STEROIDAL STUDIES IN VASCULAR DISEASE

BY

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II. Abbreviations.

Adrenocorticotrophin	ACTH
Adenosine-5[1]-triphosphate	ATP
2-Amino-2-hydroxymethyl-1,3-propanediol	TRIS
1,4 Bis-2(4-methyl-5 phenyl-oxazolyl)-benzene	Dimethyl POPOP
Correlation coefficient	r
2,5-Diphenyl-oxazole	PPO
Glucose-6-phosphate	G-6-P
Glucose-6-phosphate dehydrogenase	G-6-P.D.
Nicotinamide adenine dinucleotide phosphate	$NADP^+$
Nicotinamide adenine dinucleotide phosphate reduced	NADPH
Percentage overweight	% O.Wt.
Standard deviation	S.D.
Serum uric acid	S.U.A.
Surface area	S.A.
Trimethylsilyl ether	TMSE
Trimethylsilyl ether derivative of a D.17-OS	TMSE-S
Trimethylsilyl ether derivative of pregnenolone	TMSE-P
Variation coefficient	V.C.

STEROIDS

TRIVIAL NAME	SYSTEMATIC NAME
Androstenedione (A)	5β-Androst-4-ene-3,17-dione.
Aetiocholanolone (E)	3α-Hydroxy-5β-androstan-17-one.
Testosterone (T)	17β-Hydroxy-5β-androst-4-en-3-one.
11β-androstenedione	11β-Hydroxy-5β-androst-4-ene-3,17-dione.
Androsterone (A)	3α-Hydroxy-5α-androstan-17-one.
Epiandrosterone (epiA)	3β-Hydroxy-5α-androstan-17-one.
Dehydroepiandrosterone (DHA)	3β-Hydroxyandrost-5-en-17-one.
Aetiocholanolone glucuronide (E.G)	3α-D-Glucopyranosiduronoxy-5β-androstan-17-one.
Aetiocholanolone sulphate (E.S)	3α-Sulphoxy-5β-androstan-17-one.
Androsterone glucuronide (A.G)	3α-D-Glucopyranosiduronoxy-5α-androstan-17-one.
Androsterone sulphate (A.S)	3α-Sulphoxy-5α-androstan-17-one.
Epiandrosterone sulphate (epiA.S)	3β-Sulphoxy-5α-androstan-17-one.
Dehydroepiandrosterone glucuronide (DHA.G)	3β-D-Glucopyranosiduronoxyandrost-5-en-17-one.
Dehydroepiandrosterone sulphate (DHA.S)	3β-Sulphoxyandrost-5-en-17-one.
Progesterone	5β-Pregn-4-ene-3,20-dione.
11β-Hydroxyprogesterone	11β-Hydroxy-5β-pregn-4-en-3,20-dione.
17α-Hydroxyprogesterone	17α-Hydroxy-5β-pregn-4-ene-3,20-dione.

TRIVIAL NAME	SYSTEMATIC NAME
Deoxycorticosterone (DOC)	21-Hydroxy-5β-pregn-4-ene-3,20-dione.
Corticosterone	11β,21-Dihydroxy-5β-pregn-4-ene-3,20-dion
11-Deoxycortisol	17α,21-Dihydroxy-5β-pregn-4-ene-3,20-dion
Pregnanediol	3α,20α-Dihydroxy-5β-pregnane.
Cortisone	17α,21-Dihydroxy-5β-pregnan-4-ene-3,11,20-trio
Aldosterone	11β,21-Dihydroxy-5β-pregn-4-ene-3,20-dion 18-al.
Cortisol	11β,17α,21-Trihydroxy-5β-pregn-4-ene-3,20-dione.
Pregnanetriol	3α,17α,20α-Trihydroxy-5β-pregnane.
Tetrahydrocortisone	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione.
6β-Hydroxycortisol	6β,11β,17α,21-Tetrahydroxy-5β-pregn-4-ene-3,20-dione.
6α-Hydroxycortisol	6α,11β,17α,21-Tetrahydroxy-5β-pregn-4-ene-3,20-dione.
20β-Hydroxycortisol	11β,17α,20β,21-Tetrahydroxy-5β-pregn-4-ene-3,20-dione.
20α-Hydroxycortisol	11β,17α,20α,21-Tetrahydroxy-5β-pregn-4-ene-3,20-dione.
Allotetrahydrocortisol	3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one.
Pregnenolone (Preg)	3β-Hydroxypregn-5-en-20-one.
17α-Hydroxypregnenolone	3β,17α-Dihydroxypregn-5-en-20-one
Pregnenetriol	3β,17α,20α-Trihydroxypregn-5-en-20-one.
Deoxycorticosterone acetate (DOCA)	21-Acetoxy-5β-pregn-4-ene-3,20-dione.

TRIVIAL NAME	SYSTEMATIC NAMES
Pregnenolone sulphate	3β-Sulphoxypregn-5-en-20-one.
17α-Hydroxypregnenolone sulphate	3β-Sulphoxy-17α-hydroxypregn-5-en-20-one.
Cholesterol	3β-Hydroxycholest-5-ene.
21-Hydroxycholesterol	3β,21-Dihydroxycholest-5-ene.
Cholesterol sulphate	3β-Sulphoxycholest-5-ene.

17-Oxosteroids	17-OS
17α-Hydroxycorticosteroids	17-OHCS
11-Deoxy-17-oxosteroids	D.17-OS
11-Deoxy-17-oxosteroid sulphates	D.17-OS.S
11-Deoxy-17-oxosteroid glucuronides	D.17-OS.G
11-Deoxy-17-oxosteroid sulphates plus glucuronides	D.17-OS.(S+G) or D.17-OS.

III. INTRODUCTION.

A. Hypertension.

Ever since Johnson(1868) advanced the concept that hypertension results from the contraction of arterioles throughout the body there has been ceaseless clinical and experimental investigation in search of the factors responsible for arteriolar constriction in hypertension. Two main facets have been considered in the mechanism of arteriolar constriction during the last century: neurogenic and humoral.

It has been known since the time of Claude Bernard (1851) that stimulation of the sympathetic nerves produces vasoconstriction (except in the heart), the intensity of the effect varying from one organ to another. Also cutting the sympathetic nerve or temporarily interrupting it by local anaesthesia increases the blood flow through the affected tissues (other than brain and kidney). When we add to these facts the marked pressor response induced by such emotions as fear and anger it does not seem surprising that a nervous pathogenesis of idiopathic hypertension has been considered from the earliest studies. As long ago as 1852, Rokitansky, the morphological pathologist, attributed idiopathic cardiac hypertrophy, doubtless predominantly our modern idiopathic hypertension to a disturbance of innervation. Laycock, another early student, suggested in 1873 that Bright's disease results from primary disturbance of the nervous system. With the establishment of evidence that increased peripheral resistance is the immediate mechanism of the rise in blood pressure, the line of thought

that neurogenic factors contribute directly to increased vascular resistance in hypertension was rendered all the more appealing. Nevertheless, the rapid progress in experimental medicine through the availability of synthetic compounds, the introduction of radio-isotopes and the various forms of chromatography, to name but a few of the advancements, has helped to throw more light on the patho-genesis of hypertension. The results have strongly supported partisans of the "humoral" aetiology of this disease.

Various vasoconstrictive substances, found in the body fluids of hypertensive patients have been described. In 1904, Elliot noted the similarities between the effect of stimulation of sympathetic nerves and that of adrenaline. It was suggested that the autonomic nerves might produce their effect by releasing at their termination compounds with specific affinity for the receptors of the motor units activated. The classical works of Dale (1933) and Von Euler (1951a) resulted in the isolation of these substances and their identification as adrenaline and noradrenaline, both of which have been shown to be secreted by the adrenal medulla. The discovery of adrenaline and noradrenaline ultimately permitted the recognition of a group of patients, albeit a small one, whose hypertension was clearly due to excessive catecholamine production as a primary cause - the phaeo-chromocytoma (Von Euler 1951b). These findings gave rise to a constantly increasing interest in the adrenal gland as an important, if not causative factor in hypertension. This stimulated an

intensive search for other humoral agents which could be responsible for elevating blood pressure. The central point of subsequent investigations has been shifted from the adrenal medulla to the adrenal cortex.

The first description of hypertensive changes secondary to an adrenocortical tumour was reported in 1897 by Neusser, and a further hint of a possible relationship between the adrenal gland and hypertension was made by Josué in 1903. Little notice, however, was given to this association until the more extensive observations of Oppenheimer and Fishberg in 1924.

In 1932, Cushing described a clinical syndrome which he thought was due to a basophil adenoma of the pituitary gland. Hypertension was one of the cardinal features of this syndrome. Subsequent work has shown that the cause of the disturbance described by Cushing was an increased production of the adrenocortical steroids by either a tumour or hyperplasia of the adrenal cortex. Various authors have commented upon the elevated blood pressure in patients with tumours and hypertrophy of the adrenal gland and contrasted it with the reduced blood pressure of Addison's disease (1855), in which "cortical atrophy" was found to be responsible for the signs and symptoms, including arterial hypotension which invariably accompanies this disease.

It is now generally accepted that the elevation of blood pressure in Cushing's syndrome is the effect of an increased production of adrenocorticosteroids whereas the low pressure in Addison's disease

is due to adrenocortical hypofunction. In 1939 Loeb, Atchley, Ferrebee & Ragan called attention to two patients with Addison's disease whose arterial pressure exceeded normal limits while being treated with deoxycorticosterone (DOC). Several years later Perera (1945) reported a hypertensive patient who developed Addison's disease and as a result the blood pressure fell to subnormal levels. Hypertension recurred on administration of steroids, but therapy with salt alone resulted in a drop in blood pressure to normal limits, even though the patient was maintained in water and electrolyte balance. "One could not help but conclude that the adrenal cortex or appropriate steroid substitution therapy was important for the development or maintenance of this man's essential hypertension." This was confirmed by Thorn, Harrison, Merril, Criscitiello, Frawley & Finkenstaedt (1952), who also reported a fall of blood pressure to normal level in a hypertensive patient who developed Addison's disease.

Conn (1955) described a new clinical entity, "primary aldosteronism". A syndrome caused by an adrenocortical tumour secreting increased amounts of aldosterone which in turn induced potassium wastage and sodium retention both in association with mild hypertension.

Countless experiments on animals have indicated that the adrenal cortex plays an essential part in hypertension. Goldblatt (1937) in his pioneer investigations showed that, while the elimination of both adrenal medulla does not interfere with the production of renal artery constriction hypertension in dogs, the blood pressure falls to

normal after bilateral adrenalectomy. Moreover the hypertension is restored by administration of cortical extracts or deoxycorticosterone acetate (DOCA) (Page & Lewis, 1951). However, doses of cortical extract or cortisone which maintain hypertension in adrenalectomized dogs or rats do not raise the blood pressure in normal animals. Adrenalectomy also interfered with the development or maintenance of hypertension produced in animals by various procedures other than renal artery constriction; Jeffers, Lindauer & Lukens (1937) demonstrated this in experimental hypertension produced by the intracisternal ingestion of kaolin in dogs; Page & Reed (1945) in hypertension evoked with dihydroxyphenylalanine and related compounds in rats; McCann, Rothballer, Yeakel & Shenkin (1948) in hypertension obtained by auditory stimulation in rats; Ledingham (1951) and Floyer (1951) in the development or maintenance of renoprival hypertension in single and parabiotic rats.

The effect of adrenalectomy on hypertension in animals has been commented upon by many workers. It has been assumed ".....this effect can be partly accounted for by salt deficiency, the extracellular fluid being depleted of sodium, and hence of water, because of the failure of the kidneys to conserve sodium" (Pickering, 1955). However, a direct action of corticoids was sought by other investigators. Ramey, Goldstein and Levine (1951) demonstrated in a series of well designed experiments that a single injection of adrenal cortical extract, but not DOC, would potentiate the response to noradrenaline in adrenalectomized dogs, though not in normal controls. Moreover,

Fritz & Levine (1951) found that the vessels of the mesoappendix of the adrenalectomized rat became refractory to the repeated application of noradrenaline while those of the normal rat did not. The responsiveness of the vessels was restored by 11-oxysteroids injected intramuscularly or when applied locally in much smaller doses. They concluded that these corticoids are necessary to obtain the optimal response of the blood vessels to noradrenaline.

