear but Bush and Willoughby (1957) have reported high values in hirsute women.

The other striking feature of these 2 cases is the high 11-oxidation index (2 and 2.06) which has been obtained by a shift of metabolism in favour of higher values for tetrahydrocortisol than tetrahydrocortisone. In the other children studied of all groups, more tetrahydrocortisone than tetrahydrocortisol was invariably isolated.

Results interpreted.

The results obtained in this analysis have been reviewed. Are they of significance in relation to the conditions in which they have been found?

As stated earlier hydrocortisone secreted by the adrenal cortex circulates in part unbound to protein. Only this fraction is free for glomerular filtration and can be detected in the urine. Resting values of urinary hydrocortisone are of no value in detecting hypofunction of the adrenal cortex but Ross (1960) has stressed its value in the diagnosis of Cushing's syndrome when it is increased.

The /

The fraction of protein-bound hydrocortisone is bound to a globulin named 'transcortin' and binding tends to inhibit both excretion and metabolism. It is assumed that the free steroid is released from the binding protein according to body requirements so that there is an available circulating pool of active material.

The main site of metabolic degradation is in the liver by the system of 5α and 5β-reductases and after reduction the tetrahydrometabolites, conjugated mainly with glucuronic acid, are excreted in the urine.

There are few abnormal protein-binding conditions and the alterations observed in the metabolism of hydrocortisone in the primordial and hypopituitary dwarfs are unlikely to be due to alterations in transcortin levels or activity.

Apart from the slight increase in the 11-oxidation index found in the group of small children in whom malnutrition might have been a causal factor no substantial difference was noted between their hydrocortisone metabolism and that of the normal group. The influence of calorie intake on human growth is well recognised. Small appetites may result in less food being made available which in turn predisposes to anorexia. Such a circumstance may well reduce the available carbohydrate required to maintain adequate blood sugar levels and as a compensatory mechanism, endogenous glucocorticoids assume a greater role in maintaining /

maintaining adequate blood sugar levels. Hence less hydrocortisone is converted peripherally to cortisone, hydrocortisone being the more active glucocorticosteroid. The net result might therefore be a diminution of tetrahydrocortisone (THE) which might account for the slightly raised 11-oxidation index.

In primordial dwarfism little is known of the pituitary/adrenal function. One case described by Hubble (1966) had a fasting growth hormone level of 12 mμg./ml. They do not respond to treatment with growth hormone (Shepherd, Waxman, Bernstein and Ferrier, 1960) and the results of this present investigation indicates that they secrete smaller quantities of free hydrocortisone than do normal children when subjected to a standard corticotrophin stress. Otherwise their metabolism of hydrocortisone does not differ from the normal pattern.

According to modern concept, growth proceeds by cell multiplication and by increase in cell size. In the young embryo multiplication is more important while in postnatal life increase in cell size is the determining factor for growth. Cheek and Cooke (1964) have suggested that in primordial dwarfism there is a decreased number of cells of normal size so the demand for growth hormone is less. If this hypothesis is applicable to the cellular content of the adrenal cortex then it is reasonable that in response to a standard stimulus the secretion /

secretion of hydrocortisone should be less than in the average normal subject.

In hypopituitarism, with growth hormone failure, hypoglycaemic unresponsiveness is characteristic often in association with evidence of thyroid and adrenocortical insufficiency. It is seldom that the complete picture is seen and it is an impression that some cases are deficient in growth hormone alone. The 2 cases investigated here, had lowered growth hormone levels (Table 3) and one (W.McI.) exhibited hypoglycaemic symptoms during an insulin unresponsiveness test. Both showed infantilism and the areas of their pituitary fossae were 30.5 sq.mm. and 42 sq.mm. (both below the 5th percentile for age) by the Silverman technique. The protein bound iodine level was normal in both. The finding of a high daily excretion of free hydrocortisone in both was unexpected although in that the total urinary metabolites were low clearly indicates that this latter finding did not indicate an increased adrenocortical activity, but rather an alteration in hydrocortisone metabolism. The evidence suggests that in these two children the body requirements for free hydrocortisone, probably for its glucocorticoid action, has resulted in a reduction in its conversion to cortisone as evidenced by the low excretion of tetrahydrocortisone and the high 11-oxidation index.

The /

The present understanding of the action of human growth hormone could support this hypothesis. Raben (1965) asserts that human growth hormone is a single substance causing both growth by protein synthesis and lipolysis. Levine and Luft (1964) however consider that two hormonal fractions compose human growth hormone, one promoting protein synthesis and increasing insulin secretion and the other having a diabetogenic effect - resisting insulin activity - and a lipid mobilising action. Randle, Garland, Hales and Newsholme (1963) have suggested that free fatty acids released from adipose tissue under the influence of growth hormone inhibits carbohydrate metabolism in muscle and thereby causes a rise in blood sugar. This mechanism for maintaining the blood sugar level may break down when growth hormone production is impaired and the alternative action of glucocorticosteroids would then be of importance. Long, Katzin and Fry (1940) have shown that the source of the carbohydrate to maintain adequate blood sugar levels by glucocorticoid activity is from increased protein catabolism. Corticotrophin then, through its action on the adrenal cortex, antagonises human growth hormone. If this is the mechanism operating in growth hormone deficient children then failure of synthesis as well as increased protein catabolism adequately explains their growth retardation but it might also account for the higher level of free urinary cortisol found.

It /

It is possible however that since it is only the free protein-unbound hydrocortisone which is filtered by the glomerulus that less protein binding takes place in growth hormone deficiency. An extensive search of the literature has cast no light on this problem although Korner (1965) has shown that one action of growth hormone is to stimulate RNA synthesis. It is conceivable that deficiency of growth hormone might result in reduction of the labile pool of proteins and that the specific α_1-globulin, transcortin, is quantitatively reduced.

Since so little contemporary work on this subject is available for comparison the full significance of these findings cannot be stated. It is clear that further study of the type already completed here in association with an evaluation of plasma steroids, free and protein-bound, is essential in the hope that it will yield information which will help to add a cubit to the height of these children of short stature.

STUDIES IN ALDOSTERONE EXCRETION.

Urinary aldosterone in cases of congenital pyloric stenosis and in nephrosis.

The isolation and characterisation of aldosterone by Grundy, Simpson, Tait and colleagues (1952 and 1954) was a remarkable achievement and subsequent investigations of its actions led to a fuller understanding of renal tubular reabsorption of sodium and potassium.

However, the concentration of aldosterone in urine is approximately one in 200 million, and in blood one in 1,000 million (Ross, 1959) which demands that methods for its quantitative estimation must not only be specific but accurate and reliable. While the paediatrician is familiar with the mineralocorticoid effects of milligram quantities of desoxycorticosterone, it is perhaps not without some reluctance that tenths of a milligram of 9α-fluorohydrocortisone, a more powerful salt-retaining steroid than desoxycorticosterone, are being introduced to the therapeutic armamentarium for the management of the salt-losing syndrome in congenital adrenal hyperplasia. It is against this background that the microgram or perhaps nanogram quantities of aldosterone, likely to be encountered in paediatric practice are to be seen. Has the estimation of urinary aldosterone, often in less than microgram amounts, a place in paediatric diagnosis or is the estimation of research value only?

Salt-losing states demand immediate correction by measures often including the administration of synthetic mineralocorticoid /

mineralocorticoid and such measures may rapidly remove the stimulus for aldosterone secretion. To delay treatment while a urine collection is made may well prove fatal. Conditions exist in which aldosterone excretion is markedly diminished, for example the salt-losing form of congenital adrenal hyperplasia, while increased excretion has been reported in nephrosis (Luetscher and Johnson, 1954). Visser and Cost (1964) have described a family with a biosynthetic defect in the production of aldosterone but diagnosis of this condition depends on more elaborate biochemical procedures than demonstrating an absence of aldosterone in the urine. These authors emphasise that the finding of aldosterone precursors in the urine is essential to the diagnosis and there are few paediatric centres where urinary tetrahydrodesoxycorticosterone, tetrahydrocorticosterone and tetrahydro-18-hydroxycorticosterone could be estimated with precision. Further the cases referred to are unique in the literature.

Conn's (1955) syndrome of hyperaldosteronism is rare in paediatric practice but exhibits the same clinical features of hypokalaemia, alkalosis, muscle weakness with attacks of tetany and raised blood pressure typical of the adult form of the disease. In Conn's syndrome the urinary excretion of aldosterone is increased but without demonstrating a raised aldosterone secretion rate no surgeon would be prepared to undertake the hazardous task of exploring /

exploring the adrenals for an adenoma which might be remarkably small. The estimation of aldosterone secretion rate requires the administration of tritium-labelled aldosterone and at present ethical considerations restrict our injecting children with radio-active material. Nonetheless, Laragh (1960) has suggested that measurement of secretion rate is a more reliable index of increased production than the quantity of the acid hydrolysable metabolite usually measured.

Conn's concept of 'primary aldosteronism' does not include oedema although it is present in those conditions which give rise to secondary aldosteronism such as cardiac failure and nephrosis. Fine, Meisalas, Colsky and Oxenhorn, (1957) have however described oedema in association with adrenal tumour and hyperaldosteronism.

Of the conditions in which secondary hyperaldosterone is seen, nephrosis is not rare in hospital paediatric practice in Glasgow. During the years 1963 to 1966, 74 children with nephrosis have been in-patients in Professor J.H. Hutchison's wards in the Royal Hospital for Sick Children, Glasgow. Some of these however are re-admissions of cases in relapse. In a selected number (6) of these the urinary excretion of aldosterone was monitored during the oedmatous and diuretic phases.

Congenital pyloric stenosis is also a condition in which the /

the excretion of aldosterone might be increased because of the loss of sodium and chloride due to vomiting.

In 15 cases of this condition the daily urinary aldosterone was estimated.

These results will be discussed.

Methods of Estimation

The early biological assay techniques for aldosterone estimation devised by Dorfman, Kotts and Feil (1947) and used by Luetscher and colleagues (1951) were displaced by physicochemical methods devised by Ayres, Garrod, Simpson and Tait (1957) and Brooks (1960). Ayres and co-workers (1957) introduced the principle of isotope dilution for the estimation of aldosterone. This involved the use of C^{14}-labelled acetic anhydride to form the diacetate of aldosterone and after separation of the diacetate by column chromatography, the specific activity of the aldosterone diacetate was determined. From knowledge of the original radioactivity present, quantitation of the separated aldosterone could be calculated. With this method, recovery rates of 37 per cent have been reported.

With the introduction of tritium-labelled aldosterone double labelling methods for aldosterone estimations were devised by Kliman and Peterson (1958) and they claim that with this method 0.01 µg. of aldosterone can be detected.

The /

The principal excretory product of aldosterone is a 3-oxo-conjugate, split by hydrolysis at pH 1 to liberate free aldosterone (Jones, Lloyd-Jones, Riondal, Tait, Tait, Bulbrook and Greenwood, 1959). Only about 10 per cent of the total aldosterone secreted is excreted in the urine in this form (Mills, 1954; Neher and Wettstein, 1955). A further 10-15 per cent is excreted as tetrahydroaldosterone conjugated with glucuronic acid (Ulick, Laragh, Lieberman and Loeb, 1958). By the method used in this work only the free and the 3-oxo-conjugate of aldosterone are estimated.

There is no large series of normal values for urinary aldosterone in infancy and childhood quoted in the literature. Minick and Conn (1964) give values for aldosterone excretion in 30 normal children ranging from 4 months to 13 years 8 months with recovery rates from 75 - 85 per cent. The range is from 1 µg. at 4 months to 9 µg. at 13 years 8 months but they also found a daily excretion of 12 µg. per day in a 22 year old male and 6 µg. in a 54 year old woman. It is clear from these findings that there is a wide range of normal values from infancy to adult life and while values greatly in excess of the accepted normal range would be significant, undue stress should not be placed on values less than 1 µg. estimated by the present physico-chemical method. In these /

these circumstances secretion rate studies using double isotope-labelling techniques are clearly indicated, although if a low secretion rate causes clinical symptoms the emphasis would naturally be on treatment in the first instance.

The investigations to be discussed in this Chapter concern the estimation of urinary free aldosterone in 15 male infants with pyloric stenosis and 6 children with nephrosis admitted to the Royal Hospital for Sick Children, Glasgow.

Urine was collected from the infants by securing a length of ½" (1.27 cm.) wide Paul's tubing over the penis with 'Sleek'. The free end was knotted and the urine which collected was transferred at intervals to suitable containers. 5 consecutive 24-hour urine collections were made using chloroform as preservative. The 6 children with nephrosis were continent of urine and co-operated in urine collecting satisfactorily. Occasionally, during the diuretic phase nocturnal enuresis resulted in incomplete collections and these are indicated. 105 urine collections involving 135 estimations of free urinary aldosterone were undertaken. The basic method used is that of Brooks (1960) as amplified in Chapter 5.

Pyloric Stenosis

The management of infants with pyloric stenosis has /

has local variations. In this department a regime of simplicity has been adopted in which the patient is disturbed as little as possible. The diagnosis is made on palpation of a pyloric 'tumour' during a test feed, and only rarely is diagnostic assistance sought from a barium meal.

A gastric lavage is done not less than four hours before operation. Pyloromyotomy is performed under general or local anaesthesia according to the surgeon's preference and the child returned to the medical ward. The feeding regime at the time of the investigation was to commence 4 hours after operation with 8 ml. half-cream National Dried Milk alternating hourly with half-strength physiological saline in unrestricted quantities. The volume of milk is gradually increased and also the interval between feeds until on the third post-operative day 45 ml. milk is being given 4 hourly. Water at this stage is given instead of saline. On subsequent days the volume of each milk feed is increased by 15 ml. until an appropriate full feed is reached. The child is discharged on the 5th post-operative day and returns on the 12th day for removal of sutures. Breast-fed infants are given expressed breast milk and allowed home usually on the 2nd post-operative day to continue breast-feeding. The sutures are also removed on the 12th post-operative day.

Biochemical /

Biochemical studies are not done routinely and parenteral fluid and electrolyte replacements are undertaken only in the infrequent cases in which dehydration is severe.

During the past 4 years 58 cases, on average, have been treated annually in this unit with one death (a mortality rate of less than 0.5%).

The present study was designed to determine the level of free aldosterone excretion in 15 of these infants. It was thought that they might shown an increased excretion of aldosterone since they also exhibit a marked tendency for renal conservation of sodium chloride. In addition to the loss of chloride in the vomit, there is also loss of sodium and potassium and the total body electolyte concentration is reduced. This further contributes to dehydration. Increased aldosterone production might be expected to combat these changes.

Table 1. Urinary excretion of free aldosterone in cases of pyloric stenosis treated by Ramstedt operation.

Case No.	Age (wk.)	Urinary Aldosterone (µg.) 5-day Total	Per Day	Per Day Corrected
1-7		Aldosterone not detected in single 24-hour collections.		
8	11	11.5	2.3	3.0
9	6	3.0	0.6	1.0
10	5	2.0	0.4	0.6
11 /				

Case No.	Age (wk.)	5-day Total	Per Day	Per Day Corrected
11	5	2.5	0.5	1.0
12	3	3.0	0.6	0.8
13	10	2.5	0.5	1.1
14	10	Nil	-	-
15	7	7.5	1.5	2.7

Aldosterone

The estimation of aldosterone by the method used involves three chromatographic systems. Small losses occur inevitably at various stages and it is necessary to measure the final percentage recovery. This is done by adding 10 muc. (millimicrocurie) 7-H^3-aldosterone to the urine before extraction and measuring the specific activity of an aliquot of the diacetate fraction. Another aliquot of this fraction containing approximately the same amount of aldosterone as the standards to be used, is applied to the final chromatogram. Since the amount of aldosterone likely to be encountered was not known, the optimum aliquot applied to the final chromatograph was found by trial and error. In Cases 1 - 7, using 24-hour urine volumes, insignificantly different galvanometer readings for blank and fluorescence were obtained, even when using three-quarters of the aldosterone extract and with satisfactory recovery rates. Subsequently, the urine from each patient was bulked for 5 consecutive 24-hour periods and the daily excretion of free /

free aldosterone taken as one-fifth of the total. Thus in only 7 of the 15 cases studied were reliable results obtained (Table 1). With recovery rates ranging from 43 - 75 per cent, the daily urinary excretion of free aldosterone ranged from 0.6 to 3 µg.

In Table 2 the calculated excretion of free aldosterone for the first 24-hour period in Cases 1 - 7 is shown.

Table 2. Daily excretion of free aldosterone calculated from recovery rates and fluorimeter sensitivity: total single 24-hour urine specimens were extracted.

Case No.	% Recovery	Aliquot of Extract Applied	Calculated Daily Aldosterone Excreted (µg.)
1	54	0.25	0.7
2	46	0.25	0.8
3	60	0.25	0.6
4	60	0.5	0.3
5	58	0.5	0.3
6	38	0.75	0.35
7	62	0.75	0.2

The average paper blank reading in each experiment was equivalent to 0.3 µg. aldosterone. Under test conditions a ratio of 1 - 4 for blank to aldosterone diacetate standard readings was consistently achieved by a minimum of 0.5 µg. amounts. While readings could be /

be obtained for 0.1 µg. aldosterone diacetate a 'noise to signal' ratio of at least 1 to 4 was accepted as a better working rule. It will be seen from Tables 1 and 2 that the calculated daily excretion of free aldosterone and that actually found from bulked 5-day collections of urine are in agreement and in each case the daily urinary excretion is 3 µg. or less. Since only two fractions are estimated by the method used, and together these fractions represent 5 - 15% of the total daily secretion of aldosterone by the adrenal cortex (Ross, 1959; Jones, Lloyd-Jones, Riondel, Tait, Tait, Bulbrook and Greenwood, 1959), daily secretion rates for aldosterone from 5 - 60 µg. might be expected in infants up to 3 months of age.

Data on the aldosterone secretion rate for infants in this age-group are scant, but Degenhart, Visser, Wilmink and Croughs (1965), using the double-isotope dilution technique of Kliman and Peterson (1960), found the aldosterone secretion rate to be 60 µg./24 hours and 13.5 µg./24 hours in two infants, aged 2 months and 4 months respectively. After 4 to 6 days of salt deprivation the secretion rates were 100 µg./24 hours and 225 µg./24 hours respectively. In the present series all the urine collections for aldosterone estimation were made after the operation and during the period when an increased salt intake was encouraged. Indeed all cases received during the 3 days after operation more salt than would have been /

been contained in standard milk feeds appropriate for the infants' ages. This might have depressed aldosterone production. The values found for free urinary aldosterone agree with those found by the others whose work has been quoted and since only very small amounts of free aldosterone appear in the urine of this age group, if more critical information is required then other methods for aldosterone secretion should probably be undertaken.

Nephrosis.

Massive oedema, hypoproteinaemia, hypercholesterolaemia and gross albuminuria were present in each of the six cases studied. In early experiments while perfecting the method for urinary aldosterone estimation I had been unable to isolate and quantitate free aldosterone in cases of nephrosis by the method used (Brooks, 1960). Furthermore, for measuring recovery rates the tritiated aldosterone (10 muc.) which had been added to the urine aliquots before hydrolysis on counting, indicated ridiculously low recovery rates. This early failure was attributed to inexperience until in a series of estimations of urinary aldosterone (urines from a group of normal children) quite fortuitously a specimen of urine from a case of nephrosis was included. Only in this latter specimen was the recovery rate unsatisfactorily low and free aldosterone was not detected. This finding was disquieting /

disquieting for it is commonly accepted that nephrosis is one of the three main conditions in which there is a high aldosterone excretion secondary to non-adrenal pathology (Luetscher and Johnson, 1954). The other two conditions are congestive cardiac failure (Stringer and Wener, 1953) and hepatic cirrhosis (Chart and Shipley, 1963).

It was therefore planned that the daily urinary excretion of aldosterone would be estimated in selected nephrotic patients (6) from the oedematous, oliguric, albuminuric phase right through to the diuretic phase when albuminuria has cleared.

Results.

The results of this investigation are shown in Tables 3 - 8. Each patient shows a progressive reduction in albuminuria and only in one case (DN) was there a continuing mild (1.5 g./litre) albuminuria when the experiment was concluded. All showed a reduction in weight which corresponded to the urinary diuresis. It will be further observed that the greatest diuresis occurred synchronously with a marked reduction in the albuminuria. In columns 4 and 5 of Tables 3 - 8 will be found the values for daily aldosterone excretion with their corresponding recovery rates. It is pointed out here that urine samples were processed in batches of 6 and since it was necessary to use the maximum quantity of /

Table 3. Daily urinary aldosterone in relation to urine volume and proteinuria in nephrosis. Table 4.

Case 1						Case 2.				
% Recovery	Aldosterone ug./day ≡	Protein g./litre	Urine Vol.(ml.)	Weight (Kg.)	Day	Weight (Kg.)	Urine Vol.(ml.)	Protein g./litre	Aldosterone ug./day ≡	% Recovery
	-	-	390	36.84	1	24.7	380	-	-	
	-	-	330	36.84	2		420	-	-	
	-	18	225	36.8	3		600	12	-	
24	-	20	-	36.82	4	25.0	340	±6	-	14
	-	-	480	36.8	5		320	6.5	-	
	-	10	285		6	24.5	400	6	-	
	-	4	690	38.28	7	34.25	380	6	-	
	0	4	975	39.15	8	23.8	420	7	-	
41	0.8	1	960	40	9	23.8	450	7	-	26
	0.5	4	1290	39.84	10		800	3	-	
	12	0	2400	38.42	11	19.25	1000	2	-	
	9	0	3000	36.2	12		1500	1	2	
	6	+	2160	35.18	13		980	1	8	
52	10	+	2280	33.24	14	19.8	900	1	9	48
	8	-	1440	33.48	15	20.0	800	0		

≡ Uncorrected values. + Trace

Case 3						Case 4.				
% Recovery	Aldosterone ug./day ✱	Protein g./litre	Urine Vol.(ml.)	Weight (Kg.)	Day	Weight (Kg.)	Urine Vol.(ml.)	Protein g./litre	Aldosterone ug./day ✱	% Recovery
	-	-	440	15.05	1	29.64		13	-	
	-	-	420	14.4	2	30.04	720	11	-	
25	-	-	540	14.5	3	30.2	570	6	-	21
	-	12	600	14.5	4	30	660	6	-	
	-	5	420	14.5	5		465	10	-	
	-	9	540		6	30.8	-	10	-	
	-		500	14.4	7	31.2	-	4	0.4	
43	-	8	540	14.48	8	32.2	2070	3	1.0	56
	0.5	2	600	13.6	9	29.88	2070	3	0.7	
	4	1	610	13.5	10	27.4	3570	1	1.0	
	6	½	810	12.75	11	24.2	2190	1	1.5	
75	12	-	800	13.0	12	-	780	1	1.6	
	20	½	810	13.2	13	22.72	1290	1	0.8	60
	16	-	900	13.0	14	22.6	840	1½	0.4	

✱ Uncorrected values.

Table 7. Daily urinary aldosterone in relation to urine volume and proteinuria in nephrosis. Table 8.

Case 5						Case 6				
Recovery %	Aldosterone ug./day *	Protein g./litre	Urine Vol.(ml.)	Weight (Kg.)	Day	Weight (Kg.)	Urine Vol.(ml.)	Protein g./litre	Aldosterone ug./day *	Recovery %
	-	12	105	16.84	1	23.2	320	1	0.5	
	-	8	240	17.4	2	23	410	1.5	1.0	
38		12	360	17.6	3	22.8	325	3	0.5	
	0.5	9	470	-	4	23.8	290	3.5	-	31
	-	6	420	17.7	5	23.2	300	5	-	
		-	960	17.92	6	23.5	460	12	-	
	-	6	930	18.0	7	24.2	480	12	-	
	-	6	1290	17.74	8	24.2	500	8	0.4	
	-	5	1680	18.0	9	24.7	550	4	1.0	
61	1.2	3	1200	17	10	23.5	1060	1	2.3	
	2.6	4	1530	-	11	21	2100	1	6.6	52
	3.2	-	1140	15.6	12	20.5	1800	1	7.0	
	3.6	-	960	15.6	13	18.2	1760	-	6.4	

* Uncorrected values

of the urine extract for the final chromatogram, in the hope that there would be adequate quantities of aldosterone to give a fluorescent spot, only one test in six was submitted for scintillation/counting, to estimate recovery. As will be seen, free aldosterone was not detected in any of these patients during the period when albuminuria was marked and it was only when the albuminuria cleared the measurable quantities of aldosterone were isolated. In none however could the values be truly considered in excess of normal.

Discussion.

What is the significance of these findings? Can they be justified on technical or biochemical considerations?

Some technical problems of the method are worthy of note. The hydrolytic process to free the Δ-4-3-oxo-conjugate of aldosterone is carried out at pH 1. With this degree of acidity the proteins in the urine are precipitated to form a thick curd. On even the most gentle agitation of the urine with chloroform for extraction there is formed a thick, creamy emulsion in which is trapped some of the chloroform. The approved technique for separation is to drain off the chloroform as much as will separate on standing and then to centrifuge the emulsion thus releasing the trapped chloroform, which is then beneath the emulsion in the test tube. The

emulsion may be further washed with chloroform and re-centrifuged in the hope that all the aldosterone likely to be adherent to the protein will be recovered. Does this process however release the aldosterone or does the albumin exercise a particular affinity for aldosterone? If this were so a possible explanation of the findings would be at hand.

To test this hypothesis human plasma was added to 500 ml. water to make protein concentrations of 2, 4 and 6 g. per litre. To a fourth flask no protein was added. It was assumed that the plasma used contained 5 g. total proteins/100 ml. To each flask 10 µg. aldosterone was then added and the procedure for hydrolysis extraction, separation and quantitation followed. It was felt that to add more than normal amounts of aldosterone would have been inconsistent with the nature of the experiment for if protein binding played a part in the low recoveries then greater quantities of aldosterone might have saturated the protein to create further unnatural conditions. In Table 9. the results of the experiment are shown.

Table /

Table 9. Recovery of aldosterone added to water containing human plasma to give protein concentrations of 2, 4 and 6 gm./litre.

Water/ml.	Protein G/litre	Added Aldosterone	Aldosterone Recovered µg.
500	2	10 µg.	3.0
500	4	10 µg.	1.5
500	6	10 µg.	2.0
500	0	10 µg.	8.0

These findings supported the hypothesis that it is with difficulty that free aldosterone is recovered from high-protein containing fluids. A further experiment was devised. One specimen of urine containing 4 gm. of albumin or more from each of the patients was selected and the pH brought to 1 with hydrochloric acid. When the precipitated proteins settled they were separated and to the remaining aqueous phase chloroform was added and hydrolysis allowed to proceed for 18 hours. The separated proteins were then suspended in water (100 ml.) and the pH re-adjusted to 1. The contents of the flasks were then boiled for exactly 10 minutes, cooled rapidly in ice-water and extracted twice with half volumes of chloroform. The extracts from each fraction were processed separately and quantitated. The results are shown in Table 10.

Table /

Table 10. Recovery of aldosterone from the protein precipitate of urine and the aqueous phase in 6 cases of nephrosis.

	Aldosterone µg. per 100 ml. urine			
Protein G/Litre	Protein precipitate	Aqueous phase	Total	Calculated daily excretion µg.
1. 4	2	0.2	2.2	2.2 x 6.9 = 15.2
2. 6.5	3.5	0.6	4.1	4.1 x 3.2 = 13.12
3. 8.0	1.8	0	1.8	1.8 x 5.4 = 9.72
4. 5.0	4.0	1.0	5.0	5.0 x 4.6 = 23.25
5. 9.0	2.1	0	2.1	2.1 x 4.2 = 9. 87
6. 6.0	3.8	0	3.8	3.8 x 3.0 = 11.4

The findings here indicate that the major portion of the aldosterone is in the protein precipitate and that it is released by boiling at pH 1.

Since /

Since the daily urine volume of these patients during the oedematous phase seldom is in excess of a few hundred millilitres the daily output of aldosterone is still within the normal range.

E.J. Ross published in 1959 his book entitled "Aldosterone in Clinical and Experimental Medicine" (Ross, 1959). He quotes 10 papers from 3 groups of investigators to support the claim that there is increased production of aldosterone in nephrosis. 6 papers from Luetscher and colleagues (1950-1954) indicate that the high sodium-retaining activity of extracts from urine of patients with nephrosis is due to aldosterone.

The methods for extraction are similar to those used in this work but their quantitation was by bioassay techniques rather than chemical.