The elevation of blood pressure following the administration of various steroids has been reported by many workers. Selye, Hall & Rowley (1943) reported wide spread lesions similar to those seen in human nephrosclerosis and in the accelerated phases of idiopathic hypertension, cardiac hypertrophy, and decreased adrenal size on treatment of several animal species with massive doses of DOCA and salt. Selye (1946) and Knowlton, Loeb, Stoerk & Seegal (1947) demonstrated that the hypertensive action of DOCA was potentiated by salt. Friedman & Nakashima (1952 & 1953), in a screening test for renal and cardiovascular effects of different adrenal hormones in rats, demonstrated that cortisol had the most pronounced hypertensive effect of all the corticosteroids tested. They suggested that this steroid might exercise a direct action on the vascular bed, in contrast with DOCA.

In man, the administration of adrenocortical hormones likewise causes elevation of arterial pressure. The rationale behind these administrations probably originates from earlier

studies on salt metabolism. Ambard & Beauyard (1905) demonstrated that the blood pressure of hypertensive patients is further elevated by ingestion of salt and lowered by a salt free diet; from this they conclude that hypertension is caused by abnormal salt retention. In support of this Allen & Sherrill (1922), Volhard (1931), Kempner (1946 & 1949), and Corcoran, Taylor & Page (1951) succeeded in markedly lowering the blood pressure in many patients with hypertension by a poor salt diet. With the detection of the electrolyte regulation properties of certain corticoids, the central point of investigations, concerned with a possibility of disturbance of the salt metabolism in hypertension, was transferred directly to the adrenal cortex. This confirmed the work of Loeb et al (1939) who reported development of hypertension in two patients suffering from Addison's disease, after treatment with DOC esters, and the work of Thorn & Firor (1940) and McCullagh & Ryan (1940) who reported similar findings. This pressor effect has also been demonstrated in uncomplicated hypertensive vascular disease but not in normotensive subjects (Perera & Blood, 1947). The prompt rise in blood pressure in the hypertensive group could not be ascribed to salt and water retention, as there were similar changes in the normal group. Finally, since the introduction of cortisone and cortisol to wide clinical use, it has become well known that over dosage or prolonged treatment with these steroids frequently results in an elevation of blood pressure, which returns to normal on withdrawal of the steroid or reduction of its dosage (first reported

by Hench, Kendall, Slocumb & Polley, 1949).

Adrenocorticotrophin (ACTH) was also shown to elevate blood pressure. Hench et al (1949) and Perera (1950) reported that ACTH may raise the blood pressure of normotensive patients. In hypertensives it causes a marked increase in arterial pressure. These observations of the pressor action of ACTH were subsequently confirmed by many clinical reports.

All these findings supported the introduction of bilateral adrenalectomy as a surgical procedure in the treatment of severe hypertension. DeCourcy, DeCourcy & Thuss (1934), Allen & Adson (1937), Crile (1938) and Neuhof (1948), performed partial or subtotal adrenalectomies together with other surgical procedures in patients with severe idiopathic hypertension. However, total adrenalectomy became feasible only with the introduction of cortisone for clinical use. Since then many reports of total or subtotal adrenalectomy for hypertension have appeared, but it is difficult to assess their value, as they were combined with various other surgical procedures, mainly sympathectomy. Green, Nelson, Dodds & Smalley (1950) were the first to describe a patient with malignant hypertension and diabetes mellitus whom they treated by total adrenalectomy alone. The operation was followed by a fall in blood pressure and a dramatic remission of the diabetes, this still continuing 15 months after adrenalectomy. Wolferth (1953), Bowers (1954), Pickering, Wright & Heptinstall (1952), Thorn et al (1952), Rosenheim (1954) and

Wilson (1955) reported the effect of subtotal and total adrenalectomy combined with, in some cases, various types of sympathectomy for the treatment of high blood pressure. However, in most of these studies the data are not sufficient to assess accurately the effect of adrenalectomy on the hypertensive state. The work of Arnott, Crooke, Donovan & Taylor (1957) furnishes more comprehensive and valuable information about the patient's condition during a long post-operative period and gives a detailed account of the electrolyte balance in the patients studied. In the light of the conclusive evidence supplied by this investigation, the results of previous workers can be reassessed and better understood. It now seems established that, in patients with severe hypertension who have had bilateral adrenalectomy, blood pressure can be maintained at normal levels if the daily cortisone dose is low enough to keep the patients in a state of mild "adrenocortical hypofunction". The authors conclude, "it is clear that the vascular disease has been retarded or, perhaps even arrested by a state of slight adreno-cortical insufficiency but it appears that the liability to excessive constriction of the arteriolar vascular bed, which is fundamental to essential hypertension, persists and a slight increase in the dosage of cortisone quickly recalls a severe hypertensive state".

In the light of all the facts and findings described above, it seems to be proved beyond doubt that the adrenal cortex plays an essential part in the development and maintenance of arterial

hypertension. However, the exact nature of this relationship, and the way in which adrenocortical secretion influences the mechanism of high blood pressure has not been as yet elucidated. All the evidence derived from previous research suggested two possible mechanisms (Kornel, 1959):

1. The role of the adrenal cortex in hypertension is of merely permissive nature. This is to be understood as follows: adrenocortical secretion, or more precisely mineralocorticoid secretion, is essential for the maintenance of the proper sodium and potassium intracellular concentrations; these concentrations are responsible for the general muscular tone, including that of the vascular musculature, thus conditioning the response of the arterioles to various vasoconstrictive agents.

2. Adrenocortical hormones per se contribute directly to the increased and normal vascular resistance, either acting as pharmacodynamically active compounds on nerve endings in the arteriolar wall (or on the muscle directly) or increasing the responsiveness of the arterioles to noradrenaline or other vaso-constrictive substances.

Bearing the above mechanisms in mind, it would be more purposeful to be selective, rather than exhaustive in further review of biochemical investigations and controversies related to hypertensive disease, with special emphasis on those aspects most closely related to the authors subsequent experimental studies. Further review will be subdivided into the following sections:- glucocorticoids,

mineralocorticoids and adrenal "androgens".

1. Glucocorticoids:

The recognition of the association of cortisol over-production with a form of hypertension at once raises the possibility that some patients with hypertension who did not have Cushing's syndrome even in mild form, might yet suffer from hypersecretion of cortisol, which for some reason was not producing clinical evidence of its presence. An added stimulus for research along these lines was the fact that exogenous cortisol will induce hypertension even in the absence of dietary sodium (Freidman, Freidman & Nakashima, 1953; Knowlotn, Leob, Stoerk, White & Heffernan, 1952).

Cooper, Touchstone, Poberts, Blakemore, Rosenthal & Kasparow (1958) studied steroid formation by slices of human adrenal glands, excised for the treatment of hypertension and incubated in autologous plasma. The results showed a negative correlation between cortisol formation per g. of tissue and diatolic blood pressure of the individuals from whom the glands were removed ($r = -0.64$, $P<0.01$). This decline in cortisol formation was also associated with a fall in the ratio of the formation of cortisol/corticosterone due to the fact that corticosterone formation declined significantly less with increasing diastolic pressure than cortisol. From this Cooper et al concluded that, "since cortisol and corticosterone arise from the same precursors, the shift of ratio indicates changes in the functional capability in the adrenocortical cells rather than in

supply of substrates. The fall of the cortisol/corticosterone ratio suggests that there develops a deficiency of 17α-hydroxylase with progressing hypertension". In support of these findings Cooper, Kasparow, Blakemore & Rosenthal (1958) reported that the cortisol output by adrenals in situ, computed from rate of blood flow and steroid concentration in the adrenal venous blood at operation prior to excision of the gland, showed a negative correlation to diastolic blood pressure of the patient similar to that in the corresponding adrenal incubates.

However, in 1959 Touchstone, Cooper & Blakemore reported increased mean level of 11-deoxycortisol in adrenal venous blood of nine hypertensive patients, which does not substantiate their theory that there is a deficiency at 17α-hydroxylase level. Further evidence against decreased cortisol or increased corticosterone output by the adrenal gland of hypertensive subjects comes from the studies of Genest, Nowaczynski, Koiw, Sandor & Biron (1961). They found no essential difference in the mean excretion of cortisone, cortisol and their tetrahydro derivatives and the tetrahydro derivative of 11-deoxycortisol in groups of patients with idiopathic, renal and malignant hypertension as compared to that of normals. Finally using the Porter Silber reaction (1950) which is specific for steroids with a dihydroxyacetone side chain, (steroids loosely grouped as 17-hydroxycorticosteroids) Vermeulen & Van der Straeten (1963) measured the

daily urinary excretion of free and total Porter Silber chromogens. They compared the daily urinary levels of these steroids in idiopathic hypertensive patients with a control goup and found no statistical difference. More elaborate studies by the same investigators where cortisol and corticosterone production rates were measured again showed no difference on comparison of the controls with patients suffering from idiopathic hypertension.

Direct support of the theory that there is progressive diminution of 17α-hydroxylase activity in the course of arterial hypertension ironically enough comes from the findings of Genest et al (1960) and Vermeulen & Van der Straeten (1963) who reported significantly decreased urinary excretion levels of pregnanetriol the catabolic product of 17α-hydroxyprogesterone.

The studies of Nowaczynski, Kiow, & Genest (1964) lend very impressive support to a 17α-hydroxylase impairment in hypertension. They estimated the excretion of pregn-5-ene-3β, 17α,20β-triol the catabolic product of 17α-hydroxypregnenolone, in a group of male and female patients with idiopathic, renal and malignant hypertension and found the mean excretion of this compound to be significantly decreased ($P<0.001$) in the three types of hypertension when compared with the mean levels in normal males and females.

For proper interpretation of urinary levels of any compound, knowledge of the precursors of that compound must be available.

Urinary pregnanetriol is mainly derived from 17α-hydroxyprogesterone but it may also derive from 17α- hydroxypregnenolone. The studies of Landau & Laves (1959) have shown that part of the urinary pregnanetriol may originate also from the testes. However, Roberts, Vande Wiele & Lieberman (1961) have shown that pregn-5-ene3β,17α,20β-triol is the main metabolite of 17α-hydroxypregnenolone and possibly derives solely from that steroid. In addition, Decourcy (1956) did not obtain any pregn-5-ene3β,17α,20β-triol from 17α-hydroxyprogesterone administered to a man. This shows that the Δ^4-3-oxo configuration is not converted in vivo into Δ^5-3β-ol configuration.

As several steroids have been shown to give rise to urinary pregnanetriol it cannot be assumed that the determination of urinary pregnanetriol necessarily reflects the production or metabolism of a single steroid. However, as 17α-hydroxypregnenolone appears to be the unique precursor of pregn-5-ene3β,17α,20β-triol, it might, therefore, be concluded that the decrease in the urinary excretion of this compound indicates an inability of the adrenal cortex of hypertensive patients to hydroxylate pregnenolone at the 17α position.