In Luetscher and Johnson's (1954) paper 5 children with a nephrotic syndrome were studied. Pooled urine (1 - 5 days) for each patient was used, the protein content ranging from 1.7 to 7.3 g./24 hours for the 5 cases. Using a bioassay technique based on the excretion of sodium and potassium by adrenalectomised rats, they estimated the urinary sodium-retaining factor as 7.8 to 8.4 µg. DOCA equivalents. The equivalent of 1/72 of the daily urinary output was used per assay. Their only comment /

comment regarding the proteinuria is to say that it is not an important factor in the sodium-retaining activity of the extract.

In another communication, Luetscher and Deming (1950) show the effect of cortisone and intravenous plasma on the course of nephrosis. On cortisone alone 3 patients, aged 5, 7 and 5½ years showed a fall in albuminuria but a less impressive fall in the urinary sodium-retaining factor (2.0 µg. - 1.9 µg.; 3.1 µg. to 1.2 µg.; 4.6 µg. - 3.1 µg. DOCA equivalents).

In graphs which they show of 2 patients given intravenous albumin (10 - 50 g./day) it appears that during the period of administration, although the body weight fell in keeping with a diuresis, the albuminuria increased to 18 and 34 g. and at this time the urinary sodium-retaining factor fell to 1.8 and 2 µg. (DOCA equivalent). There was no appreciable change in the plasma protein level during the administration of albumin.

The interpretation of the fall in urinary sodium-retaining corticoids in these two patients is summed up in the author's words that 'it is of interest that assays of sodium-retaining corticoid showed a decrease in normal levels during or after diuresis induced by albumin'. It is of interest if it is accepted that /

that albuminuria is not an important factor in the estimation of the sodium-retaining activity of nephrotic urine.

In another paper by Luetscher and colleagues, (Axelrod, Cates, Johnson and Luetscher, 1955) the urine extracts from 3 nephrotic children (aged 10, 4 and 4 years) are compared with a urine extract from 1 normal child. Comparable figures are given as 6.3 and 10.8 µg.; 8 µg., 14.1 and 15.2 µg. (µg. DCA equivalents per 33 minute urine volumes). These are compared with the one normal value of 0.8 and 1.14 µg. (µg. DCA equivalent per 33 minute urine volume).

It must be remembered however that in alkali ion tests aldosterone possesses more than 100 times the mineralocorticoid activity of desoxycorticosterone while in Addison's disease aldosterone is only 20 - 30 times as potent as desoxycorticosterone.

If then the values in µg. DCA equivalents are expressed as true µg. aldosterone, there is no gross increase in urinary aldosterone output in the patients quoted.

Further aldosterone lacks the property of promoting water retention, which is so characteristic of desoxy-corticosterone so that increased aldosterone alone cannot be the cause of the oedema in nephrosis.

Two further points require consideration. The plasma /

plasma binding of hydrocortisone is to the protein 'transcortin' and this is an α_1-globulin (Slaunwhyte and Sandberg, 1959). In nephrosis plasma α_1-globulin is reduced in quantity and the deficit is found in the urine. These authors have shown that the 'transcortin' binding capacity is reduced in nephrosis.

The binding of aldosterone however is less well documented although from well planned experiments by Daughaday, Holloszy and Mariz (1961) it would appear that albumin is the chief medium for transporting aldosterone in the circulation. At lower temperatures than 37°C aldosterone is bound partly to albumin and partly to α_1-globulin and these are just the conditions prevailing in the nephrotic urine, namely a high albumin and α_1-globulin content. Aldosterone then is likely to be bound to both protein fractions in the urine from cases of nephrosis and it would appear that this fact has not received much consideration in the estimation of urinary aldosterone in nephrosis.

Bartter (1956) while reviewing the role of aldosterone in normal homeostasis cites a case of idiopathic hypoproteinaemia whose daily urinary aldosterone during the hypoproteinaemic phase was in excess of 120 µg./day (range over 10 days, 60 to 204 µg./day). When given albumin intravenously (50 g. daily) over 7 days the /

the urinary excretion of aldosterone fell to 10 - 20 µg./day, there was a sodium diuresis and the patients's weight fell from 61 kg. to 57 kg. The author argues that there was an expansion of the vascular compartment resulting in a lowering of aldosterone secretion with marked urinary loss of sodium.

On the basis that only unbound circulating cortisteroids are free both to exert their physiological activity and for glomerular filtration, it is clear that with a low plasma albumin, which would normally bind aldosterone, more free hormone will be circulating to cause sodium retention and more will be available for excretion. Intravenous albumin restores the level of the binding protein, to which circulating aldosterone has an affinity and it is thereby inactivated and its excretion inhibited. This would seem to be at least an alternative explanation of Bartter's findings.

Of the factors which determine the excretion of aldosterone, in the light of present knowledge, no dogma is justifiable for it would seem that here again it is probably incorrect to assume that a high excretion rate results from increased secretion or conversely that a low excretion rate indicates a reduction in the production of aldosterone. That the excretion of free aldosterone in nephrosis is high is quoted freely in the literature but /

but few papers consider the part played in this alleged finding of the hypoproteinaemia (hypoalbuminaemia) and the massive albuminuria characteristic of the oedematous phase of the disease.

It appears from Luetscher's work that extraction with chloroform should release the bound aldosterone although Ross (1967) and Grant (1967) have been unable to clarify this point. In discussion with Klopper (1966) I have found that he, too, has considerable difficulty in extracting steroidal material from high protein-containing biological fluids and has a scepticism which doubts reports wherein no special extraction procedures have been employed.

There are few references to investigations aimed at the separation of free aldosterone from high protein-containing urine, but this work demonstrates that with ordinary extraction procedures free aldosterone was not obtained from the high protein-containing urine of nephrotic patients but when the protein precipitate was boiled at pH 1 before extraction, free aldosterone was then found. Such strong acid hydrolysis would normally degrade most corticosteroids but Axley, Ingall, Norymberski and Woods, (1961) have shown that 17-deoxycorticosteroids do withstand boiling in strong acids for at least 10 minutes. They used this method /

method of hydrolysis for the block estimation of urinary 17-deoxycorticosteroids of which aldosterone is an example.

Summary

Once again it may be that in this delicate type of analysis secretion rate studies based on the isolation of aldosterone from plasma will give the answer to this and other unsolved problems regarding this powerful mineralocorticosteroid in cases of nephrosis.

CHAPTER FIVE

METHODS

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Determination of 17-oxosteroids in urine.

After measuring the daily urine volume, duplicate aliquots (10 ml.) are measured into 8 inch B 24 tubes. The tubes are placed in a boiling water bath and concentrated hydrochloric acid (1 ml.) added to each tube and the contents mixed. Boiling is continued for exactly 10 minutes to effect hydrolysis of conjugated 17-oxosteroids.

The tubes are then cooled in ice water immediately. Chloroform (10 ml.) is added to each tube, which is stoppered and inverted sharply twenty times to extract the steroids into the organic (chloroform) phase. After the layers separate, the upper urine layer is removed by suction and the chloroform washed successively, once with 2.5 N - sodium hydroxide (2 ml.) and twice with distilled water (2 ml.), the aqueous phases being removed by suction after each wash.

While effecting complete removal of the aqueous phases there are small losses of the organic phase but always 5 ml. chloroform can be obtained from each of the duplicates. At no time has automatic pipetting been used. The chloroform (5 ml.) is then placed in B 10 test tubes and evaporated under a stream of nitrogen in

a /

a water bath at 40°C. The residues are subjected to a modified Zimmermann reaction.

Once weekly recovery experiments have been included by adding to 2 additional aliquots (10 ml.) of urine, 20 µg. of a typical 17-oxosteroid - (dehydroepiandrosterone DHEA). This is a convenient 17-oxosteroid for use also as a standard in the Zimmermann reaction.

Zimmermann colour reaction.

This reaction is carried out in B 10 test tubes. The steroid residues from the above extractions are dissolved in the Zimmermann reagent (0.25 ml.). The tubes are stoppered and gently rotated to ensure that all the steroid extract is in solution. They are then placed in the dark at 20°C for 1 hour. Thereafter, ethanol (3 ml.) is added to each tube and after mixing the optical densities are read at 440, 520 and 600 mμ. on an Unicam S.P.600 spectrophotometer using a 10 mm. light path. The Allen (1950) correction formula is applied. 2 standards of DHEA (10 μg.) and 2 blanks containing only the Zimmermann reagent are treated in the same way. After subtracting the blank readings from both standards and test readings the microgram equivalents of the test samples are calculated and appropriately adjusted for the total daily urine volume. The final result is expressed as mg. 17-oxosteroids per 24 hours.

From 1963, instead of reading the optical density of the Zimmermann chromogens in ethanol they have been extracted into ether and the optical density of the ether layer measured. This is achieved by adding to each tube aqueous ethanol (2 ml. 50%) followed by diethyl ether (3 ml.) Fig. 1 shows the improved colour obtained /

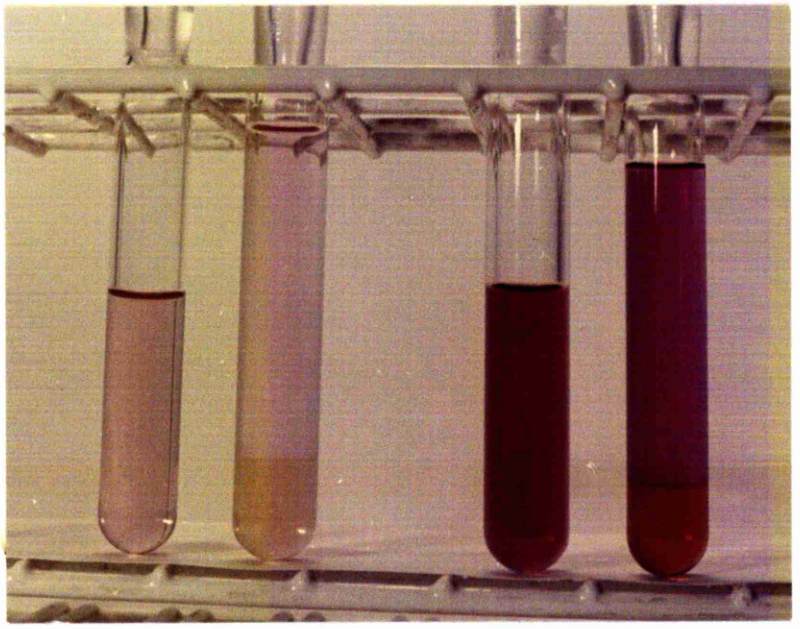


Fig. 1. Zimmermann chromogens in ethanol (left of pairs) and in diethyl ether(right of pairs).

obtained by this step.

The Zimmermann reagent is made immediately before use by adding 0.5 per cent purified m-dinitrobenzene in ethanol (2 ml.) to 25 per cent tetramethyl ammonium hydroxide (B.D.H.: 1 ml.). Both reagents are stored at 5°C and the m-dinitrobenzene is made freshly each week. Ethanol is aldehyde-free and the diethyl ether is peroxide free.

Girard T Reagent.

This reagent will combine with ketones to form water soluble complexes. By partitioning the reaction mixture between water and benzene non-steroidal non-ketonic material will be extracted into the organic phase which may then be discarded. The ketonic-Girard complexes in the aqueous phase may then be hydrolysed by strong acid at room temperature to release the free ketones. These may then be extracted into organic phase for quantitation. Theoretically, by this process, 17-oxosteroids may be extracted from urine extracts freed from non-steroidal chromogenic material. The procedure is carried out as follows.

Urine aliquots (10 ml.) are hydrolysed, extracted and blown to dryness as earlier indicated. Ethanol (2 ml.), Girard Reagent T (0.2 g.) and glacial acetic acid (0.5 ml.) are added to the dried extracts and refluxed for 20 minutes. /

minutes. After cooling, 3 N-sodium hydroxide (3 ml.) and water (50 ml.) are added and further extraction with benzene (50 ml.) is carried out. After separation the benzene is discarded. Concentrated hydrochloric acid (5 ml.) is added to the aqueous phase and hydrolysis allowed to proceed at room temperature for 1.5 hours. The freed ketones are then extracted with benzene (50 ml.), the benzene separated, washed and evaporated to dryness in vacuo. The Zimmermann reaction is then performed on the residue as before.

The Allen formula (1950).

This correction formula has been applied to all spectrophotometric readings and to facilitate understanding of the scientific data, shortly to be given, I include here a note on its significance. Allen studied the absorption curves of the colours produced by dehydroepiandrosterone (50 µg.), pregnanediol (500 µg.) and an urinary extract treated separately with ethyl alcohol (90%) and concentrated sulphuric acid in the ratio: 1:4 v/v. Both dehydroepiandrosterone and pregnanediol gave absorption maxima the former at 600 mµ. and the other at 480 mµ., while between these wave lengths a urine extract alone gave an absorption curve which approached a straight line, decreasing from 480 mµ. to 600 mµ.

By /

By developing the colour reaction in a mixture of dehydroepiandrosterone, pregnanediol and urine extract he found that the resulting absorption curve contained the elements of the absorption curve of each substance. He was therefore able to devise a formula which permits the analysis of mixed absorption curves provided the absorption curve of the contaminating substance closely approaches a straight line. The equation is:

$$CDX_x = OD_x - \frac{OD_a + OD_b}{2}$$

where OD_x is the observed density at wave length x, at the absorption maximum; OD_a and OD_b are the observed densities at wave lengths a and b; a and b being equidistant from x; CD_x is the calculated density due to substance X having an absorption maximum at wave length x.