The most impressive evidence implicating glucocorticoids with essential hypertension comes from the work of Kornel, who, in a very thorough study described in a series of papers covering a decade unequivocally established a relationship between glucocorticoid metabolism and idiopathic hypertension (Kornel, 1957; Kornel, 1960; Kornel, 1962; Kornel, 1964; Kornel, 1965; Kornel & Motohashi, 1965, and

Kornel & Takeda, 1967). Studying the urinary excretion of 17-hydroxycorticosteroids (17-OHCS) as free, glucuronide and sulphate fractions in normotensive and hypertensive patients he demonstrated that:-"1) excretion of 17-OHCS glucuronides was lower in hypertensive patients; 2) excretion of 17-OHCS sulphates was higher in these patients; 3) the higher excretion of sulphates in hypertensives was mainly due to an increase in the more polar steroids, mainly 6α-hydroxycortisol, 6β-hydroxycortisol and another still more polar steroid in this fraction; 4) the lower excretion of glucuronides was due only to a decrease in the less polar steroids (mainly allotetrahydrocortisol, tetrahydrocortisol and tetrahydrocortisone) in the glucuronide fraction." These results closely correlated with those of an analogous study in plasma. In support of this Touchstone, Kasparow & Blaemore (1965) have shown in vitro that the biosynthesis of 20α and 20β-hydroxycortisol are increased in the adrenal tissue of hypertensive subjects: also Besh, Brownwell, Hartman & Watson (1962) demonstrated in dogs with experimental hypertension a 50% increase in the formation of 20β-hydroxycortisol. Kornel suggests that the more polar sulphated 17-OHCS, which are increased, may be directly involved in the mechanism of increased arteriolar resistance, the fundamental finding leading to arteriolar hypertension. He also suggests the possible secretion of these sulphated steroids directly by the adrenal gland as happens with dehydroepiandrosterone sulphate (DHA.S) and possibly corticosterone sulphate (Vande Wiele, MacDonald, Gurpide

& Lieberman, 1963; Lebeau & Baulieu, 1964).

Kornel suggested that the increase in sulphate fraction of the 17-OHCS in the hypertensive patients was due to a decreased ability of the patients to reduce ring A of the steroid nucleus (necessary for the formation of C-3 glucuronide conjugates) with the resulting increase in a compensatory sulphation pathway which does not require ring A reduction. Interpretation of this theory would suggest an increase in the sulphation of all the 17-OHCS, not just the more polar 17-OHCS and through this a decrease in all glucuronide conjugated 17-OHCS. However, this was not reported by Kornel, but it may possibly be that inhibition of ring A reduction applies only to the more polar steroids. Therefore, one would expect an increase in only the more polar sulphate steroids. Another explanation could be the further hydroxylation of cortisol sulphate, as happens in the further metabolism of DHA.S.

2. Mineralocorticoids:

Interest in a possible role of aldosterone in the pathogenesis of at least some forms of hypertension sprang originally from two main facts. Firstly aldosterone was found to be an extremely potent sodium retaining hormone, with properties similar to, but much stronger than deoxycorticosterone which, as already mentioned, could produce hypertension in animals. Secondly, there was the recognition by Conn in 1955, of the hypertensive state associated with cortical adenoma producing aldosterone in excess.

However, the role played by aldosterone in essential hypertension has never been clear. Genest, Lemieux, Davignon, Koiw, Nowaczynski & Steyermark (1956); Garst, Shumway, Schwartz & Farrell (1960); Genest, Koiw, Nowaczynski & Leboeuf (1958); Venning, Dryenfurth, Dossetor & Beck (1961) and Genest et al (1960) all found elevated urinary levels of aldosterone in patients with idiopathic hypertension: most of these reports did not give clinical details of their patients to indicate the severity of the hypertension and whether or not steps had been taken to exclude renal factors. In particular, information was lacking as to whether or not patients had been treated with hypotensive drugs or diuretics and as to the amount of dietary sodium permitted.

Elevated urinary levels of aldosterone were also found in patients with severe and malignant idiopathic hypertension and it was suggested by Genest et al (1956) that "human arterial hypertension could be caused by a mild and chronic hyperaldosteronism'. In a subsequent report Genest et al (1958) confirmed their previous findings, but were more cautious in their interpretation.

On the other hand Hernando, Crabbe, Ross, Reddy, Renold, Nelson & Thorn (1957) and Cottier, Muller & Schmid (1959) failed to find elevated urinary aldosterone levels in untreated mild hypertensive patients on a normal sodium intake. In the evaluation of urinary aldosterone levels it must be remembered that the amount of aldosterone appearing in the urine is only a

portion and often a variable portion, of the amount secreted. Therefore, it does not always mirror the secretion rate. Thus studies reporting secretion rates will tend to be.more. authoratative. Laragh, Ulick, Ganuszewicz, Deming, Kelly & Lieberman (1960) measured aldosterone secretion rates in 7 untreated idiopathic hypertensive patients and found them to be within the normal range. Two years later Cope, Harwood & Pearson (1962) confirmed these findings.

More recent reports confirm the findings that many patients in the advanced stages of hypertension secrete and excrete increased amounts of aldosterone in relation to sodium balance (Laragh, Sealey & Sommers, 1966). However, the more recent reports do not completely clarify the role of aldosterone in idiopathic hypertension. Genest (1965) still claims to find increased urinary aldosterone excretion in patients with idiopathic hypertension, whereas Ledingham, Bull & Laragh (1967), estimating aldosterone secretion rates in 113 patients with uncomplicated idiopathic hypertension and 56 controls present very convincing evidence to the contrary. This group also demonstrated a very close relationship between rate of salt excretion and the rate of aldosterone secretion over a very wide range of urinary sodium in 58 normal subjects and showed that in general, a strikingly similar relationship is apparent in patients with essential hypertension; only 5 out of 113 showed abnormal large amounts of aldosterone in relation to urinary sodium.

Laragh, Cannon & Ames (1963) have concluded that "increased aldosterone secretion does not occur in essential hypertension, unless renal involvement has reached a stage when secondary aldosteronism is induced." In this context Cade, Shires, Barrow & Thomas (1967) describe very unexpected steroid patterns in renovascular hypertension, a condition generally associated with elevated aldosterone rates (Gowenlock & Wrong, 1962; Laidlaw, Yendt, Bird & Gornall, 1963 and Slaton & Biglieri, 1965). They found grossly abnormal diurnal rhythm of plasma cortisol concentration with high late evening levels and low to normal norming levels. However, the aldosterone excretion was normal in all the 13 patients studied.

The role of aldosterone in experimental hypertension is as uncertain as its role in idiopathic hypertension in man. Aldosterone produces hypertension in rats but not in dogs, cats or rabbits, and like DOC it produces hypertension only if certain minimal quantities of sodium are present in the diet. Hypertension could not be produced in adrenalectomized, unilaterally nephrectomized rats by the administration of 40 µg of d-aldosterone in sesame oil (Gross, Loustalot, Meier, 1955); this amount had sodium retaining activity equal to 1 mg of DOCA, the administration of which resulted in hypertension. Later the same authors succeeded in producing hypertension in unilaterally nephrectomized salt loaded rats given 0.5 mg of dl-aldosterone monoacetate daily (Gross, Loustalot & Meier, 1957). Hypertension has been produced in rats by the

prolonged administration of only one-thousandth of this dose, that is 0.5 µg of d.aldosterone administered sub-cutaneously in ethanol every second day, increasing to 1 µg daily after 3 months (Kumar, Hall, Nakashima & Gornall, 1957). Hypertension appeared after 100 days. These investigators surprisingly found that the substitution of 1% saline for drinking water made no difference in the rate at which blood pressure became elevated. When a similar dose of aldosterone was given to adrenalectomized rats, hypertension developed earlier than when given to intact rats. Singer, Losito & Salmon (1963) working with rats with their renal artery clipped were able to demonstrate that elevated aldosterone secretion was unnecessary for development of persistent hypertension. In rats with a clip on one renal artery and an opposite normal kidney, the aldosterone secretion rate was elevated. After removal of the normal kidney, though the blood pressure remained elevated, the aldosterone secretion rate dropped to normal.

The mechanisms by which mineralocorticoids are implicated in the pathogenesis of hypertension are complex. There is evidence that whenever vascular smooth muscle contracts more than normal, such as produced by chronic infusion of noradrenaline, the muscles tend to accumulate sodium, and as the muscle hypertrophies it accumulates more sodium in proportion to the increased volume of vascular tissue as well as because of increased concentration. Gross (1961) and Mendlowitz, Naftchi, Bobrow, Wdf & Gitlow (1963)

have shown that mineralocorticoids not only favour renal retention of sodium, but also may have a direct effect on the tissues in terms of a shift of ions into or out of smooth muscle cells. Aldosterone causes more renal retention of sodium in normotensives than in patients with idiopathic hypertension, however, aldosterone produces a greater increase in vascular reactivity in the hypertensive group, which Mendlowitz et al (1961) suggest is a direct effect on vascular smooth muscle. On the other hand glucocorticoids have little effect on the retention of sodium by the kidney, yet they increase responsiveness of blood vessels in normotensive subjects, but not in hypertensive patients. (Mendlowitz, Naftchi, Weinreb & Gitlow, 1961).

3. Adrenal "Androgens":

Bruger, Rosenkrantz & Lowenstein (1944) were the first to estimate 17-oxosteroid (17-OS) excretion in urine (24-hour specimens), using a method based on the Zimmerman's colour reaction. They selected only female subjects for their investigation, in an attempt to eliminate the contribution of androgenic steroids of testicular origin. Fourteen normotensives and 40 hypertensive subjects were investigated. The urinary excretion of the 17-OS was found to be significantly lower in the hypertensive group, although it was still within the lower limit of the excretion of the normal subjects. The authors suggested that their findings may indicate an altered pattern of corticoid production or a hypofunction of adrenal cortex due to exhaustion under the constant

strain imposed upon it by the persistent elevation in blood pressure. A low urinary excretion of 17-OS was also reported by Selye (1947) in 12 of 18 hypertensive patients. However, Sayers (1950) thought the determination of "androgenic" steroids (17-OS) in hypertension did not seem to have a strong rationale as their concentration in urine or blood hardly reflects adrenocortical activity, although they partly constitute the metabolic product of the glucocorticoids.

Hetzel & Hine's (1952) investigation of urinary 17-OS level showed no difference from normotensive controls. The authors concluded that "there is no evidence for a significant disturbance of the adrenal cortex in hypertension". In a series of 19 patients with severe types of renal disorders and probably all exhibiting an elevated blood pressure, Wallace, Christy & Jailer (1955) measured urinary 17-OS and blood urea nitrogen. In 1 case of acute glomerulonephritis, 3 cases of chronic glomerulonephritis and 1 of 2 hypertensive patients low urinary 17-OS were found.

Warter, Schwartz & Block (1960) looked at 17-OS levels in patients with idiopathic hypertension, who were split into two groups; patients with elevated urinary aldosterone levels and patients with normal urinary aldosterone levels. They found no difference in 17-OS between the two groups. However, they point out that urinary dehydroepiandrosterone (DHA) and 17-OS were often

subnormal in patients with idiopathic hypertension, but that the variation of the aldosterone DHA ratio was not significant. The authors interpreted the often subnormal urinary DHA and 17-OS levels found in their studies as alterations met with in all deficiency states.

While investigating the range of values for individual 17-OS in normal subjects Feher (1966) and Keutmann & Mason (1967) have shown urinary DHA to be absent or very low in childhood and old age, and present in varying amounts in normal adults. In seeking to determine factors which could influence both uricosynthesis and blood pressure Kölbel, Gregorova & Sonka (1964, 1965a) found a relationship between the absence of urinary DHA and elevated serum uric acid levels in hypertensive patients. They found that 18 out of 23 (74%) hypertensive males and 12 out of 23 (52%) hypertensive females had no DHA in their urine. Full details were not supplied but in almost all the patients showing an absence of urinary DHA the serum uric acid was elevated.

Hypertension is not the only disease in which an absence of urinary DHA has been described. Sonka, Gregorova & Krizek (1964) reported the absence of urinary DHA in patients suffering from gout, a disease in which one of the cardinal features is grossly elevated serum uric acid levels. In female obese diabetics Sonka & Gregorova (1964) reported the absence of urinary DHA in 27 out of the 32 patients. The same authors have also reported lowered

urinary DHA levels in patients with toxaemia of pregnancy and have related this decrease to the elevated plasma lipids normally displayed in this disease (Kotasek, Fassati, Sonka, Brestak, Gregorova & Fassati, 1967).