In Fig. 2 I illustrate this correction calculation using dehydroepiandrosterone (DHEA) and a urine extract. It will be seen that the optical density of the Zimmermann colour at 520 mμ. for urine plus steroid (237) is greater than the optical density at 520 mμ. for the steroid alone (206). The difference is the contribution by the impure substances in the Zimmermann reagent and urine extract. By taking optical density readings at /

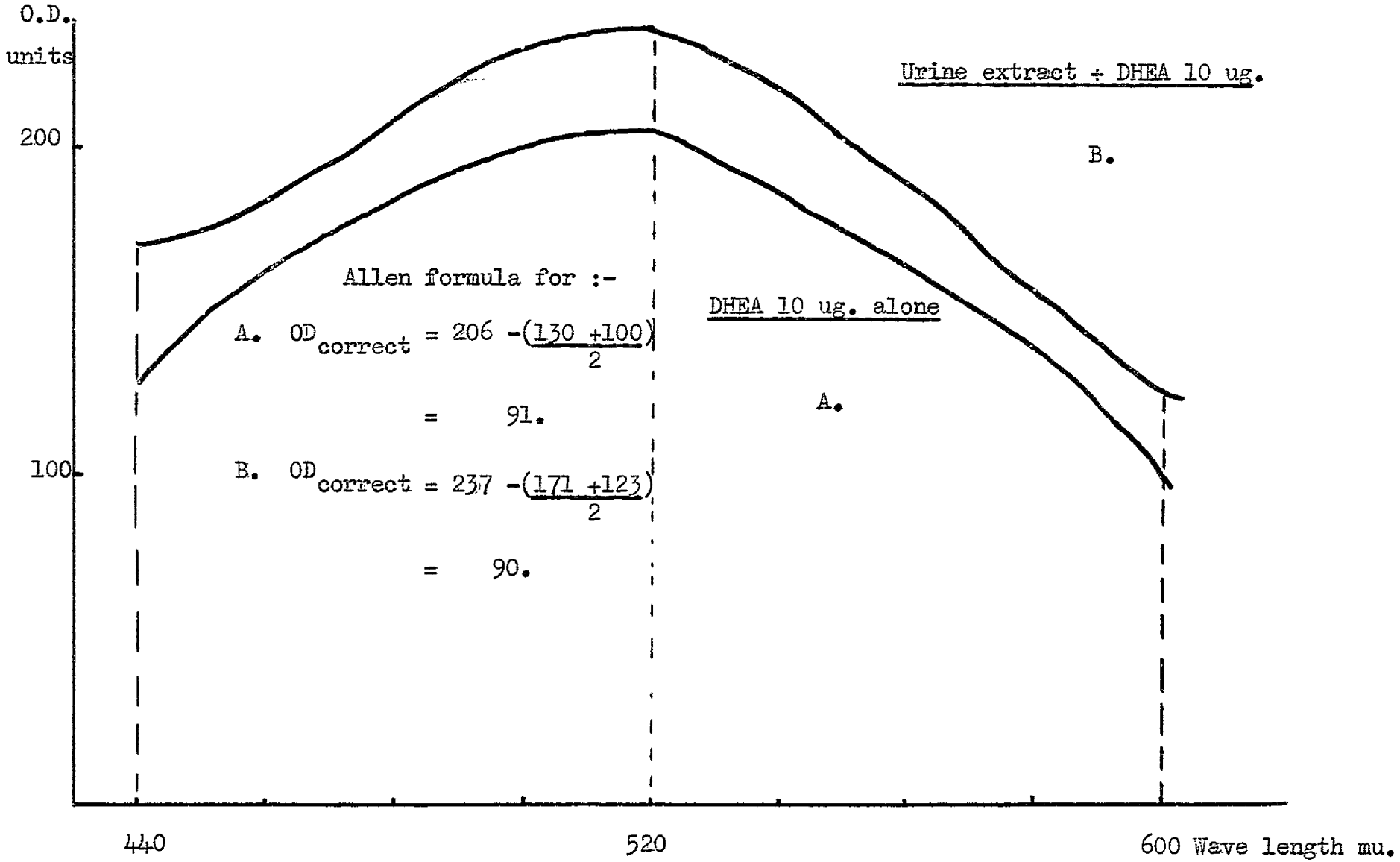


Fig. 2. Absortption curves of dehydroepiandrosterone (A) and a urine extract containing dehydroepiandrosterone (B).

at 440, 520 and 600 mμ. and applying the formula the true contribution of dehydroepiandrosterone to the curve of the mixture may be calculated.

Discussion on 17-oxosteroid method.

2,000 estimations, each one in duplicate have been done in the course of this work. Duplicates differing by more than plus or minus 5% from their mean were rejected and the test repeated.

Table 1 shows the typical results of an experiment to test the recovery rate of dehydroepiandrosterone (DHEA) from adult urine. The steroid was added to separate aliquots of urine before and after hydrolysis. Table 2 shows the results of a similar experiment using 'infant' urine.

From Tables 1 and 2 it will be noted that the recovery rates of dehydroepiandrosterone are much lower (65.30% and 53.8%) after acid hydrolysis than without acid hydrolysis (91.48% and 92.8%). This has been my constant finding. Acid hydrolysis therefore alters the steroid molecule in such a way as to destroy Zimmermann positivity. It must be remembered that the DHEA added to the urine before hydrolysis is free and therefore does not have the protection of the glucuronide moitié. While some freed steroid is probably degraded by hydrolysis further consideration of the /

Table 1. Recovery experiment of dehydroepiandrosterone (DHEA) from adult urine with reproducibility data.

1. Aliquot of urine
2. Chloroform to extract 10 ml.
3. Aliquot Chloroform taken 5 ml.
4. DHEA 10 ug. as standard and added to urine aliquots.

Wave length mu	440	520	600	A.C.	AC-RB	Mean	% Recovery
Reagent Blank (RB)	066	060	014	020			
Standard DHEA (10ug)	190	275	109	126	106		
" "	192	275	112	123	103	104	
Urine 1 - 5 ml.	404	658	318	297	276		
Urine 1' - 5 ml.	420	654	309	290	270	273	
* Urine 2 - DHEA ≡ 5 ug.	424	702	341	320	300	307	65.38
Urine 2'- DHEA ≡ 5 ug.	452	738	355	335	315		
** Urine 3 - DHEA ≡ 5 ug.	415	710	328	339	319	320	90.38
Urine 3'- DHEA ≡ 5 ug.	400	700	320	340	321		

* DHEA (10 ug.) added to urine before acid hydrolysis
**DHEA (10 ug.) added to urine after acid hydrolysis.

per cent recovery

90.38	90.20	97.0	
91.24	90.65	93.20	
90.86	89.21	90.61	Mean 91.48%
94.21	88.46	92.80	
93.01	91.34	89.00	

Recovery rates from a series (15) of recovery experiments, the DHEA having been added to the urine after acid hydrolysis.

Table 2. Recovery experiment of dehydroepiandrosterone (DHEA) from infant urine.

1. Aliquot of urine; 10 ml.
2. Chloroform to extract; 10 ml.
3. Aliquot Chloroform taken: 5 ml.
4. DHEA: 10 ug. as standard and added to urine aliquots.

	Wave Length mu	440	520	600	A.C.	AC-RB	Mean	% Recovery
	Reagent Blank (RB)	066	060	014	020			
	Standard DHEA (10 ug.)	190	275	109	126	106	104	
	" DHEA	192	275	112	123	103		
	Urine 1 - 5 ml.	271	282	118	088	068	067	
	Urine 1' - 5 ml.	286	290	122	086	066		
*	Urine 2 - DHEA ≡ 5ug	440	424	176	116	96	095	53.8
	Urine 2' - DHEA ≡ 5ug	408	413	178	115	95		
**	Urine 3 - DHEA 5 ug	456	459	186	136	116	115	92.8
	Urine 3'- DHEA 5 ug	485	474	193	135	115		

* DHEA (10 ug.) added to urine before acid hydrolysis

** DHEA (10 ug.) added to urine after acid hydrolysis.

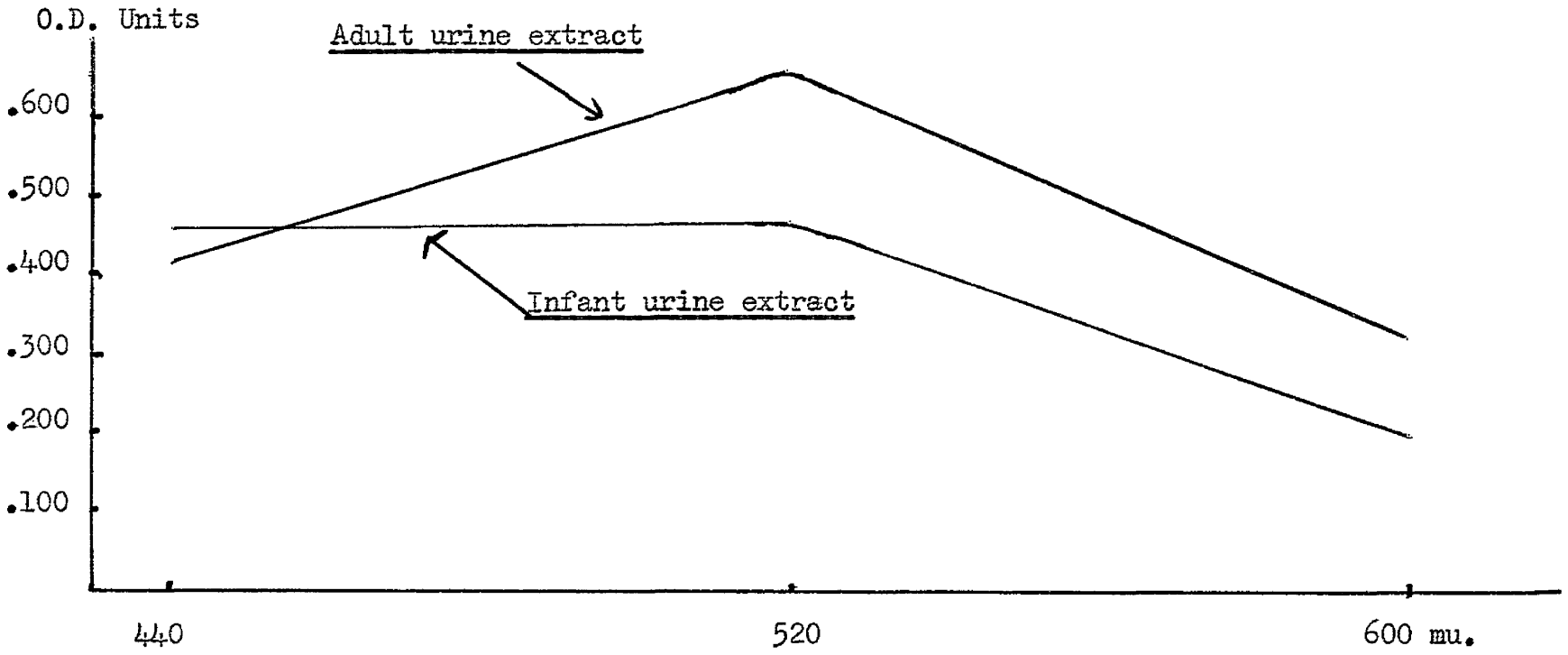


Fig. 3. Absorption curves of Zimmermann chromogens extracted from adult and infant urine. Note that there is no peak from infant urine extract.

the possible by-products of strong acid hydrolysis is outside the scope of this work.

A further point of note from Tables 1 and 2 is the difference in the optical density readings at 440, 520 and 600 mμ. obtained for adult urine and infant urine. This is represented in Fig. 3 graphically. It is clear that an undoubted peak at 520 mμ. is obtained with adult urine but not so in 'infant' urine when no peaks are recorded. This failure to obtain a satisfactory peak in the latter might have been due to the small amount of steroidal material in the urine or to the presence of considerable amounts of non-steroidal chromogenic material native to infant urine. While in health, the urinary excretion of 17-oxosteroids is normally low an attempt to improve the specificity of the general method was made by introducing the Girard process for separating ketonic material from crude extracts. This was carried out as earlier indicated. While satisfactory recovery rates were obtained by the Girard process comparable to those obtained when omitting it, there was no improvement in the optical density peak at 520 mμ. from infants' urine alone. This is illustrated in Table 3.

The introduction in 1963 of repartitioning the Zimmermann chromogens between aqueous ethanol and diethyl ether has given a more impressive peak at 520 mμ. from extracts /

Table 3. Girard T separation of native 17-oxosteroids from 'infant' urine. Aliquots (10 ml.) of urine used according to method (page).

Wave-length mμ.	440	520	600	A.C.
Infant A	493	496	278	116
	480	485	242	124
Infant B	641	658	492	092
	623	620	430	096
Infant C	326	350	110	132
	325	325	100	113

Note the absence of distinct peaks at 520 mμ.
Zimmermann chromogens were read in ethanol.

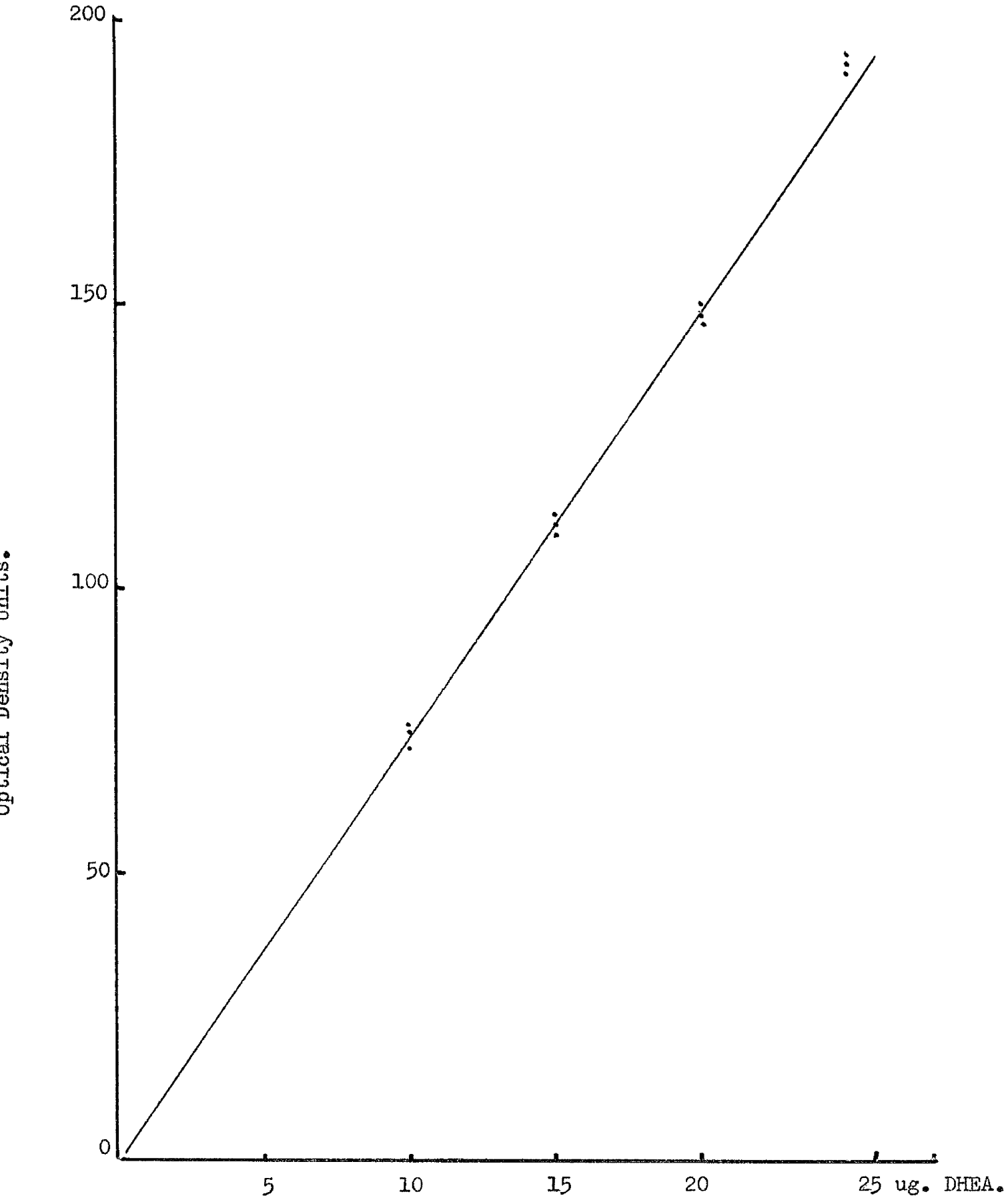
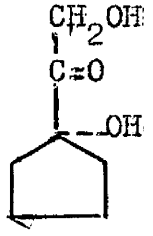


Fig. 4. The Zimmermann chromogens developed from dehydroepiandrosterone obey Beer's Law in diethyl ether.

extracts obtained from the urine of children of all age groups (Fig. 1). Fig. 4 shows that the Zimmermann chromogens in the ether layer obey Beer's Law.

Determination of 17-oxogenic steroids and total 17-hydroxycorticosteroids in urine.

The term 17-ketosteroid was first used by Brooks and Norymberski (1952; 1953) to indicate those corticosteroids which form 17-ketosteroids when subjected to oxidation (the terms 17-oxogenic steroid and 17-oxosteroid are now preferred). They showed that 17-hydroxycorticosteroids with an α-ketol (5,1) or a glycol (5,2) sidechain would yield 17-oxosteroids (5,3) on oxidation with sodium bismuthate.



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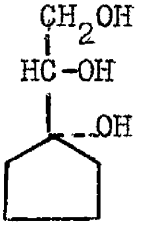

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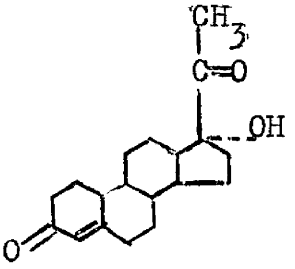


(5,3)

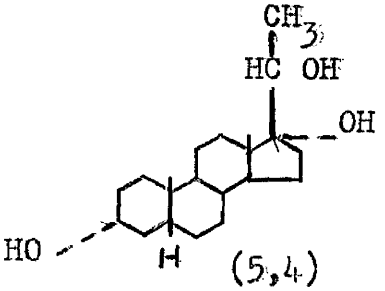
In health, 17-oxogenic steroids are the normal metabolites of hydrocortisone, cortisone and 11-deoxycortisol, the latter being an immediate precursor of hydrocortisone.

Urine which has been subjected to this oxidation procedure will, of course, contain native 17-oxosteroids as well as 17-oxosteroids derived from 17-oxogenic steroids. It is therefore necessary to estimate 17-oxosteroids in the urine before and after oxidation and by subtraction, the true value of 17-oxogenic steroids may be calculated.

There are also however in health, small amounts and

in /

in some disease processes large amounts of a precursor of hydrocortisone which is not 17-oxogenic on oxidation. This substance is 17α-hydroxy progesterone (5,3) and it is present in large amounts when 21-hydroxylation is completely or partially absent (this is seen in some cases of congenital adrenal hyperplasia). A metabolite of 17α-hydroxyprogesterone, pregnanetriol (5,4) appears in the urine in increased amounts when 21-hydroxylation is defective.



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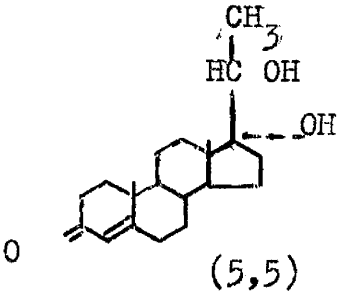
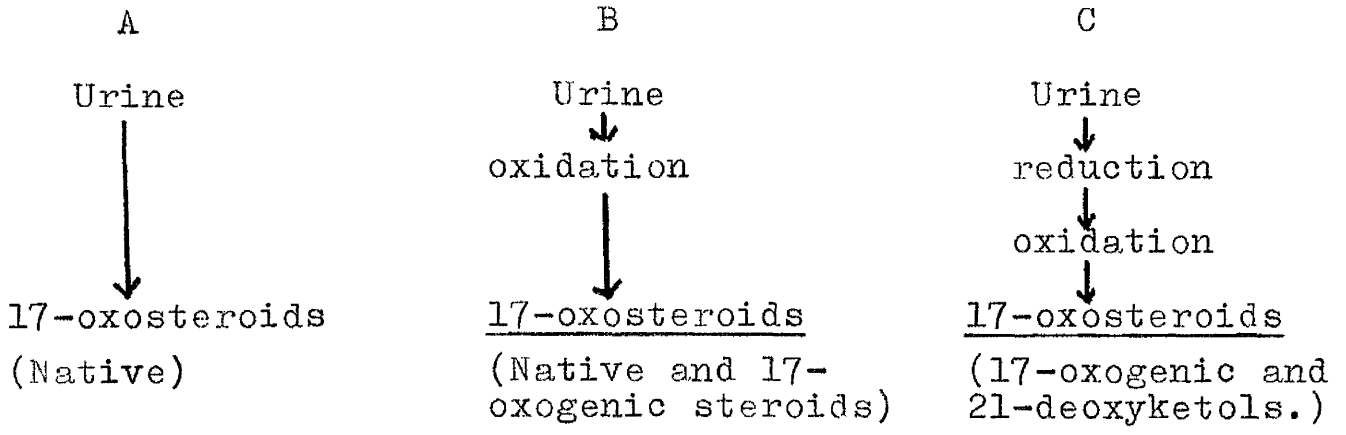


(5,5)

If 17α-hydroxyprogesterone is reduced in vitro with sodium or potassium borohydride, a glycol (5,5) is formed which when oxidised will result in the formation of a 17-oxosteroid. It follows that if a sample of urine is reduced then oxidised the 17-oxosteroids will have come from 17-oxogenic steroids and 21-deoxy-17-hydroxycorticosteroids (5,1; 5,2; 5,4; 5,5), the native 17-oxosteroids having been reduced to alcohols which do not then give the Zimmermann reaction. These former two estimated together rep resent the total 17-hydroxycorticosteroids. It will be seen that by a combination of 3 steps as outlined, it is possible to /

to estimate 21-deoxy-17-hydroxycorticosteroids and this I have used for screening purposes as a rapid test for 21-hydroxylation.



21-deoxyketols = C - (B - A).

Normally in infancy and childhood the urinary total 17-hydroxycorticosteroids should not be greater than the 17-oxogenic steroids by more than 200 micrograms/day and if it was found to be so then the more time-consuming estimation of pregnanetriol - the metabolite of 17α-hydroxyprogesterone - was undertaken.

Appleby, Gibson, Norymberski and Stubbs (1955) and Appleby and Norymberski, (1955) first published their method for the oxidative removal of the α-ketol and glycol sidechains of 17-hydroxycorticosteroids and the removal, after reduction of 21-deoxy-17,20-ketol sidechains. They used sodium borohydride for reduction and the surface oxidisant sodium bismuthate. I have followed their method without modification from 1960 to 1963

The /

The procedure is as follows.

Four aliquots (10 ml.) of urine are placed in B 24 test tubes, sodium borohydride ($NaBH_4$); (20 mg.) is added to 2 tubes and allowed to stand overnight at room temperature. Glacial acetic acid (1o ml.) and sodium bismuthate (2.5 gm.) are added to all four tubes and all are shaken for ½ hour in the dark. After the addition of 6% sodium thiosulphate ($Na_2S_2O_5$; 25 ml.) shaking is continued until all the sodium bismuthate is reduced - indicated by a change from orange to creamy white. 10 N. hydrochloric acid (15 ml.) is added to each tube and all placed in a boiling water trough for 10 minutes and thereafter cooled in ice water. The extraction is effected with ethylene dichloride (2 x 20 ml.) and the ethylene dichloride washed with water (10 ml.) and 3 N. - sodium hydroxide (10 ml.). A maximum aliquot of ethylene dichloride (30 ml.) is blown to dryness and the Zimmermann reaction performed. In conjunction with a straight estimation of 17-oxosteroids, performed on the same urine, the appropriate calculations are made. Experiments using 17-hydroxyprogesterone to test reduction and oxidation, gave satisfactory recovery rates (Table 4).

The method however has drawbacks, the most significant /

Table 4. Recovery of 17-hydroxyprogesterone from urine using sodium borohydride for reduction and sodium bismuthate for oxidation. 17α-hydroxyprogesterone added to the urine in varying concentrations.

Wave length mμ 440	440	520	600	A.C.	AC-RB	Mean	%Recovery
Reagent Blank (RB)	078	070	026	018	018		
Standard DHEA (10 ug.)	190	275	109	126	109	107	
" " "	192	275	112	123	106		
Urine 1 - 5ml.	259	383	156	176	159	158	
Urine 1' 5 ml.	234	364	144	175	158		
Urine 2- 17-P 12.5ug.	451	600	292	293	276	278	89.6
Urine 2'-17-P 12.5ug.	469	670	256	298	281		
Urine 3 -17-P 10.0ug.	469	630	254	279	262	263	98.0
Urine 3'-17-P 10.0ug.	555	701	286	281	264		
Urine 4 -17-P 7.5ug.	424	585	242	252	235	232	92.0
Urine 4'-17-P 7.5ug.	452	600	264	247	230		

Uncorrected for varying chromogenicities.
17α-P 17α-hydroxyprogesterone.

significant of which are the length of time required for oxidation in the dark, the variable oxidising capacity of different batches of sodium bismuthate and the unpleasantness of working with strong acetic acid. In 1961 Few published his experience in the use of sodium metaperiodate as an oxidising agent to replace sodium bismuthate. While the method was no saving in time, it had the advantage of being cleaner and the oxidative capacity of sodium metaperiodate is more reliably constant from batch to batch. Few's method (1961) has been followed from 1963 until the present, in the following way.

To 2 aliquots of urine (10 ml.) sodium borohydride (200 mg.) is added and reduction allowed to take place for 2 hours or overnight according to convenience. Reduction is stopped by the addition of 25 per cent acetic acid (0.5 ml.) added drop by drop. After 15 minutes, 10 per cent sodium periodate (4 ml.) and N. sodium hydroxide (1 ml.) are added to each tube. The pH is adjusted to 7 and the mixtures incubated for 1 hour at 37°C to effect oxidation. The glucuronide residues of the steroid conjugates are also converted to formates by this oxidation step.

The formates are hydrolysed by the addition of 2.5 N. sodium hydroxide (1 ml.) and incubation continued for a further /

further 15 minutes. After cooling, dichloromethane (10 ml.) is added to each tube and extraction effected by inverting the stoppered tubes 20 to 30 times.

The layers are allowed to separate and the supernatant urine is removed by suction. 2.5 per cent sodium dithionite in 5 per cent sodium hydroxide (5 ml.) is added to the dichloromethane and the tubes shaken vigourously. After separation, the upper aqueous layer is removed by suction and the organic phase filtered through Whatman No. 1 paper to remove droplets of any remaining aqueous phase. Aliquots (5 ml.) from each tube are collected and evaporated in a water bath at 40°C under a stream of nitrogen.

The residues are subjected to the Zimmermann reaction and estimation of 17-oxosteroids made against standard dehydroepiandrosterone. After an adjustment for the daily urine volume, the results are expressed as mg. total 17-hydrocorticosteroids per 24 hours.

A typical recovery experiment using 17α-hydroxy-progesterone as the model is shown in Table 5.

Table 5. /

Table 5. Recovery of *17α-hydroxyprogesterone after reduction and oxidation by Fews' (1961) method. Dehydroepiandrosterone used as standard and no correction made for differing chromogenicity.

Wavelength mμ.	440	520	600	Ac	AC-RB		%Recovery
R.B.	082	078	038	18	17		
R.B.	087	077	038	15			
DHEA 10 μg.	186	242	113	93	76	77	
DHEA 10 μg.	184	240	104	96	79		
*17αOHP 20 μg.	314	367	164	128	111		72%
17αOHP 20 μg.	290	364	156	129	112		72.5%
17αOHP 20 μg.	305	366	168	130	113		73%

Preparation of β-Glucuronidase.

This enzyme is abundant in the dorsal hump of the limpet (Patulla Vulgata). A method for preparing an acetone precipitate powder from homogenised dorsal humps, high in β-glucuronidase activity, has been recommended by the M.R.C. (1963). Fortunately the rocky foreshore of the river Clyde south of Gourock provides a breeding ground for these molluscs. It is my practice to harvest limpets from the rocks immediately beyond the village of Seamill. I choose those, 4 - 5 cms. in diameter across the base, and pick them immediately the high tide ebbs. It is most convenient to pick about 11 a.m. carrying them back to Glasgow immersed in sea water. Dissection of the dorsal humps is carried out immediately on return and the first acetone precipitation reached the same evening. The calculated Fishman units prepared in this way has been valued, according to current prices, at £325.

The method is as follows:

The limpet is removed from its shell and the swollen dorsal hump dissected from the muscular pad. Batches of humps (290 g.) are collected and homogenised with ice water (300 ml.) for 5 minutes. The homogenate is then centrifuged for 10 minutes at 1500 r.p.m.

The /

The residue is homogenised a second time with the same volume of ice water, and again centrifuged. The combined supernatant is treated with acetone to bring the concentration to 60 per cent acetone in water. The mixture is placed in the refrigerator (2 - 4°C) overnight and the supernatant removed by centrifugation. The acetone precipitate is washed 4 times by thorough stirring with acetone, followed by centrifugation. The resulting powder is dried in air. This crude preparation is re-processed as above using 30 ml. ice water per g. of powder and a fine flaking residue is obtained. This final powder is assayed for β-glucuronidase activity by the following method (Talalay, Fishman and Higgins, 1946).