An unifying theory to encompass the above conditions, all of which are associated with a deficiency of urinary DHA, was postulated by Sonka, Gregorova, Jiranek, Kolbel & Matys (1965b). It stems from the studies of Marks & Banks (1960) who demonstrated that DHA, pregnenolone, epiandrosterone and to a lesser degree several other 17-OS at concentrations of $1x10^{-6}$M or less, inhibit mammalian glucose-6-phosphate dehydrogenase (G-6-P.D.) activity. All the steroids with inhibitory action had a ketone group at C-20 or C-17; progesterone, oestrogens, testosterone, corticosteroids and steroids with hydroxyl groups at C-17 or C-20 had little or no inhibitory action. The inhibition was shown to be non-competitive and left the steroid unchanged at the end of the experiment. Similar findings have been reported by McKerns & Kaleita (1960). G-6-P.D. is the rate limiting enzyme in the pentose phosphate pathway which is very important for the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$).

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is necessary for the reduction synthesis of fatty acids (Gibson, Jacob, Porter, Tietz & Wakil, 1957; Bressler & Wakil, 1961; Wakil & Bressler, 1962), unsaturated fatty acids (Bloomfield & Block, 1958), sphingomyelin (Brady & Koval, 1958), cholesterol (Popjak, Gosselin, Gore & Gould, 1958;

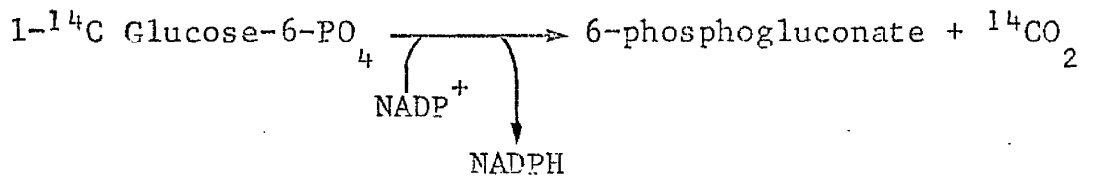
Tchen & Bloch,1957), and steroids (Hayano,Saba, Dorfman & Hecter, 1956; Ryan & Engel,1957). It also serves as a coenzyme in reduction of folic acid to tetrahydrofolic acid (Hatefi, Osborn, Kay & Huennekens, 1957) and is capable of reducing disulphidic bonds (Lynn, Earnhardt & Brown, 1961).

Thus through a reduced level or a complete absence of DHA in the urine which is extrapolated to low secretion rates Kolbel et al (1965b) suggest that certain biosynthetic pathways are stimulated, resulting in:- 1) increased fat synthesis and deposition causing obesity; 2) elevated plasma lipids as displayed in toxaemia of pregnancy; 3) elevated blood glucose levels through excessive reduction of the disulphidic bridges (-S-S- → -S-H, H-S-) joining chains A and B of the insulin molecule, therefore, deactivating the hormone; 4) elevated plasma uric acid levels, caused by excess tetrahydrofolic acid stimulating overproduction of purine the precursors of uric acid.

The above responses, if mediated through a lack of DHA will all be, to a certain extent, dependent on blood levels of DHA. Yet Kolbel et al in all their communications give little or no reference to DHA blood levels, which are very small relative to blood dehydroepiandrosterone sulphate (DHA.S) levels, and would appear to be inadequate for the inbitory task suggested. For, according to Lopez-S, Krehl & Woon (1967), they only obtained 13% reduction of red blood cell G-6-P.D. activity after oral administration of 100 mg of DHA per day for 3 days, and from their

calculation based on observations of Sjovall, Sjovall, Maddock & Horning (1966) oral consumption of 100 mg would elevate blood DHA levels 80μmoles/100 ml to a blood level very unlikely to be met with under normal or pathological conditions. Kischner, Lipsett & Collins (1965) reported DHA levels of 1.0 and 1.3μg/100 ml for females and males respectively, while Plager (1966) reported DHA.S levels of 46 and 100 mg/100 ml for females and males respectively. Thus, although DHA.S does not inhibit G-6-P.D. (Tomkins, Yielding & Curran, 1961) it is interconvertible with DHA (Chapdelaine, MacDonald, Gonzalez, Gurpide, Vande Wiele & Lieberman, 1965) and, therefore, is a potentially more powerful inhibitor of G-6-P.D. than DHA.

Tsutsui, Marks & Reich (1962) neglected blood levels and examined inhibition of G-6-P.D. by DHA at the site of its biosynthesis, namely the adrenal cortex. They studied the effect of exogenous DHA on whole rat adrenal homogenates incubated with $NADP^+$ in concentrations which did not limit the rate of oxidation of 1-^{14}C glucose or 1-^{14}C glucose-6-phosphate to $^{14}CO_2$ oxidation of 1-^{14}C glucose or 1-^{14}C glucose-6-phosphate to $^{14}CO_2$ and found a decrease in the rate of formation of $^{14}CO_2$ and generation of NADPH.



When the concentration of $NADP^+$ was sufficiently low to limit the

rate of oxidation, addition of DHA stimulated the rate of formation of $^{14}CO_2$ and NADPH. The authors conclude that it is possible that DHA and perhaps related steroids which can influence the rate of generation of NADPH, may, thereby affect the biosynthesis of steroids in the adrenal gland.

On the question of the relationship, if any, of absence of urinary DHA to the aetiology of hypertension Kolbel et al (1965a) offer two explanations. Firstly, "NADPH is essential in the synthesis of aldosterone. Cohen & Crawford (1963) showed that a sodium free diet enhanced the intensity of the pentose phosphate cycle in the adrenal cortex, and that overproduction of NADPH is connected with overproduction of aldosterone." Secondly, "over-production of NADPH in DHA deficient tissues could also maintain a larger quantity of sulphydryl groups of amino acids in the reduced state in the smooth muscle of the arterial wall. If these groups are important for the action of sympathomimetic amines (Trinus, 1961), oversensitivity to physiological amounts of these compounds could result".

Villee & Hagerman (1959) have shown that 20α and 20β-hydroxy-cortisol all stimulating G-6-P.D. activity, the hypertensive patient will have adrenals particularly efficient at hydroxylating steroids. One would, therefore expect hypertensive patients to display excessive production of polar steroids. This is substantiated by the work of Kornel & Motohashi (1965) and Kornel & Takeda (1967) who demonstrated elevated levels of polar steroids in plasma and urine of hypertensive patients.

B. Salt Metabolism.

1. Salt retention:

It is not the author's intention to review the literature pertaining to the influence of the mineralocorticoid aldosterone on salt metabolism, but to point out that in any investigation or discussion relating to salt metabolism, the role of aldosterone must surely always merit major consideration. Aldosterone, secreted by the zona glomerulosa of the adrenal cortex, plays a major role in the hormonal control of sodium and potassium levels in the body. Its secretion is under the control of the renin-angiotensin system, plasma potassium level and to a lesser extent pituitary ACTH. All aspects of the present knowledge concerning aldosterone, including biosynthesis, regulation of secretion, mechanism of action and influence on salt metabolism are amply covered in the following reviews - Poss (1964), Baulieu & Robel (1964), Peart (1965), Mulrow (1967) and ~~Colgan~~ Coghlan & Blair-West (1967).

2. Salt excretion:

The isolation of the salt retaining compound aldosterone in 1953 by Simpson, Tait, Wettstein, Neher, E~~we~~ & Reichst~~ie~~ein at once stimulated a renewed search for a hormonal steroid with the reverse action, that is, one which stimulated sodium diuresis. As early as 1948, Wilkins & Lewis suggested the existence of such a steroid to explain the excessive salt loss seen in some cases of congenital adrenal hyperplasia. However, as yet, there has been no success

in finding such a hormone, despite active research by many investigators. Nevertheless, a great many and often independent observations all suggest that such a substance does exist.

It is well known that patients with Addison's disease are sensitive to small doses of DOC, and readily develop excessive sodium retention if the dose is too large, whereas normal subjects will frequently resist very large doses of DOC with only slight increases in sodium retention. This suggests the presence of some substance in the normal subject which is capable of antagonising the sodium retaining properties of DOC. Since patients with Addison's disease have virtually no adrenal glands, the source of the substance or some precursor of it is probably the adrenal gland.

Fraser (1967) in a normal male subject showed that sodium restriction induced a gradual decrease in urinary sodium well before increases in plasma aldosterone levels were observed. This confirms the work of Leutscher & Lieberman (1958) who followed urinary sodium and aldosterone levels of normal subjects on low salt intake and observed salt retention before urinary aldosterone levels increased, suggesting a decreased production of some sodium excreting factor prior to the increase in aldosterone production.

An inverse relationship exists between the amount of sodium excreted in the urine and the excretion (Thorn, Ross, Crabbe & Van't Hoff, 1957) or secretion (Laragh et al, 1966) of aldosterone. This relationship, however, ceases to be significant at physiological

levels of aldosterone exretion that cover the range of normal circumstances of salt and water equilibrium (Thorn et al, 1957). This suggests that, under normal conditions, aldosterone does not regulate the amount of sodium excreted into the urine. A further observation lending support to this conclusion is the two hour delay before an effect of aldosterone on the kidney is observed in man. This data suggests that aldosterone is not responsible for quick changes in sodium excretion. Further, Crabbe, Ross & Thorn (1959) have shown that the amount of aldosterone excreted in the urine, and presumably the amount secreted, rises to a maximum in response to a low sodium diet, but often falls again towards, and in some subjects to, the level found before the diet was administered. Despite this, sodium excretion falls exponentially and continues at a level equal to, or below, the intake. These observations suggest a dual control of salt homeostasis with aldosterone regulating retention and some other substance regulating excretion of sodium.

In the adrenal gland of the newborn baby the ratio of C_{19} to C_{21} steroids produced is very high. This is thought to be due to high production of C_{19} steroids by the large foetal zone and minor production of hydroxycorticosteroids by the small adult zone. This production ratio of C_{19}:C_{21} steroids very quickly decreases over the first three weeks of life as the foetal zone involutes and the adult zone grows (Prader, 1967). Yet, under these circumstances of minimal C_{21} steroid production, New, Miller &

Peterson (1966) using a double isotope derivative technique report very high urinary levels of free aldosterone and normal levels of the tetrahydroglucuronide and the 3-oxoconjugate of aldosterone, all calculated per square metre of body surface area and compared with the respective levels in adults measured in the same units. The very high free urinary aldosterone levels could possibly be explained by postulating active secretion of a salt excreting hormone by the foetal adrenal zone and the elevated aldosterone necessary as a compensatory mechanism to maintain salt balance, with the urinary aldosterone level decreasing to normal as the excessive secretion of the salt excreting hormone declines with the involution of the foetal zone. Since the foetal zone is producing primarily C_{19} steroids the salt excreting hormone could possibly be one of these.

From a different direction comes suggestive evidence for the existence of a sodium excreting hormone. In the earlier days of aldosterone investigation, bioassay was necessary for the estimation of aldosterone levels. Several groups at the same time including Leutscher, Deming, & Johnson (1952), Singer & Venning (1953) and Cope & Garcia-Llaurado (1954) all experienced difficulty in carrying out satisfactory bioassays of the sodium retaining factor present in urine extracts. Typical of these are the findings reported by Singer & Venning (1953). Urine, collected from two patients with nephrosis, over time periods ranging from

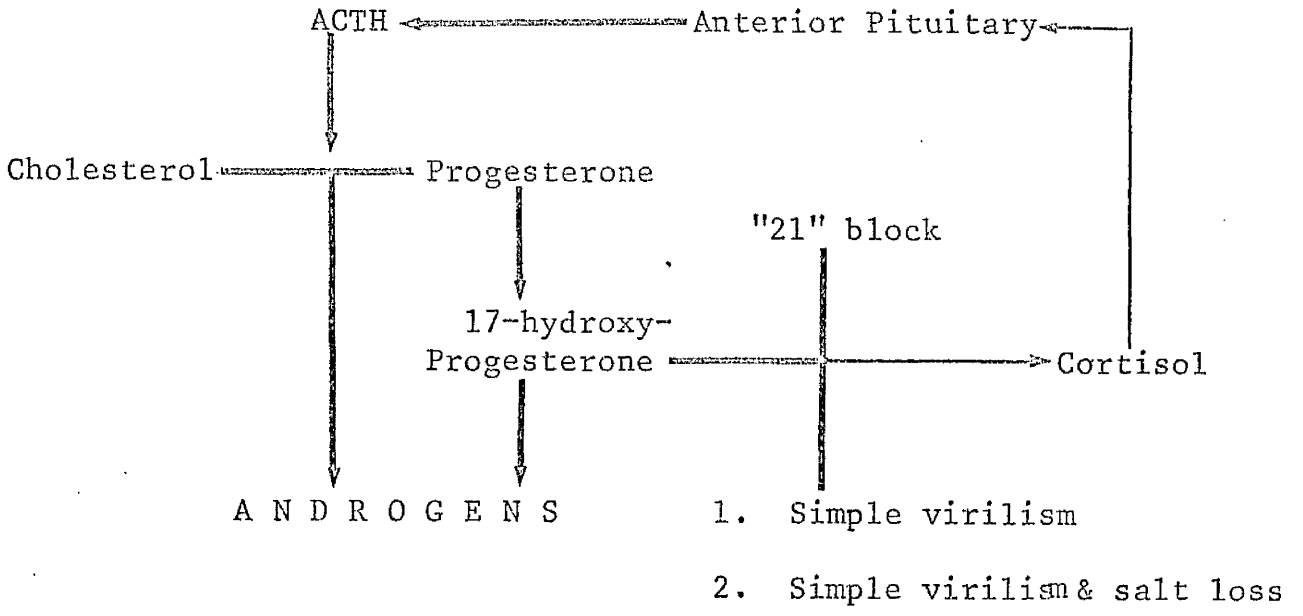
5 to 40 minutes, was extracted and injected into sodium ^{24}Na loaded, adrenalectomized rats and the ^{24}Na excretion measured. The results indicated that the effect of the sodium retaining factor extracted from the urine was being inhibited. For example, of the extracts of 10, 20 & 30 minute urine collections from one patient the extract of the 20 minute urine volume showed maximum sodium retaining ability while the 10 and 30 minute urine extracts showed equal sodium retaining ability.

To some extent in these earlier studies difficulties were due to impurities in the urinary extracts, and a better dose response curve could be obtained if the extracts were partially purified. However, this was not always so and Dryenfurth & Venning (1959) suspected that the interference was caused by a substance antagonising the action of aldosterone present in the extracts, and preventing the development of a proper dose response curve. As further evidence they found some urine extracts which seemed actually to increase sodium output when injected into adrenalectomized mice.

The original concept of a salt excreting hormone stems from studies on infants with congenital adrenal hyperplasia of the salt-losing type. Congenital adrenal hyperplasia, a rare disease appearing in infants, has virilism as one of its cardinal features, caused by excessive androgen production. The association of a clear and severe salt losing state with some cases of congenital adrenal hyperplasia had been recognised by

clinicians for many years. It seems to have been first reported by Butler, Ross & Talbot (193) and Wilkins, Fleischman & Howard (1941). Darrow (1944) drew attention to the paradoxical situation in some of these patients, who while manifesting a severe hypoadrenal state had grossly hyperplastic adrenal cortices and he also described a patient in Addisonian crisis who needed large quantities of DOC for full restoration. Darrow suggested that the excessive androgen production characteristic of this disease may in some way be interfering with sodium and potassium metabolism. In 1949 Lewis & Wilkins were able to show that ACTH administration to children with congenital adrenal hyperplasia, resulted in a large rise in the already high urinary excretion of 17-oxosteroids, but there was only slight increase in the already low excretion of corticosteroids. This together with the tendency to enter Addisonian crisis, strongly suggested an inadequate production of corticosteroids in this disease. Until 1950 attempts to treat congenital adrenal hyperplasia by medical or hormonal means had no success, then Wilkins, Lewis, Klein & Rosemberg in 1950 discovered that the characteristically elevated urinary 17-oxosteroid levels found in this condition could be promptly lowered to normal levels by treatment with cortisone. Kelley, Eik & Raile (1952) reported that plasma 17-hydroxycorticosteroids were abnormally low in untreated cases of this disorder and that no rise in plasma corticosteroids levels resulted from ACTH administration. Syndor,

Kelley, Raile, Elk & Sayers (1953) were able to show that elevated and measurable amounts of ACTH were present in the plasma of untreated patients, but could not be detected after cortisone treatment. Credit for the first recognition of the nature of the defect in this disease goes to Bartter, Albright, Forbes, Leaf, Dempsey & Caroll (1951) who explained that excessive ACTH is being produced in these infants and inducing adrenal hyperplasia which results in grossly elevated urinary 17-oxosteroid levels, presumably from excessive adrenal androgen production. However, despite this hyperactivity insufficient cortisol is being produced to suppress pituitary ACTH secretion and the pituitary, in turn, secretes more ACTH and induces even greater adrenal hyperplasia, but still very little cortisol. A form of vicious circle is thus created. After treatment with cortisone plasma cortisol levels rise and the secretion of pituitary ACTH is inhibited, adrenal hyperactivity declines as does urinary 17-oxosteroid levels and on continued cortisone therapy adrenal function apparently returns to normal. This transition can take place in 24 to 36 hours, but unfortunately does not always cure salt-loss. Elucidation of the actual metabolic block responsible for the failure to synthesise cortisol is attributed to Dorfman (1954) and Eberleim & Bongiovanni (1955) who suggested that the primary defect in the disease was a lack of the "21 hydroxylase" enzyme. This might be illustrated as follows:-



About a third of the patients with congenital adrenal hyperplasia are salt-losers. The pathogenesis of this phenomenum is not fully understood. Three major hypotheses have been advanced to explain it.

The first was suggested by Crigler, Silverman, & Wilkins (1952), who observed an increase in sodium diuresis in patients treated with ACTH (Wilkins, Klein & Lewis, 1950), as well as a decreasing requirement for DOCA when treated with cortisone. These findings supported the earlier observation of Darrow (1944) who had noted that patients with salt-losing congenital adrenal hyperplasia required amounts of DOCA or salt, or both, which were larger than those needed by Addisonian patients and of Barnett

& McNamara (1948) who had shown that the electrolyte disturbance in adrenal hyperplasia was different from that observed in patients with Addisons disease. All these findings were interpreted as suggesting the secretion of a salt-losing hormone or the secretion of a pattern of steroids conducive to salt loss.

Another explanation was advanced by Bongiovanni & Eberlein who pointed out that both the salt-losing and the non-salt-losing forms of congenital adrenal hyperplasia are caused by the same hydroxylation defect of the adrenal cortex, however, in the salt-losing form the defect is almost complete (Eberlein, 1958). They suggested that "a minimal amount of hydrocortisone is required for aldosterone to exert its metabolic action in man" (Eberlein & Bongiovanni, 1958). Thus, although untreated salt-losing patients may be producing normal amounts of aldosterone, its action is not manifested, because only negligible amounts of cortisol are being produced.

Blizzard, Liddle, Migeon & Wilkins (1959) studied aldosterone excretion in patients with congenital adrenal hyperplasia and found low to normal aldosterone excretion levels in salt-losing patients with no increase on salt deprivation. Most of the patients with the non-salt-losing form of the disease had an increased excretion of aldosterone with a further increase on salt deprivation. These findings were interpreted as indicating that the salt loss was due to inadequate aldosterone production.

Numerous investigators have studied the excretion and secretion of aldosterone in patients with congenital adrenal hyperplasia, but the results are as yet inconclusive. Von Prader, Spahr & Neher (1955) reported two cases of simple virilizing adrenal hyperplasia in adults and four cases of salt-losing adrenal hyperplasia in children, all of whom showed normal or greater than normal excretion of the 3-oxo conjugate of aldosterone. Leutscher & Curtis (1955) found normal or slightly increased excretion of aldosterone in patients with the salt-losing syndrome. Jailer, Ulich & Lieberman (1959) estimating aldosterone secretion rates by an isotope dilution technique, found two salt-losers to be within the normal range and two out of three simple virilisers to have elevated secretion rates and in the salt-losers sodium loss and adrenal crisis occurred after ACTH administration. Rosemberg, Dufault, Bloch, Budnitz, Butler & Brem (1960) described a child with salt-losing adrenal hyperplasia studied at 18 months and again at $4\frac{1}{2}$ years who excreted normal to high levels of aldosterone, which increased on sodium restriction and ACTH administration, and yet was still coincident with a negative sodium balance. Recently Visser & Degenhart (1967) described a new born infant with salt-losing adrenal hyperplasia who had an elevated aldosterone secretion rate (280 µg/day) on normal sodium intake with an increase on sodium restriction to 570 µg/day. Fortunately treatment with

cortisone corrected the salt loss and probably induced a decrease in aldosterone secretion.

On the other hand, Lieberman & Leutscher (1960) demonstrated subnormal aldosterone excretion in infants with salt-losing adrenal hyperplasia while clinical signs of salt depletion were evident and Bryan, Kliman & Bartter (1962) using a double isotope derivative method found extremely low aldosterone production rates in 5 salt-losers. Mattox and Lewbart (1959) found excretion of the 3-oxoconjugate of aldosterone in 25 children to range from 0.7 to 18 mg per m^2 per day. Six patients with simple virilising adrenal hyperplasia and six out of 7 salt-losers fell within the normal range, however, the salt-losers showed no change on salt deprivation. Kowarski, Finkelstein, Spaulding, Holman & Migeon (1965) measured aldosterone secretion rates in two adult simple virilizers and five salt-losers. In the adults, aldosterone secretion rates were above normal and could be further increased by salt restriction, though treatment with glucocorticoids brought the secretion rates within the normal range. In the patients suffering from salt-loss,aldosterone secretion rates were below normal in severe cases and normal in milder cases. However, they showed no increase on salt restriction. Finally, New et al (1966) in an extensive study measured the excretion of free aldosterone, the 3-oxoconjugate of aldosterone and tetrahydro-aldosterone glucuronide in 40 normal infants and 28 infants and

children suffering from congenital adrenal hyperplasia, 12 of whom were salt-losers. When corrected for surface area, the excretion of the 3-oxoconjugate of aldosterone and tetrahydro-aldosterone glucuronide for normal infants and children with simple virilism were within the range for normal adults, though in several cases of simple virilism values for the two aldosterone metabolites were quite high. In the cases of adrenal hyperplasia with salt-loss values tended to be very low, though for 5 of the 18 cases the excretion of the 3-exoconjugate of aldosterone was well within the normal range.

Without forming any definite conclusions as regards the secretion and excretion of aldosterone in congenital adrenal hyperplasia, it would appear that in many of the salt-losing patients there is normal production of aldosterone and in some of these patients there is also a normal aldosterone response to sodium restriction. The abnormal salt metabolism in these patients provides strong evidence for a salt-losing steroid. Further, in many of the cases of simple virilism there appears to be a higher than normal secretion of aldosterone, this is also suggestive of the existence of a salt-losing steroid.

Several steroids have been proposed for the role of a salt-losing hormone. Two of them are progesterone and 17α-hydroxyprogesterone and the following theory on their mode of action in this disease has been cited by several authors (Jacobs, Van der Poll, Gabrilove & Soffer, 1961; Degenhart, Hendrick, Visser,

Wilmink & Croughs, 1965 and Kowarski et al, 1965). There is a 21-hydroxylation defect in both the salt-losing and non-salt-losing forms of congenital adrenal hyperplasia, and it affects both cortisol and aldosterone formation because they both require a 21-hydroxylation for their biosynthesis. Since cortisol secretion is many times greater than that of aldosterone, a limited 21-hydroxylation deficiency might lower the production of the former without affecting the secretion of the latter. The 21-hydroxylation deficiency is always more marked in the salt-losing patients, although never complete. This has been satisfactorily demonstrated by the estimation of cortisol production rates (Kenny, Malvaux & Migeon, 1960 and Degenhart et al, 1965).