Solutions: One litre of 0.1 M acetate buffer, at pH 4.5 is prepared from sodium acetate (5.785 g.) and glacial acetic acid (3.25 ml.) This is stored in the 'deep freeze'. One litre 0.4 M glycine buffer (pH10.45) is prepared from aminoacetic acid (16.3 G), sodium chloride (12.65 G) and sodium hydroxide (10.9 ml. of 100 g. NaOH in 100 ml. water).

Method: Phenolphthalein solution (0.1 per cent is prepared in 80 per cent ethyl alcohol.) Two hundred milligrams of the enzyme is suspended in 40 ml. distilled water. One millilitre of the suspension is made up to 12.5 ml. with distilled water of which 0.5 ml. is /

is used for the test (=200 µg. enzyme powder).

The determinations are carried out in duplicate.

Acetate buffer 0.1 M (4 ml.) is placed in each tube. Phenolphthalein glucuronide (50 mg.) is added to the two experimental tubes but not to the control tubes. The tubes are then placed in water bath at 38°C and allowed to come to temperature. The enzyme solution (0.5 ml.) is added to each tube at timed intervals and mixed, stoppered and incubated for exactly 60 minutes. Glycine buffer 0.4 M (5 ml.) is then added to each tube, including the control tubes and then enzyme solution (0.5 ml.) is added to the control tubes. The optical densities are read at 540 mµ. against standards of phenolphthalein (5 - 40 µg.) using an Unicam S.P. 600 and cuvettes with 10 mm. light path. Typical results are as under:

OD_{540} Blank	000
OD_{540} Standard 30 µg. phenolphthalein	299
OD_{540} Test	217

Calculation 30 µg. phenolphthalein = OD 299

21.7 µg. " = OD 217

200 µg. enzyme powder liberates 21.7 µg. phenolphthalein

1 g. enzyme powder liberates $\frac{21.7 \times 1000 \times 1000}{200}$

= 108500 µg. phenolphthalein.

1 g. enzyme powder contains 108,500 Fishman Units.

Estimation of Urinary Pregnanetriol.

Pregnanetriol is the metabolite of 17α-hydroxy-progesterone and it appears in the urine in excess amounts when 21-hydroxylation is defective. (One form of congenital adrenal hyperplasia is due to 21-hydroxylase deficiency, partial or complete). In the urine, pregnanetriol is conjugated with the highly water-soluble sugar acid, glucuronic acid. Hydrolysis of the conjugate is effected by incubating urine with glucuronidase and after extraction of the free pregnanetriol with organic phase, it is separated by column chromatography and quantitated. Goldzieler and Nakamura's (1962) method with slight modification has been used. Two aliquots of urine (20 ml.) are pipetted into B 24 test tubes and the pH adjusted to 4.5 with 5 N. sulphuric acid or 10 per cent sodium hydroxide; acetate buffer pH 4.5 (2 ml.) and 6000 units of β-blucuronidase are added and incubation is carried out at 37°C for 24 hours. Extraction is effected with chloroform (2 x 20 ml.) and separation by centrifugation if necessary. The chloroform extracts are bulked quantitatively using additional small volumes of chloroform for rinsing. The combined extracts are washed with 0.1 N sodium hydroxide (15 ml.) and then with water (15 ml.). Anhydrous sodium sulphate (10 g.) is added to each tube and allowed to stand for

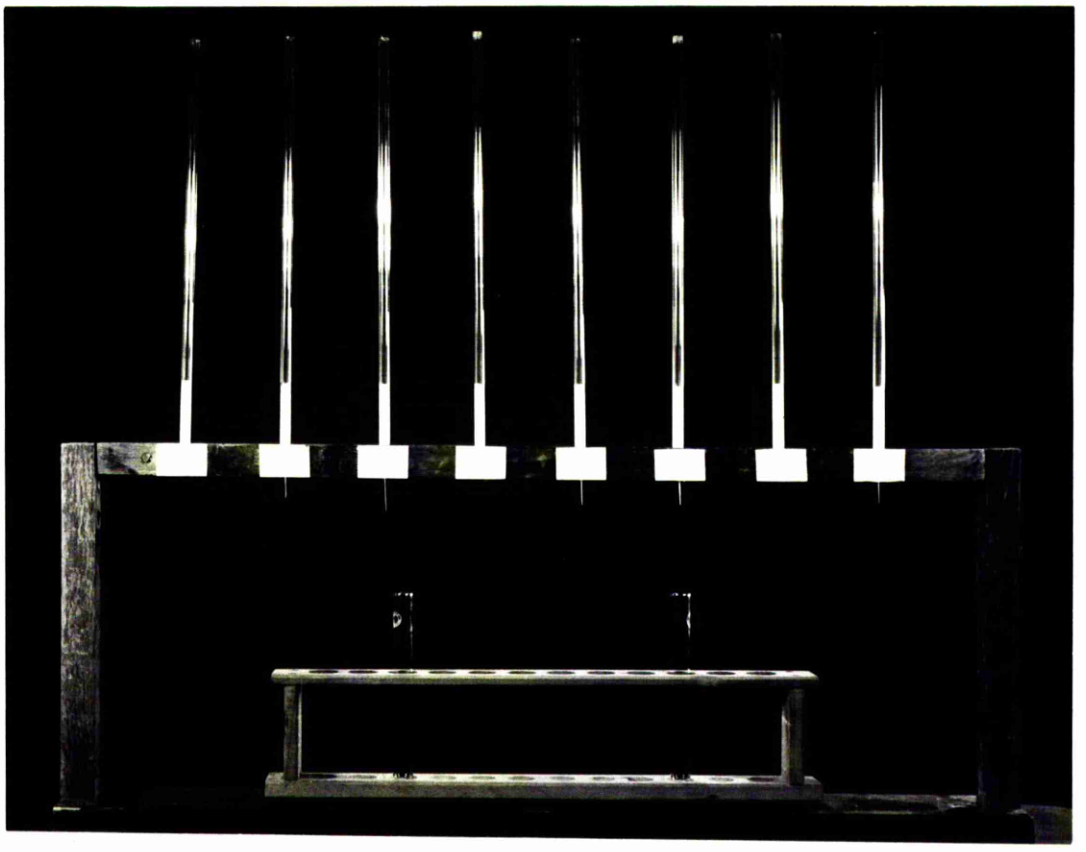
10 /

10 - 15 minutes with occasional swirling. The dried chloroform and 2 chloroform washings of the sodium sulphate are filtered through Whatman No. 1 filter paper into appropriate tubes and blown to dryness in a water bath at 40°C using a stream of nitrogen.

The residues are transferred quantitatively to 10 ml. volumetric flasks and again blown to dryness. They are then made up to volume with benzenes for chromatographic separation.

Chromatographic columns are prepared from acid-washed borosilicate glass (O.D. 8 mm.) tubing. Lengths of this (20 cm.) are heated about 3 cm. from one end and drawn out to a long fine capillary tip. The tip is sealed leaving about 10 cm. of capillary. Benzene (2 ml.) is added to each column and a small plug of cotton is stamped into the bottom of the column with a glass rod so that its upper surface is flat and its depth 4 mm. All air bubbles are expelled and exactly 1 g. silica gel (100 - 200 mesh, without preconditioning) added as a slurry in benzene (3 ml.) The column is filled to the top with benzene and the gel settled by tapping the column gently with a glass rod. The tip of the capillary is then broken off to allow a flow of 1 drop per second. The supernatant benzene at the top of the column is drawn off and discarded (See Fig. 5.).

An /



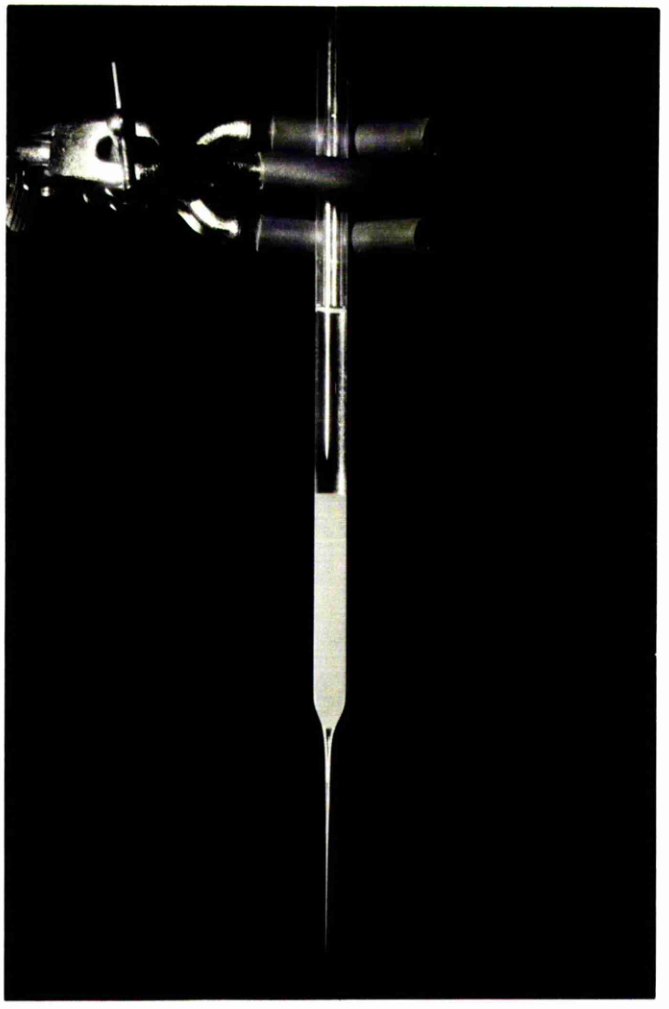


Fig. 5. Chromatographic columns used in estimation of pregnanetriol.

An aliquot (5 ml.) of the benzene solution containing the test sample is transferred quantitatively to the column without disturbing the surface of the silica gel. A small amount of benzene (4 ml.) is used to rinse the sample on to the column. The eluate from this fraction is discarded. The second phase (10 ml.; 25 per cent ethyl acetate in benzene) is run through the column allowing the first 1 ml. portion through the column separately. This eluate is also discarded. The third phase (7 ml.; 50 per cent ethyl acetate in benzene) is run through as above and the eluate discarded. Finally ethyl acetate (10 ml.) is run through and collected - this eluate contains the pregnanetriol fraction. It is blown to dryness with a stream of nitrogen in a water bath of 40°C. The residue is dissolved in alcohol (0.5 ml.) and water (10 ml.) added. Oxidation of the pregnanetriol to a 17-oxosteroid (etiocholanolone) by Few's (1961) method follows and the Zimmermann colour reaction is performed. This oxidation step is my own modification to the original method which utilizes charring of the pregnanetriol fraction with sulphuric acid. This latter reaction I have found wholly unsatisfactory when applied to extracts from childrens' urine because of a high content of nonsteroidal chromogenic material. High background and no absorption maximum have been obtained

so /

so frequently that I have preferred the well proven estimation of Zimmermann chromogens in ether.

Table 6 gives the scientific data of the method.

Table 6. Data concerning the recovery of pregnanetriol estimated as etiocholanolone. Low recovery rates around 60%, especially when dealing with small quantities of pregnanetriol (5 µg.) which are encountered in this field, are inevitable.

Wave length mµ.	434	515	595	AC	AC-RB
R.B.	023	024	010	8	
R.B.	021	025	015	8	
Etio. 10 µg.	055	097	047	46	38
"	050	092	042	46	
Columned Pregnanetriol 5 µg.	036	048	024	18	12
Oxidised 5 µg.	016	036	020	18	12

$$\text{Recovery} = \frac{100 \times 12 \times 2}{38} = 63.1\%$$

Estimation of free aldosterone in urine

The method of Brooks' (1960) has been followed almost entirely, the only modification being that, in the final chromatogram, aldosterone rather than corticosterone diacetate was used as the standard and the solvent system E_2B (isooctane, tert. butanol, water: 10:5:9) replaced the Bush A system.

Method:

Urine (500 ml. or the total daily output if less than 500 ml.) is adjusted to pH 1 with 0 1 N. hydrochloric acid. The urine is transferred to a separating flask (1 litre capacity) and tritiated aldosterone (10 muc.) added for recovery estimations. Chloroform ($\frac{1}{2}$ vol.) is run into the flask and after gentle shaking, a stream of nitrogen is bubbled through the urine for 5 minutes and the air above the urine displaced by nitrogen. The flask is then stoppered and allowed to stand for 18 hours. After gentle agitation, the chloroform extract is separated and the urine extracted a second time with chloroform ($\frac{1}{2}$ vol.) for 6 hours. Any emulsion that forms is broken by centrifugation. The urine is discarded and the combined chloroform extracts are washed with water (1/20th vol.), 0.1.N. sodium hydroxide (1/20th vol.) and water (2 x 1/20th vol.). The washings are backwashed with chloroform and the total extract /

extract is evaporated to dryness in vacuo at 40°C. The dry extract is then weighed and a sheet of Whatman No. 2 paper cut so that the loading does not exceed 1 mg. per cm. Side lanes (1.5 cm.) are allowed for standards.

First Chromatogram.

The extract is quantitively applied to the start line of the paper which has been impregnated with formamide in acetone (30:70) and allowed to dry, suspended in a dust-free atmosphere for exactly 10 minutes. Cortisone (20 µg.) and hydrocortisone (20 µg.) are applied to each side lane and in addition, cortisone (20 µg.) is applied to the middle of the centre lane. The paper is allowed to equilibrate for 30 minutes in the tank before the mobile phase (chloroform saturated with formamide) is added. After development for 3 hours at 22°C the paper is removed from the tank and allowed to dry for a few minutes in air. Aldosterone in this system has a mobility slightly less than that of cortisone. From the leading front of cortisone, a zone 5 cm. back to the origin is outlined and cut out of the chromatogram. This is cut into strips (1 cm. wide) and these are eluted together overnight in 80 per cent methanol in water. The eluate is evaporated in vacuo to about $\frac{1}{4}$ volume. Water (10 ml.) is added and the mixture extracted with chloroform (3 x 30 ml.)

The /

The chloroform is then washed once with water (10 ml.) to remove traces of formamide, and is evaporated to dryness in vacuo at 40°C.

Second Chromatogram.