An increased production of various cortisol precursors is a constant feature of congenital adrenal hyperplasia. Two of these, progesterone and 17α-hydroxyprogesterone are aldosterone antagonists (Landau, Lugibihl, 1958 and Jacobs et al, 1961) and have been shown to be capable of bringing about negative sodium balance in DOC treated patients suffering from Addison's disease. The non-salt-losing patients have a relatively milder 21-hydroxylase defect and are, therefore, able to compensate for these antagonists by an increased secretion of aldosterone. However, in patients with a greater enzyme deficiency, aldosterone production is also affected, and they are unable to compensate

for the presence of these antagonists, and thus develop negative sodium balance.

Cortisone treatment of patients with non-salt-losing congenital adrenal hyperplasia lowers the secretion of these cortisol precursors and through this brings about a decreased aldosterone secretion in these patients. Similarly, in the mild salt-losers, cortisone treatment alone can sometimes control the electrolyte disturbance (Wilkins, 1965). From these facts one could theorise that mild salt-losers have an aldosterone secretion in the normal range when untreated and treatment with cortisone, by lowering the secretion of the antagonists, enables them to achieve sodium homeostasis, whereas in patients with an almost complete 21-hydroxylase deficiency, aldosterone secretion is insufficient even when the production of antagonists are lowered by treatment with cortisone. Further, an increased urinary sodium excretion after administration of ACTH has been observed in both salt-losing and non-salt-losing forms of congenital adrenal hyperplasia (Lewis & Wilkins, 1949; Wilkins, Klein & Lewis, 1950; Jailer, Louchart & Cahill, 1952 and George, Saucier & Bartter, 1965) and even during therapy with DOCA, patients of the salt-losing type show this increased excretion of sodium after ACTH administration (Wilkins et al, 1950). This sodium loss induced by ACTH administration is attributed to an even greater overproduction of cortisol precursors, which antagonise

the more modest ACTH induced increase in aldosterone.

The above theory holds only if the production of the aldosterone antagonists progesterone and 17α-hydroxyprogesterone are high enough to inhibit the sodium retaining properties of aldosterone. An estimate of how high this production would need to be may be calculated from the results of Landau & Lugibihl (1958) who, in acute studies on two adrenal deficient subjects demonstrated that 150 mg of progesterone nullified the sodium retaining effect of 20 μg of aldosterone and from the results of Kowarski et al (1965) who reported the mean aldosterone secretion rate in patients with non-salt-losing congenital adrenal hyperplasia as 290 μg/day and in normal as 100 μg/day. Thus application of the estimate of the former group to the aldosterone secretion rates of the latter group indicates that 290-100 = 190 μg of aldosterone are secreted by the non-salt-losing patients to ameliorate the inhibition caused by the overproduction of the cortisol precursors. If we equate the inhibitory potency of progesterone and 17α-hydroxyporgesterone then the combined total secretion necessary to nullify 290 μg of aldosterone would be 1,425 mg. Further, in the studies reported where the patients are salt-losers, secreting normal amounts of aldosterone and who continue to lose sodium during salt restriction even though their aldosterone secretion has increased, such as the case by Visser & Degenhart (1967), the secretion of these two

steroids progesterone and 17α-hydroxyprogesterone would have to be very high to inhibit the action of aldosterone which in the case cited was 380 mg secreted per day.

Of the two corticosteroids progesterone and 17α-hydroxyprogesterone, secretion rates have been estimated, in this disease, for only 17α-hydroxyprogesterone. Fukushima, Bradlow, Hellman, Zumoff & Gallagher (1961) reported the daily secretion rate of 17α-hydroxyprogesterone to be 240-280 mg/day in congenital adrenal hyperplasia and 3 mg/day in normals. Assuming progesterone to have a secretion rate comparable with 17α-hydroxyprogesterone, and this is probably an overgenerous assumption, then, the combined secretion rates are still insufficient to achieve the degree of antagonism of aldosterone postulated by the above theory.

A search for the active steroid in urine of a patient with salt-losing type of congenital adrenal hyperplasia was made by Neher, Desaulles, Vischer, Wieland & Wettstein (1958). Large volumes of this patient's urine were extracted, concentrated and fractionated, the successive stages of concentration of the hypothetical sodium excreting factor being controlled by bioassay on rats. From this and from hog adrenals they finally obtained a potent fraction from which a crystalline steroid was eventually isolated. It proved to be 3β, 16α-dihydroxyallopregnane-20-one. Cope & Parry (1959) tested this compound on patients recovering from congestive cardiac failure with oedema, this state being

considered as one of the most favourable for the demonstration of any sodium excreting action possessed by this steroid. No effect on the sodium or potassium excretion of these patients could be detected. Negative results were also obtained by Coppage, Grant & Liddle (1960) who tested the compound on 5 subjects using doses up to 400 mg.

It is possible that the active substance was lost in the latter stages of isolation, and that the steroid isolated was an impurity having similar chromatographic properties. Nevertheless, it is of interest that the steroid contained a 16-hydroxyl group for this functional group is known to influence the sodium retaining actions of the synthetic analogues of cortisone. It is the basis, for instance, of the reduced sodium-retaining action of triamcinolone. A methyl group at C-16 also seems to be equally potent in affecting sodium retaining properties, this being seen in dexamethasone. It is possible, therefore, that the steroid isolated by Neher et al (1958) was structurally similar to some active steroid present in the extracts which also had sodium excreting action and also possessed a 16-hydroxyl group.

Klein, Taylor, Papadatos, Laron, Keele, Fortunato, Byers & Billings (1958) collected urine from premature ACTH treated infants and from two new born infants with salt-losing congenital adrenal hyperplasia. When chloroform extracts of the urine, after hydrolysis, were run on toluene: propylene glycol system,

the eluate from the zone between the levels of tetrahydro-A and 11-deoxycortisol was the only one which significantly affected sodium excretion. A significant increase in sodium excretion which followed a dose response relationship, could be induced, and in addition to this, the extracts seemed to be more potent after ACTH stimulation. Unfortunately the rats used in these experiments were intact, not adrenalectomized, so that interpretation of observations made is obscured. More reliably assayed, because adrenalectomized rats were used, were the extracts investigated by Rosemberg et al (1960). They used the urine of a $4\frac{1}{2}$ year old child with salt-losing congenital adrenal hyperplasia. The urine was hydrolysed enzymatically, extracted and run on paper in a toluene: propylene glycol solvent system, the zone between corticosterone and 11β-hydroxy-androst-4-ene-3, 17-dione was eluted crystallised and identified as being mainly 17α-hydroxypregnenolone. Twenty mg were obtained, 50µg of which nullified the sodium retaining properties of 25µg of DOC in the rat. However, it was believed that the DOC antagonising effect was due to an accompanying impurity.

All the steroids so far considered for the role of the salt-losing hormone in congenital adrenal hyperplasia have been C_{21} steroids. Yet the disease's most prominent characteristic is virilism, caused by excessive production of adrenal "androgen" (C_{19} steroids). Darrow (1944) as a result of studies on a severe

salt-losing infant with congenital adrenal hyperplasia suggested that the excessive androgen production may in some way be interfering with sodium and potassium metabolism. However, this suggestion was treated lightly as it had been known since the work of Thorn & Engel (1938) that male sex hormones cause salt retention, not excretion. However, this does not exempt all C_{19} steroids. Further, if the hypothetical steroid is present in normals it will only clearly manifest itself when overproduced, and in congenital adrenal hyperplasia where there is a 21-hydroxylase block the compounds shown to be markedly elevated in the urine are the C_{19},17-OS. Speculating that one of the steroids in the adrenal "androgen" biosynthetic pathway does possess salt excreting properties it would have to satisfy the available clinical evidence indicating the presence of a salt-losing hormone. Assuming that changes in total urinary 17-OS mirror changes in the hypothetical salt-losing steroid, this can be shown to be the case. In congenital adrenal hyperplasia it would be produced in amounts greatly in excess of normal; this has been known since earliest investigations into this disease, where urinary 17-OS were always markedly increased. In fact Klein proposed that elevated urinary 17-OS be one of the criteria for diagnosis of this disease. Further 17-OS are decreased to normal levels by cortisone therapy, treatment which is known to bring salt-losers into sodium balance and lower the elevated aldosterone

secretion rates in the non-salt-losers to within the normal range. Also 17-OS are produced in greater amounts after ACTH stimulation, treatment which has been shown to aggravate the salt-loss in the salt-losing types and to induce salt-loss in the non-salt-losing type.

Evidence other than from the congenital adrenal hyperplasia syndrome which indicates a C_{19} steroid as the salt excreting factor is available. As mentioned earlier it is possible that the elevated urinary levels of free aldosterone in the new born infant could be explained by secretion of a salt excreting C_{19} steroid by the foetal adrenal zone, the elevated aldosterone acting as a compensatory mechanism to maintain salt balance.

Added to this evidence are the findings of Williamson (1965) who reported that the two C_{19} steroids DHA and androstenedione could increase sodium excretion when administered to water loaded rats and when natiuretic doses of these steroids were given to saline-loaded adrenalectomized rats they were still effective as natiuretic agents suggesting an action independent of aldosterone. However, very large doses, 80 mg of DHA or 100 of androstenedione per kg dissolved in sesame oil and injected subcutaneously were necessary to nullify the sodium retaining properties of 0.13 µg and 0.12 µg of aldosterone respectively. The type of antagonism involved here in the

inhibition of aldosterone is probably physiologic, that is having the same target organ as the compound they inhibit, but not necessarily the same site in this organ or even the same cell type. That it was physiologic antagonism was concluded from the fact that DHA and androstenedione increased sodium excretion in adrenalectomized rats.

C. Uric Acid, Lipids, Steroids and Vascular Disease.

1. Hyperuricaemia, gout and hypertension:

Random population sample studies of the prevalence of gout have shown an incidence of 0.3% and 0.05% for Caucasian males and females (Kellgren, Lawrence & Aitken-Swan, 1953); 2.5% for male Filipinos (Decker & Lane, 1959); and 4.2% for Maori males and females (Lennane, Rose & Isdale, 1960). The incidence of hyperuricaemia in healthy populations is similarly racial dependent, tending to be higher in certain pacific races (Bremner & Lawrence, 1966). In Caucasians the incidence has been estimated between 4.5% and 15% (Talbott, 1957; Hauge & Harvald, 1955; Popert & Hewitt, 1962 and Decker, Lane & Reynolds, 1962).

A striking association between hyperuricaemia and hypertension was recognised in a family with a unique pedigree (Duncan & Dixon, 1960). The father and six of seven siblings had hyperuricaemia , while the mother and all the siblings had raised blood

pressure. One must, therefore, enquire whether a high serum uric acid (S.U.A.) is common in patients with hypertension. Breckenridge (1966) reported elevated S.U.A. levels in 274 (58%) of 470 hypertensive patients and of 333 of the 470 not on anti-hypertensive therapy 90 (27%) were hyperuricaemic. This prevalence of hyperuricaemia in patients with idiopathic hypertension has been cited by several other authors. Kinsey, Walther, Sise, Whitelaw & Smithwick (1961) found that 183 (46%) of 425 hypertensive patients had a raised S.U.A. level. Approximately half of these patients were untreated and of these 33% had a raised S.U.A. Cannon, Stason, Demartini, Sommers & Laragh (1961) found that 43% of 141 patients with idiopathic hypertension, 44% of 52 with renal hypertension, 75% of 24 with malignant hypertension, and 38% of all untreated hypertensives had hyperuricaemia. Itakovitz & Sellers (1963) observed gout in 11 (10.5%) of 105 patients, and hyperuricaemia in 67% of 56 patients, after all of them had been subjected to adrenalectomy and sympathectomy as treatment for severe hypertension. Also, as previously mentioned a high per cent of hypertensive patients studied by Kolbel et al (1965a) were hyperuricaemic and 5 of the hyperuricaemic hypertensives reported by Kolbel et al (1964) had manifest gout and another had a typical acute gouty arthritic attack during treatment with chlorothiazide.

Some of the drugs used in the treatment of hypertension,

particularly benzothiadiazine diuretics, will elevate S.U.A. levels (Laragh, 1958). These diuretics, however, affect uric acid excretion in two ways. At low plasma levels, they act on the renal tubules to cause uric acid retention (Dinon, Kim & Van der Veer, 1958) but at high plasma levels they cause increased uric acid excretion and lower the S.U.A.(Demartini, Wheaton, Healey & Laragh, 1962). Of the other drugs used in the treatment of hypertension, only ganglion - blocking drugs raise S.U.A. (Dollery, Duncan & Schumer, 1960). Neither guanethidine (Fry & Barlow, 1962) or methyldopa (Daley & Evans, 1962), which are widely used in treatment of hypertension, will induce a rise in S.U.A.

Three possible conclusions can be drawn from the association of hypertension with hyperuricaemia: first hypertension may arise as a result of hyperuricaemia; second, hypertension may cause hyperuricaemia; and third, there may be some other factor common to these two conditions (Breckenridge, 1966). The first view was put forward by Duncan & Dixon (1960) who described a family comprising a father, mother, and seven children. The father had hyperuricaemia but not gout; the mother was hypertensive, 6 of the 7 children had raised S.U.A.; all seven developed renal disease and hypertension from which three of the children later died. This is a rare pedigree and one hesitates to think of the problem of hypertension in hyperuricaemic individuals in such dramatic terms.

Progressive renal disease is a common cause of death in patients with advanced tophaceous gout, the incidence was shown to vary from 22% to 30% (Mayne 1956; Talbott & Terplan, 1960) and many of these patients were also hypertensive. In gouty patients without advanced tophi, however, renal failure and hypertension are rare. Gout is widely recognised by life assurance companies as a benign condition (Talbott & Lilienfeld, 1959). Thus, it has been shown that physicians treating hypertension frequently observe hyperuricaemia, but those concerned with treatment of gout rarely find severe hypertension.

As regards the possibility that hypertension is the cause of the raised S.U.A., it is thought that hyperuricaemia can result either from overproduction or from underexcretion of uric acid. Reliable evidence of overproduction is gained from measurement of miscible uric acid pool size and uric acid turnover rates using isotope dilution techniques (Benedict, Forsham & Stetten, 1949). Of 4 hyperuricaemic hypertensive patients studied by Breckenridge (1966) none had evidence of overproduction of uric acid. In all four patients the turnover rate of labelled uric acid was within normal limits, while the miscible uric acid pool size was increased. Breckenridge suggested that these findings indicated a failure in uric acid excretion and that this was responsible for the elevated S.U.A. levels.

Blood uric acid is completely filtered by the kidney glomeruli, then reabsorbed by the proximal tubules and is finally secreted by the distal tubules to give the uric acid which appears in the urine (Gutman, Yu & Berger, 1959; Poulsen & Praetorius, 1954 and Lathem, Davis & Rodnan, 1960). The results of studies by three independent groups indicate that the mechanism of the hyperuricaemia in hypertension is a renal tubular abnormality of uric acid handling (Itskovitz & Sellers, 1963; Cannon et al, 1966 and Breckenridge, 1966). The latter author studied renal handling of uric acid by three groups, each of 8 subjects, the first group was normouricaemic normotensive individuals, the second group normouricaemic hypertensive patients and the third group hyperuricaemic hypertensive patients. All the hypertensive patients showed reduced glomerular filtration rates. The excretion of uric acid and the uric acid clearance were lower in all the hypertensive patients than in the normal group. In the hypertensive hyperuricaemic patients, however, although the glomeruli filtered load was higher than in the other two groups of patients, the uric acid clearance was lower. When the uric acid clearance was expressed per 100 ml of glomerular filtrate, there was no significant difference between the normal subjects and the normouricaemic hypertensive patients, but the difference between those groups and the hyperuricaemic hypertensive patients was significant and suggested

to Breckenridge a renal tubular abnormality. These results basically agree with Cannon et al (1966), but these investigators further observed that, though many hyperuricaemic hypertensive patients had relatively normal rates of glomerular filtration, they still had reduced uric acid clearances and the investigators suggested renal tubular transport as the cause and most probably reduced tubular secretion.

Tissue hypoxia has been suggested as the cause of the abnormal tubular handling of uric acid in hypertension (Itskovitz & Sellers, 1963 and Cannon et al, 1966). In support of this: high levels of lactic acid have been demonstrated in the venous and arterial blood of patients with renal and idiopathic hypertension (Demartini, Cannon, Stason & Laragh, 1965); infusion of lactic acid was shown to reduce renal excretion of uric acid in normal subjects. In addition, elevated blood lactic acid has been associated with reduced uric acid excretion and hyperuricaemia: in normal subjects during exercise (Ouick, 1935 and Nichols, Miller & Hiatt, 1951); in glycogen storage disease (Holling, 1963); and in toxaemia of pregnancy (Handler, 1960). However, Cannon et al (1966) could not satisfactorily correlate hyperuricaemia with hyperlacticacidaemia.

There is abundant evidence that elevated blood levels of uric acid in primary and secondary gout (Gutman & Yu, 1957 and Gonick, Rubini, Gleason & Sommers, 1965), familial hyperuricaemia

(Duncan & Dixon, 1960), and glycogen storage disease (Holling, 1963) may induce or accelerate kidney damage possibly through the effects of filtration and reabsorption of increased concentrations of uric acid. In addition, uric acid loading in a variety of animal studies has impaired renal function (Epstein & Pigeon, 1964 and Duncan, Wakin & Ward, 1965), and produced renal damage (Smith & Lee, 1957 and Duncan, Wakim & Ward, 1963). It has, therefore, been suggested that, once hyperuricaemia is established in hypertension, it contributes to the deleterious effects of hypertensive vascular disease in the kidney.

Support of this comes from several studies including Cannon et al (1966) who described changes in renal tissue obtained at post-mortem examination from selected patients who had clearly become hyperuricaemic during the course of their hypertensive disease. These changes were similar to those described by Gonick et al (1965) for "gouty" kidneys and they included glomerular capillary thickening and sclerosis, tubular atrophy, pigment deposits, and degeneration especially in the loops of Henle and extensive interstitial deposits of uric acid in renal medullary tissue. These lesions are not ordinarily seen in kidney sections from hypertensive patients without hyperuricaemia (Sommers, Relman & Smithwick, 1958).

Of possible interest in the aetiology of hyperuricaemia in hypertension are the recent findings of Ferris & Gorden (1968), who studied the effect of angiotensin and noradrenaline upon

urate clearance in man and found that quantities of these vasoconstrictors capable of raising the diastolic pressure 20 mmHg caused a marked reduction in uric acid clearance which could not be attributed to a reduced glomerular filtration rate, but appeared to be related to a decrease in effective renal blood flow. They suggested that these findings may be casually related to hyperuricaemia found in the various forms of hypertension.

Gout is classified among the inborn errors of metabolism (Garrod, 1931), but unlike the majority of such disorders no single specific metabolic defect has yet been found to account for hyperuricaemia characteristic of this disease (Seegmiller, Laster & Howell, 1963). Although impaired renal excretion of uric acid is responsible for the hyperuricaemia of some patients, an increased rate of purine biosynthesis de novo contributes to the hyperuricaemia in the majority of gouty patients (Seegmiller, Grayzel, Laster & Liddle, 1961). In affected members of certain families excessive production of purines is the sole cause of their hyperuricaemia (Seegmiller, 1962).

2. Urinary 17-oxosteroids in relation to gout and hyperuricaemia:

In 1949 Wolfson, Guterman, Levine, Conn, Hunt & Rosemberg, described 11 cases of gout who had urinary 17-OS levels of less than half of the minimal normal values. The decrease could not be related to the stage of disease, or the age of the patient

and since it was not uniformly present in non-gouty hyperuricaemia the authors concluded that it was not a necessary consequence of hyperuricaemia. Neither could the low urinary 17-OS levels be attributed to the arthritides since normal levels were found in mild rheumatoid arthritis. Two of the 11 patients were females and they had urinary 17-OS levels of less than 1 mg/day, suggesting that the deficiency in gout was of adrenal origin. The authors concluded that "in gout, biologic androgen activity is maintained by an androgenic hormone which does not make an important contribution to urinary 17-OS when metabolised".

Their results were confirmed by Robinson, Conn, Block, Louis & Katz (1949), but not by the studies of Butt & Marson (1952) who studied 33 gouty patients and found the urinary 17-OS levels to show no significant deviation from normal values. Sonka et al (1964) studied total and individual 17-OS in males and females suffering from gout. The total urinary 17-OS levels in all cases were in the lower limits of normal. Of the individual 17-OS, DHA was unique by its complete absence from the urine of 26 of the patients as compared to levels of 2.5 $\pm$ 0.46 mg/day in controls. A further 3 patients with uric acid stones, but no arthritis showed normal S.U.A. and urinary DHA levels, while one patient

with the same complaint had a high uric acid level and no urinary DHA. The only patient with gout who also excreted DHA had a normal S.U.A. level. All the patients were overweight and excreted much larger volumes of urine than controls. Sonka et al (1964) explained the relationship of high serum uric acid to absence of urinary DHA as follows:- " High uric acid content may be considered as a manifestation of enhanced purine biosynthesis ------ DHA acts as a remote inhibitor of purine biosynthesis by exerting its known inhibitory effect on G-6-P.D. This enzyme is utilised in the pentose phosphate cycle which produces NADPH. This in turn is required for the production of tetrahydrofolate, a necessary coenzyme in purine biosynthesis." The same authors (Kolbel et al, 1964) also reported low levels of total urinary 17-OS and the absence of urinary DHA in 26 hypertensive hyperuricaemic males (14) and females (12), 5 of whom had manifest gout and another had a typical gouty arthritic attack during the study.

The findings of Sonka et al (1964) and Kolbel et al (1964) were partly confirmed by Beck, Casey, Solmon & Hoffman (1967). In their studies 7 of 13 men with gout excreted no DHA, 4 excreted only a trace and the other 2 excreted DHA in normal concentrations. A further 5 men with other joint diseases, 3 of whom had normal S.U.A. levels, showed as a group very low levels of urinary DHA, only one of normouricaemic patients showing a normal urinary DHA level.

Evidence other than that from urinary 17-OS levels in gout suggests the existence of a relationship between uric acid metabolism and adrenal steroids. In 1948 Forsham, Thorn, Prunty & Hills demonstrated the now well known fact that ACTH administration markedly increases the urinary uric acid levels. In 50 normal subjects the increase in uric acid: creatinine ratio amounted to 87%; whereas in 30 patients with Addison's disease the mean increase was only 16%. Twenty mg of cortisol administered to patients with Addison's disease elicited a mean increase in uric acid: creatinine ratio of 48%. However, their experiments did not determine whether the increased uric acid excretion was through increased production or increased clearance of uric acid.

Thus it would appear that the bulk of the evidence would suggest some form of adrenal insufficiency in association with gout. Though in argument against this, it is noteworthy that gout has been reported on only one previous occasion in a patient with Addison's disease (Rudner & Bale, 1959), and not one case out of 77 patients with Addison's disease was found by Itskovitz & Sellers (1963).