The extract is applied to a strip (5 cm. ide with 1.5 cm. side lanes) of Whatman No. 2 paper using cortisone and hydrocortisone as before for markers. The chromatogram is equilibrated overnight in the Bush C tank and developed in that system for 3 hours. (Bush C solvents: toluene, ethyl acetate, methanol, water: 90: 10: 50: 50). Aldosterone has a mobility equal to hydrocortisone in this system. A zone corresponding to 2.5 cm. on either side of the centre of the hydrocortisone zone is cut out and eluted in 90 per cent ethanol overnight. The eluate is evaporated to dryness in vacuo at 40°C.

Third Chromatogram.

Whatman No. 1 paper is cut into lanes 1.5 cm. wide and 3 cms. long. Four lanes are cut for each test sample. On 3 lanes aldosterone (1 mg.) is spotted and on the fourth lane the test extract is applied. The spots are washed in with ethylacetate delivered from a fine pasteur pipette so that finally the samples occupy a disc 1 - 2 mm. in diameter. Only by so doing is the area occupied by the steroid after development of the chromatogram kept within 1 sq.cm. The chromatograms are then equilibrated for 3 hours and developed for 3 hours in /

in the E_2B system (isooctane, tert.butanol, water: 10: 5: 9).

Sodium fluorescence: development and measurement.

After development in the E_2B system, the chromatograms are dried in air and clamped to a sheet of glass. They are then sprayed uniformly with a solution of 0.004 per cent (w/v) blue tetrazolium chloride in 2.4 N-dosium hydroxide. The sprayed chromatogram is then covered with a second sheet of glass, the edges being sealed to prevent drying, and heated for 10 minutes by a 750 watt Radisil heater at a distance of 66 cm. The top glass sheet is elevated and com,lete drying is effected in a gentle current of air blown across the chromatogram. By preventing drying while initially heating the chromatogram, it is thought that more reproducible results are obtained.

The individual lanes of the chromatogram are then detached from one another and examined under UV light (235 mm.) to locate the sodium fluorescent spots. Sections of each lane with the fluorescent spot are removed and placed in the fluorimeter carrier. A galvanometer reading is taken for the fluorescent spot and then a blank reading is taken from the area immediately in front of the spot. Such readings are taken for the 3 standards of aldosterone (1 µg.) and for the test sample(s). /

sample(s). By deducting the blank readings and comparing 'test with standard' readings the microgram equivalents in the test sample may be calculated.

Discussion on method.

This method for the estimation of free aldosterone in urine embodies several important physico-chemical principles, each of which I have used separately in various parts of the original work for this thesis. I shall discuss these points emphasising their significance and application to the estimation of aldosterone in the urine of the paediatric patient as well as to their separate application elsewhere.

After the second chromatogram, Brooks acetylated the residue at room temperature using two drops of pyridine and one drop of acetic anhydride. Following precisely this procedure I found frequently using aliquots of the same urine that two sodium fluorescent spots could be obtained on one lane of the third chromatogram while a second lane had only one fluorescent spot. This finding suggested that despite the ostensible similarity of conditions for acetylation of the two samples, a mixture of the mono- and diacetate of aldosterone was being obtained from one of the samples. I have not diverted to analyse this finding further for at the time when I met these difficulties, pure

aldosterone /

aldosterone became commercially available and it seemed reasonable to use aldosterone itself as the standard, rather than corticosterone monoacetate which had almost the same mobility ($R_{s(aldosterone)}$ 1.2) as aldosterone diacetate on the Bush A system used by Brooks (1960).

However, acetylation has the other advantage that aldosterone diacetate gives a more intense fluorescence compared with free aldosterone (Tait, 1960). This fact seemed less important to me since the fluorimeter in use here has been constructed by Mr. D. Rowan of the Regional Physics Department, Western Regional Hospital Board, modelled on the Brooks (1960) principle, but with a more critical selection of primary and secondary filters. A primary filter of 3650A° and a secondary filter of 5975A° are used. The secondary filter was chosen because it gave the best signal to noise ratio (i.e. ratio of the fluorescence to be measured to the fluorescence from background). The machine had a further advantage that with a system of gains (increase in KV) maximum galvanometer deflection could be set for the standards of free aldosterone so that quantities in the test samples less than the standards could be more precisely estimated.

Table 7. Compounds with R_F values similar to aldosterone in the 3 solvent systems.

Solvent system	Chloroform/ formamide	Bush C	E_2B
Hydrocortisone	1.0	1.0	1.0
Cortisone	2.39	1.38	1.10
Aldosterone	2.25	0.97	0.77
17-iso-aldosterone	2.22	0.92	0.73
18-hydroxy-11-dehydrocorti-costerone.	2.2	1.08	0.74

E_2B solvent system

Having omitted the acetylation step it was necessary to ensure that there were no other steroids which had the same mobility in the three solvent systems used (chloroform/formamide; Bush C and E_2B). There were 2 possibilities and these are shown in Table 7 with their respective mobilities in the 3 systems. In overrun extracts only one fluorescent spot on the 3rd chromatogram has been encountered. Experience from running many samples of aldosterone (1 µg.) has shown that the final area is seldom more than 1.0 sq.cm. with a length of 1 cm. (The carrier aperture on the fluorimeter is 2.25 sq.cm. - i.e. the area of the paper exposed to the photomultiplier). If the test sample gives an area greater than 1.0 sq. cm. and its length exceeds 1.5 cm. a second specimen from the same urine is overrun to give a running distance for hydrocortisone on the side-lanes of 15 cm. Contaminated substances have not been accounted.

Only recently have other authors published values for daily urinary free aldosterone in infancy and childhood. In my early researches this information was not available so that much trial and error was used to determine (a) the optimum aliquot of the daily urine volume to be extracted and (b) the proportion of the extract to be spotted on the final chromatogram so /

so that the quantity of aldosterone estimated would be approximately equal to the aldosterone standard (1 µg.). It was found that where the daily volume of urine was less than 500 ml. the whole volume must be taken and the whole extract had to be spotted on the final chromatogram to obtain the maximum fluorescence. This prevented an aliquot of the extract being kept for estimation of recovery rates. (It will be recalled that tritiated aldosterone (7-H^3-aldosterone) 10 mµc. is added to the urine before extraction). Since this was so in the majority of the samples estimated with every estimation a recovery experiment was made from water. Recovery rates ranged from 43-75 per cent when no emulsion was present.

Proof of Method.

Fig.7 shows the proof of the method. The charts are the 1st and 2nd chromatograms with their corresponding scan of radio-activity as obtained from a strip scanner. Small peaks of radio-activity are seen in the 1st chromatogram corresponding to cortisone and in the 2nd, to hydrocortisone.

Emulsions.

In the outline of the method, I stated that emulsions which form in the urine during extraction are broken /

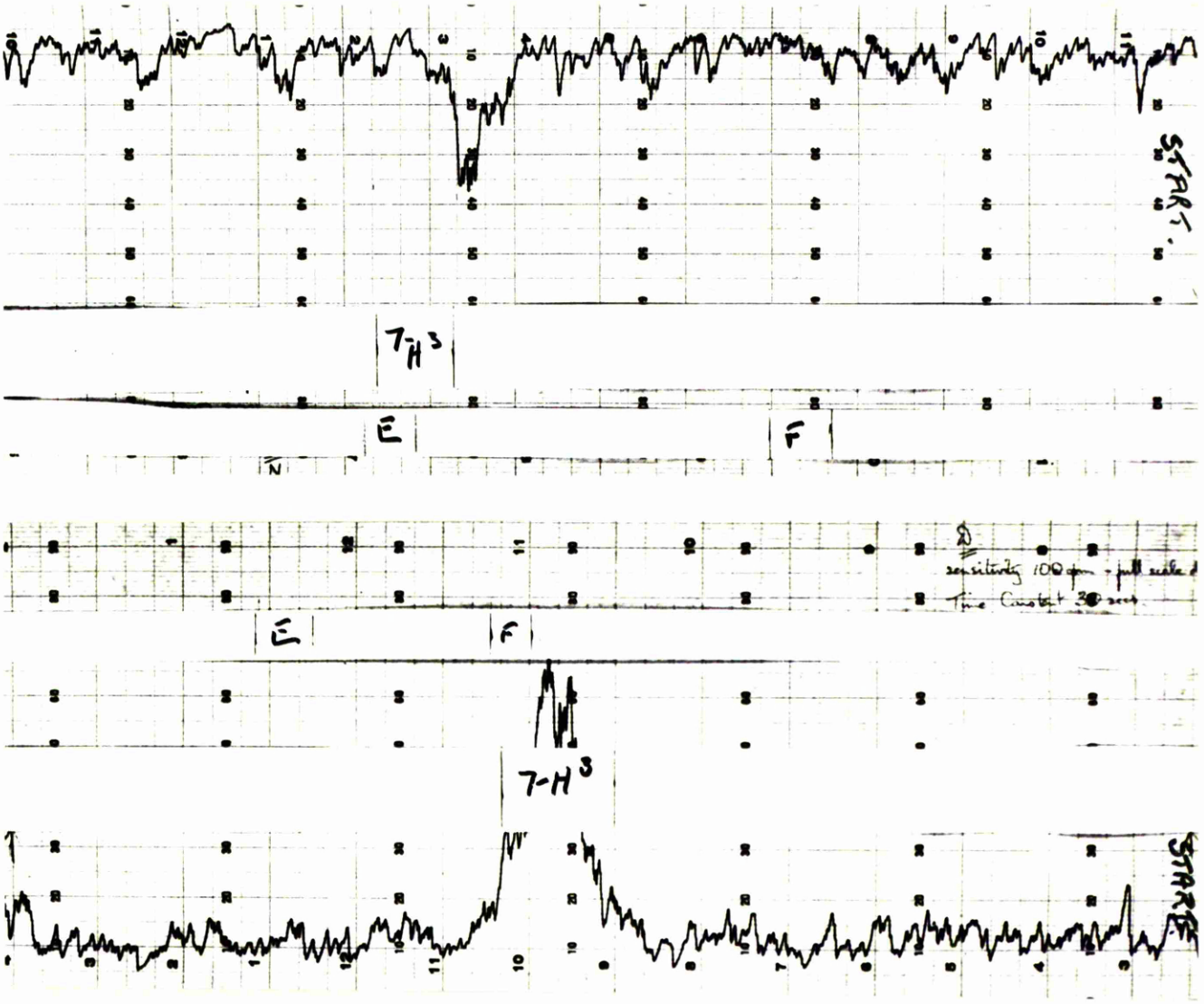


Fig. 7. Aldosterone-7-H^3 on Zaffaroni (top) and Bush C (bottom) chromatograms.

broken by centrifugation. Emulsions have been a problem and in particular when dealing with high protein-containing urines from cases of nephrosis. While many authors report high levels of urinary aldosterone in this condition, for a time I was unable to detect free aldosterone in nephrotic urine during the period of albuminuria. As soon as the diuretic phase commenced and the albuminuria cleared- usually between the 10th - 14th day of steroid treatment - aldosterone was extractable in high normal amounts.

The fluorimeter.

In this instrument (Fig.6) a mercury lamp is used as a source for excitation of fluorescence. The 3650A° line of the mercury spectrum is selected by means of the primary filter. The fluorescence light from the sample being tested is made into a parallel beam by a quartz lens and a secondary 5975A° interference filter with a band width of 100 A° is interposed between the lens and a photomultiplier which measures the intensity of the fluorescent band. The resultant photoelectric current is directly proportional to the intensity of fluorescence. For maximum stability the photomultiplier is supplied from a highly stabilised EHT power supply. The mercury vapour lamp is supplied from /

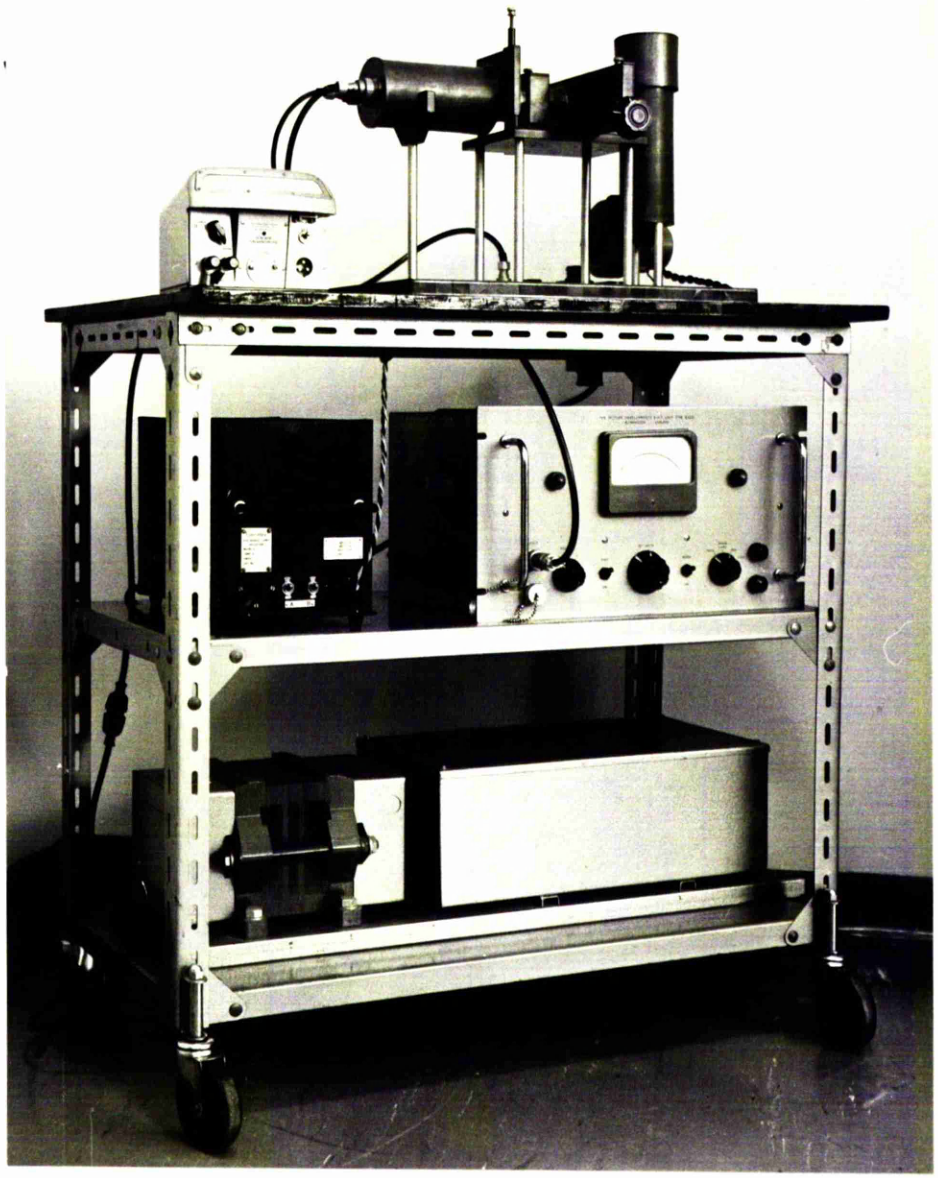


Fig. 6. Fluorimeter for measuring sodium fluorescence on paper.

from a constant voltage transformer.

Linearity of Galvanometer readings.

The average paper blank reading routinely obtained was equivalent to 0.3 µg. aldosterone. Signal to noise ratio of 4 to 1 were consistently obtained with a minimum of 0.5 µg. amounts, although galvanometer readings could be obtained for 0.1 µg. of aldosterone or its diacetate. Linearity was obtained from 0.5 µg. to 3.5 µg. aldosterone but above this quenching caused flattening of the curves.

Radio-activity counting.

The specific activity of aliquots from the final extracts was counted for me by Dr. J.K. Grant in the Department of Steroid Biochemistry at the Royal Infirmary, Glasgow. The counting was done on a Tri-Carb Liquid Scintillation Spectrometer. With a gain setting of 50 per cent and full window, the efficiency for H^3 is 28 per cent on this instrument. A background rate of 24 c.p.m. is registered.

Estimation of tetrehydroaldosterone in urine.

The method of Pasqualini, Legrand and Jayle (1963) has been used without modification.

A suitable aliquot of urine is adjusted to pH 4.8 using acetic acid/sodium acetate buffer (0.2 M). Sulphetase and β-glucuronidase are added to make the concentration of each 1500 units per ml. of urine. Hydrolysis is allowed to proceed for 48 hours and the liberated steroids extracted 3 times with 1 volume of dichloromethane. The extract is washed once with sodium carbonate (w/v - 1/20 vol.) and once with water (1/20 vol.) and then evaporated to dryness in a water bath at 40°C. The residue is then dissolved in dichloromethane (10 ml.) and adsorbed on to a Florisil column (20 g. activated by heating at 650°C for 1½ hours). Elution from the column is in three phases: 80 ml. dichloromethane containing 2.5 per cent methanol; 150 ml. dichloromethane containing 12 per cent methanol; and finally 80 ml. methanol.

Tetrahydroaldosterone is eluted in the 12 per cent methanol fraction which is evaporated to dryness and the residue spotted on a Whatman No. 1 chromatogram impregnated with formamide/acetone (30/70) as for aldosterone. As before standards of cortisone and hydrocortisone /

hydrocortisone are spotted on the side lanes. After equilibration for 30 minutes the chromatogram is developed for 3 hours at 22°C using chloroform saturated with formamide as the mobile phase. Tetrahydroaldosterone in this system has a mobility slightly less than that of hydrocortisone. From the leading front of hydrocortisone, a zone 2 cm. back to the origin is outlined and cut from the chromatogram. This is further cut into strips (1 cm. wide), and these are eluted together overnight in chloroform containing 30 per cent methanol. The eluate is evaporated and the residue is applied to a strip (8 cm. wide with 1.5 cm. side lanes) of Whatman No. 1 paper impregnated with formamide/acetone (40/60).

Hydrocortisone 2 µg. is applied to the side lanes. After equilibration for 30 minutes it is developed 5 hours in the system n-butyl acetate: formamide: water (50:10:10). Tetrahydroaldosterone in this system has a mobility of 0.45 times that of hydrocortisone. A zone is outlined between $R_{F(\text{hydrocortisone})}$ 0.4 and 0.55 and cut from the chromatogram. This is eluted in chloroform containing 30 per cent methanol overnight and evaporated to dryness.

Third Chromatogram.

The residue is dissolved in water (10 ml.) and extracted /

extracted 3 times with dichloromethane (10 ml.). This removes the formamide eluted from the previous chromatogram. The dichloromethane is evaporated to dryness and applied to a strip (2 cm. wide with 1.5 cm. side lanes) of Whatman No. 1 paper. The chromatogram is equilibrated for 3 hours and developed for 3 hours in the system isopropyl; methanol: water (2:1:1). In this system tetrahydroaldosterone has a mobility of 0.8 times that of hydrocortisone. A zone is outlined between R_F (hydrocortisone)$^{0.76}$ and 0.89, and removed and eluted and evaporated to dryness.

Quantitation.

The dried residue, in a B 10 test tube, is dissolved in ethanol, (0.3 ml.) 0.5 per cent aqueous blue tetrazolium (0.1 ml.) and 1 per cent tetramethyl-ammonium hydroxide in ethanol (0.1 ml.) are added and the tubes incubated at room temperature for 0.5 hours. Glacial acetic acid (0.2 ml.) is added to each tube and the optical density of the samples read at 460, 510 and 560 mμ. The Allen formula is applied. Cortisone (0.5 μg. and 5 μg.) standards are treated similarly.

Standard tetrahydroaldosterone was not available for testing recovery rates but it was found that aldosterone from the Florisil column appeared in the 3rd eluate.

5, /

5, 10 and 15 µg. of aldosterone were put through the columns and after chromatography the following recoveries were obtained.

Table 8. Recovery of aldosterone from Florisil columns and the 3 chromatograms of the method.

Aldosterone µg.	Recovery mean of (3) µg.	% Recovery
5 (3)	3.2	64
10 (3)	7.6	76
15 (3)	12.1	80.6

Estimation of Urinary hydrocortisone, cortisone and their metabolites, tetrahydrocortisol (THF), allotetrahydrocortisol (allo-THF) and tetrahydrocortisone (THE).

Urine (50 - 100 ml.) aliquots are hydrolysed and extracted as in the method for pregnanetriol (p.166). The urine is then brought to pH1 and the extract is quantitatively applied to a Zaffaroni chromatogram as prepared in the method for the estimation of free aldosterone (p.172), and developed for 3 hours in the solvent system chloroform/formamide.

Three zones are removed from the chromatogram and eluated. Zone 1 corresponding to $R_{F(cortisol)}$ 0.2 to 0.45 containing tetrahydrocortisol (THF) and allo-tetrahydrocortisol (allo-THF); Zone 2 corresponding to standard hydrocortisone including 0.5 cm. on either side - this contains hydrocortisone and tetrahydrocortisone (THE); Zone 3 corresponding to cortisone.

The extract from each zone is quantitatively applied to separate lanes (1.6 cm.) of a chromatogram (Whatman No. 1) using hydrocortisone and cortisone as standards and developed in the E_2B system (isooctane, tert. butanal, water: 10: 5: 9) for 3 hours. Scanning of the Zaffaroni chromatogram under UV light (Hanovia lamp = 245 mv) gives an indication of the quantities of /

of UV absorbing compounds present (particularly hydrocortisone and cortisone) thus allowing an appropriate aliquot (1 - 5 µl. containing 1 to 2 µg. UV-absorbing material) to be applied to the lanes of the E_2B chromatogram so that the fluorescence when developed will be within the sensitivity range of the fluorimeter. The appropriate strips containing hydrocortisone and cortisone are sprayed as indicated for aldosterone (p.175.) and the fluorescence measured against standard hydrocortisone and cortisone.

The areas containing tetrahydrocortisone (THE; R_F 1.92), tetrahydrocortisol (THF; R_F 1.51), and allo-tetrahydrocortisol (allo-THF; R_F 1.37) are cut out, eluted and quantitated by the micro-blue tetrazolium method (p.187.). The actual areas for these latter 3 compounds are defined by comparison with a duplicate lane stained with blue tetrazolium. Recovery experiments using hydrocortisone and cortisone gave recovery rates from 50 - 70 per cent (hydrocortisone mean 61.5 per cent; cortisone mean 60 per cent).

Table 9. Recovery rates of hydrocortisone and cortisone obtained by the method. 10 µg. of each added to urine before hydrolysis.

	Recovery µg.	
Added (µg.)	Hydrocortisone	Cortisone
10 µg.	6.5	7.0
10 µg.	5.9	6.5
10 µg.	6.2	5.5
10 µg.	6.0	5.0

These recovery rates are better than 40 - 50 per cent obtained by Harris and Crane (1964) but I am of the opinion that the improved recovery is related to the practice of effecting hydrolysis in an atmosphere of nitrogen. By so doing oxidative degradation of hydrocortisone, cortisone and their metabolites at pH1 is reduced to a minimum.

Hydrolysis of 17-oxosteroid conjugates and estimation of the extracted 17-oxosteroids after chromatographic separation.

Urine (100 ml.) is brought to pH4 with sulphuric acid. Ammonium sulphate (50 g.) is added and extraction of the steroid conjugates effected with ethyl acetate (300 ml.). The ethyl acetate is then evaporated under a stream of nitrogen. To the residue, ethyl acetate (100 ml.) containing 0.01 M perchloric acid is added, and hydrolysis allowed to proceed at room temperature for 3 hours. The ethyl acetate is then washed twice with 10% potassium hydroxide (10 ml.), once with water (10 ml.) and thereafter evaporated under a stream of nitrogen. The residue is again taken up in ethyl alcohol (3 ml.) containing 1 per cent potassium hydroxide to hydrolyse acetates formed during extraction. Hydrolysis is allowed to proceed at room temperature for 1 hour. After hydrolysis the alcohol is diluted with water (10 ml.) and the free compounds are then extracted with petroleum ether and benzene (2 x 20 ml. 1:1 v/v), and evaporated. This method is recommended by De Paoli, Nishizawa and Eik-Nes (1963) for the hydrolysis of both sulphates and glucuronides of 17-oxosteroids. The final extract obtained is sufficiently clean to allow application directly to the chromatogram without further purification.

Separation of 11-deoxy-17-oxosteroids.

This was done by the method of Morris (1959) as modified by Hill (1960).

The extract is dissolved in a mixture of 25 per cent (v/v) ethyl acetate in petroleum ether and applied to a column (1 cm. diameter) of silica gel (100 - 200 mesh, 2 g.). A volume of this solvent (20 ml.) is passed through the column and collected. 35 per cent ethyl acetate in petroleum ether (20 ml.) is then passed through and collected. Finally 65 per cent ethyl acetate in petroleum ether (20 ml.) is used to elute 11-oxygenated-17-oxosteroids. The eluates are evaporated to dryness in a stream of nitrogen and applied separately as required to the chromatograms. Androsterone appears in fraction 1 and etiocholanolone in fraction 2.

Chromatographic separation.

The extracts are dissolved in ethyl alcohol (250 µl.) and 3 aliquots (5-50 µl.) are applied to 1.6 cm. lanes. The remainder of the extract is oxidised with chromic acid (Kiliani reagent) and aliquots (5-50 µl.) are applied to 2, 1.6 cm. lanes. Similar quantities of uncolumned extract are spotted on 3, 1.6 cm. lanes. Desoxycorticosterone on the side lanes is used as a reference /

reference standard. The chromatogram is then developed for 3 hours in the Bush 3 system (petroleum, ether, benzene, methanol, water: 33:17:40:10) at 22°C.

Estimation of 17-oxosteroids on paper.

Test strips for each fraction are stained with Zimmermann reagent 2.5 N and sodium hydroxide. The appropriate areas for etiocholanolone and androsterone are cut out and eluted. Testosterone and Δ4-androstenedione fluoresce on paper and separate well on Bush 3 (testosterone R_{DOC} 0.95: Δ4-androstenedione R_{DOC} 1.45) from uncolumned extracts. Their fluorescence is measured against corresponding standards. Dehydroepiandrosterone occupies an intermediate position (R_{DOC} 1.18) between testosterone and androstenedione and an area corresponding to standard dehydroepiandrosterone is cut out and eluted. Androsterone, etiocholanolone and dehydroepiandrosterone are estimated by the Zimmermann reaction.

Recovery. /

Recovery.

From mixtures of the six 17-oxosteroids separated and estimated in the above way the following recoveries were obtained. (Table 10).

Table 10. Recovery of 6, 17-oxosteroids from the Bush 3 chromatogram. Δ4-3-ketones estimated by sodium fluorescence and 17-ketones by the Zimmermann reaction against dehydroepiandrosterone. No correction made for the different chromogenicities.

Substance	µg. spotted.	µg. estimated.
Δ4-androstenedione	(3)* 2	1.8; 2.0; 1.7; 4.1; 3.5
Testosterone	(3) 2	1.3; 2.0; 1.9
Andrenosterone	(3) 2	1.7; 1.6; 1.9
Dehydroepiandrosterone	(3) 10	8; 11; 8.3
Androsterone	(3) 10	9; 9.2; 9
Etiocholanolone	(3) 10	12; 10; 8.4

*( ) number of estimations.

In the chromatograms of the oxidised extracts for Case 4 of Chapter 2 a Zimmermann positive spot appeared at $R_{(DOC)}$ 3.07 which was not seen before oxidation. In Chapter 2 evidence is given that this compound may be androstane-17β-ol-3-one.

Other methods referred to in the text.

Determination of pregnenetriol.

The estimation of this compound was made during the investigation of Case 4 in Chapter 2.

Bongiovanni suggested (in personal communication) that the use of differential hydrolysis, first with β -glucuronidase followed by solvolysis with extraction after each procedure would give an indication of the quantities of sulphate conjugates. In this particular case 3β,-hydroxy-Δ5, and 3β,21-dihydroxy-Δ5 compounds appear in the urine in increased quantities.

Urine is therefore hydrolysed as for pregnenetriol, extracted (A) and then subjected to pH1 hydrolysis, followed by further extraction (B). The residues from each extraction are reduced, then oxidised by Few's method and estimated as 17-oxosteroids. The excess of 17-oxosteroids in extract B over extract A is taken to represent pregnenetriol.

Oxidation /

Oxidation by Kiliani reagent (Kiliani and Mark, 1901).

Kiliani reagent (5 µl.) is added to the steroid sample dissolved in acetone (0.5 ml.). The mixture is allowed to stand at room temperature for 20 minutes. Water (2.5 ml.) is then added and the steroids recovered by extraction with benzene/chloroform (6:1 v/v 2 x 2 ml.).

Solvents.

Absolute ethanol (Burroughs) and n-heptane (B D H) were used without purification. Acetone and formamide were likewise used as supplied by British Drug Houses Ltd., Poole. Ethyl ether was rendered peroxide free. All other solvents were A.R. specification and were redistilled.

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