3. Vascular disease:

The existence of a correlation between gout and obesity has been accepted since ancient times, the alleged common denominator being gluttony. The validity of this belief has

not been adequately tested and has been questioned (Talbott, 1964). Despite the long appreciated associations between obesity and atherosclerosis, studies of relationships between hyperuricaemia, with or without gout, and atherosclerosis have been undertaken only in recent years. The first systematic investigation of this kind was reported be Gertler, Garn & Levine (1951). They found hyperuricaemia to be present four times as frequently in a group of 92 men who had suffered a myocardial infarction than in control subjects. Subsequently, various investigators demonstrated correlations between hyperuricaemia and diseases caused by or associated with atherosclerosis: arteriosclerosis obliterons (Kramer, Rarilstein & De Madeiros, 1958), myocardial infarction (Eidlitz, 1961; Kohn & Prozan, 1959) cerebral thrombosis (Hansen, 1965; Meyer, Kypros & Gilroy, 1964), idiopathic hypertension (Breckenridge, 1966: Cannon et al, 1966; Kolbel et al, 1965a; Kinsey et al, 1961), diabetes mellitus (Weiss, Segaloff & Moore, 1957; Beckett & Lewis, 1960; Whitehouse & Cleary, 1966).

Elucidation of questions pertaining to relationships among hyperuricaemia, gout and these diseases requires more knowledge of roles played by other variables such as serum lipids and possibly steroids, than is now available.

(a) <u>The relationship between plasma lipids and vascular disease.</u>

It has been known for centuries that degenerative changes

in arteries are associated with the appearances of lipids in the lesions and Vogel (1847) discovered that cholesterol was a constituent of atheromatous plaques. Antischkow (1913) performed the experiment that has been performed countless times since, namely the feeding of cholesterol to rabbits, which produces lesions bearing a close similarity to certain lesions occurring spontaneously in man. Much of the older literature on this aspect of the subject is reviewed in a monograph (Cowdry, 1933).

The administration of cholesterol to the rabbit results in a marked plasma hypercholesterolaemia, and the severity of the arterial lesions produced in this species correlates fairly well with both the plasma cholesterol level and with the length of sustained hypercholesterolaemia. Using modified dietary techniques, this generalisation is true for various other species such as the rat (Wissler, Eilert, Schroeder & Cohen, 1954), dog (Steiner & Kendal, 1946), chick (Kesten, Meeker & Jobling, 1936) and many other birds and animals. Experimental atherosclerosis has been reviewed by Katz & Stamler (1953).

There is a great deal to support the general thesis that the plasma cholesterol concentration tends to be higher in individuals presenting with coronary artery disease than in normals matched for age, sex, weight, etc. (Morrison, Hall & Chaney, 1948; Steiner, Kendall & Mather, 1952; Technical group of the Committee on Lipoproteins and Atherosclerosis, 1956;

Stamler, 1960 and Kagan, Dawber, Kannel & Revotskie, 1962). The difference between the mean plasma cholesterol levels of the two groups is highly significant, but the overlap is great, and hence the plasma cholesterol level has a limited value as a prognostic test when applied to a given individual. Variations of this simple test have been proposed such as the cholesterol/phospholipid ratio, but once again there is little gain, if any, in discriminating between the total cholesterol and the cholesterol/phospholipid ratio (Ahrens & Kunkel, 1949).

Since almost all the plasma cholesterol is carried as lipoprotein complexes with α- and β- globulins, this led to the estimation of cholesterol in the α and β fraction and results expressed as the α/β ratio. This ratio was found to be reduced in patients with coronary artery disease (Barr, Russ, & Eder, 1951 and Oliver & Boyd, 1953), but the α/β lipoprotein ratio was no better as a predictor of atherosclerosis than the plasma cholesterol or plasma cholesterol/phospholipid ratio.

Perhaps one of the most significant contributions made by recent investigators is the demonstration that an increased sensitivity to simple carbohydrates may be quite widspread among apparently healthy persons (Brown & Doyle, 1966), while it is exaggerated in the great majority of atherosclerotic patients (Kuo, 1967). This increased sensitivity to dietary carbohydrate is frequently manifested by an increase in the lipids of the pre, β-lipoprotein fraction (Levy, Lees, & Fredrickson, 1966).

Since the endogenously synthesised pre,β-lipoproteins of carbohydrate origin are rich in both cholesterol and triglyceride, high concentration of these very low-density lipoproteins would account for much of the hypercholesterolaemia and hyperglyceridaemia found in association with atherosclerosis (Brown, Kinch & Doyle, 1965; Keys, Taylor, Blackburn, Brozek, Anderson & Simonson, 1963; Kannel, Dawber, Friedman, Glennon & McNamara, 1964 and Albrink, 1962).

b) Steroids and lipid metabolism.

The body of circumstantial evidence that has grown over the past years implicating elevated serum lipid levels as a factor in the development of human atherosclerosis and coronary disease has stimulated considerable research on steroids that alter lipid metabolism. Indeed many of the reported studies on the effects of adrenocortical steroids on lipid metabolism have been correlated with their effects on experimentally induced atherosclerosis. For example, the retardation of the development of atherosclerosis in cholesterol fed rabbits by administering cortisone has been reported by several workers (Duff & McMillan, 1949; Oppenheimer & Bruger, 1952; Cook, Ray, Davisson, Feldstein, Calvin & Green, 1952; Gordon, Kobernick, McMillan & Duff, 1954; Dury, 1956; and Fisher & Tapper, 1960). Although the results may be explained on the basis of the effects of cortisone on permeability or cellular reaction of the arterial wall, one could also assign a significant role to the changes in the lipid metabolism such as

the decrease in serum cholesterol/phospholipid ratio.

The fact that there are differences in metabolic responses of the various animal species to adrenocortical steroids has led to difficulty in correlating metabolic studies in man with those in experimental animals. For this reason further review of steroids in relation to lipids will be restricted to the human species and the steroids reviewed restricted to adrenocortical and androgenic steroids.

i. Adrenocortical steroids:

Since the observations of Aldersberg, Schaefer & Drachman (1950) that prolonged cortisone therapy frequently causes an elevation of serum cholesterol and phospholipids, numerous reports have appeared that have substantiated the effects of this steroid on lipid metabolism in man. Lipid alterations induced by cortisone apparently are more dependent upon duration of therapy and the clinical state of the patients than on the size ~~of the size~~ of the dose. That cortisone does not always induce a rise in serum cholesterol is indicated by the observations of Perera, Pines, Hamilton & Vislocky (1949), O'Connel & Burns (1950) and Oliver & Boyd (1956) who noted a fall in serum cholesterol. Mann & White (1953) suggested that the rise in serum cholesterol during cortisone therapy in acutely ill patients may be due to the removal of the disease stress with cortisone which then would allow a return to normal

cholesterol levels. Depression of thyroid activity has been observed in some patients that may have contributed to the rise in cholesterol during cortisone therapy (Wolfson, Beirwaltes, Robinson, Duff, Jones, Knorpp & Eya, 1950). Evidence for depression of thyroid activity included a decrease in basal metabolic rate, decrease in plasma protein-bound iodine and diminished uptake of radioactive iodine.

It has been suggested that with ageing, there may be a decrease in adrenal 11-oxosteroid production that stimulates production of ACTH. This increase in ACTH stimulates adrenocortical activity and restores the 11-oxosteroid level, but at the same time raises the adrenal output of androgenic 17-OS precursors that account for the β-lipoproteinaemia. Evidence for this mechanism was reported by Danowski & Moses (1962). The administration of small doses of cortisol to healthy men was followed by a decrease in both the urinary excretion of 17-OS and serum β-lipoprotein cholesterol levels. This hypocholesterolaemic response was maintained for 2 years. This would appear to be contrary to the findings of Kotasek et al (1967) who, while studying urinary steroids in relation to plasma lipids in late toxaemia of pregnancy found an inverse relationship between urinary DHA and β-lipoproteins. These findings are covered more fully under androgenic steroids in relation to lipids.

Oliver & Boyd (1956) reported daily intramuscular injections of DOCA for 5 days lowered the plasma cholesterol levels, cholesterol/phospholipid and the β-lipoprotein cholesterol and increased the α-lipoprotein cholesterol in 6 hypercholesterolarmic patients.

ii. Androgenic steroids:

The reports of the effects of androgenic steroids on the blood concentration of cholesterol are less consistent than those for oestrogens. Thus, testosterone or methyltestosterone has been reported to have no effect on serum cholesterol (Looney & Romanoff, 1940; Engleberg & Glass, 1955; Furman, Howard, Smith & Norcia, 1956), to increase cholesterol (Randall, 1940; Federman, Robbins & Rall, 1958) or to decrease cholesterol (Berezin & Von Studnitz, 1957). These variations could probably be attributed to differences in dosage, duration of treatment or in analytical methods used to estimate total cholesterol. The effect of testosterone on serum lipoprotein levels appears to be more consistent. In a study of the influence of gonadal hormones on protein-lipid relationships in human plasma, Russ, Eder & Barr (1955) noted that the androgens produced a decrease in the relative and absolute amounts of cholesterol and phospholipid in α-lipoproteins and an increase in the β-lipoproteins. Similar alterations in the lipo-protein composition on the electrophoretic pattern have been reported by Oliver & Boyd (1955, 1956); Berezin & Von Studnitz (1957); Furman, Howard, Norcia & Keaty (1958) and Eder (1959).

Studies on the lipid effects of combinations of oestrogens and androgens in attemps to overcome some of the feminizing properties of oestrogen therapy and yet retain the possible beneficial effect on lipid metabolism have ~~this~~ so far been disappointing. The use of simultaneous physiological doses of 17β-oestradiol and testosterone gave no consistent changes in serum lipids or low density lipoproteins (Englesberg & Glass, 1955).

The reports by Hellman, Bradlow, Zumoff, Fukushima & Gallagher, 1959a,b) on the hypocholesterolaemic effects of androsterone in hyper- and normo-cholesterolaemic patients stimulated interest in androsterone and androsterone like steroids for the treatment of hypercholesterolaemia. The hypocholesterolaemic activity of androsterone was most striking in myxoedematous patients. In these patients, the daily excretion of androgen metabolites, aetiocholanolone and especially androsterone, were very low. Also the ratio of urinary aetiocholanolone:androsterone for exogenous testosterone and endogenous precursors of androsterone and aetiocholonalone was extremely high. Administration of triiodothyronine to myxoedematous patients altered this defect in metabolism of endogenous and exogenous "androgens" by restoring the normal ratio of aetiocholanolone: androsterone, but not correcting the low urinary level of the two 17-OS. Administration of triiodothyronine to a euthyroid subject also produced an increase in aetiocholanolone:androsterone ratio.

From these results Hellman et al (1959a) postulated that some of the peripheral actions of thyroid hormones might be mediated by androsterone. Thus, the hypo-cholesterolaemic action of this steroid was referred to as a "thyromimetic" activity. The observations of Hellman et al were confirmed by Cohen, Higano, Robinson & Lebeau (1961) and Furman & Howard (1962). The former workers observed that androsterone induced an average serum cholesterol reduction of 36 mg/100 ml, the major portion of which was the result of a decrease in the cholesterol of the β-lipoprotein fraction. They also reported a reduction in serum triglyceride levels. The latter investigators found essentially the same type of response, namely, a reduction of serum cholesterol of 16% with the greatest decrease in the cholesterol content of the very low density lipoprotein fraction. In all the above studies androsterone was administered parenterally.

Subsequent studies with androsterone as a hypocholesterolaemic agent was with the androsterone dissolved in clofibrate-atromid, and administered orally. Atromid was shown to effectively lower serum cholesterol and triglyceride (Oliver, 1962; Carlson, Hogstedt & Oro, 1963 and Carson, McDonald, Pickard, Pilkington, Davies & Love, 1966). However, further studies showed that the active principle was the clofibrate (Atromid-S) and this administered alone was as effective as when administered with androsterone (Oliver, 1963 and Hellman, Zumoff, Kessler, Kara, Rubbin & Rosenfeld, 1963). Studies on the mode of action of clofibrate indicate that

it acts by potentiating the effects of endogenous hormones particularly thyroxine and androsterone (Thorp, 1962; Thorp, 1963a,b, and Thorp & Waring, 1962).

Certain recent urinary studies have suggested possible relationships between lipids and urinary steroid levels. Patients with toxaemia of pregnancy generally have elevated plasma phospholipids and β-lipoproteins (Boyd, 1935 and Kotasek, Fassati, Brestak & Link, 1965). Kotasek et al (1967) studying such patients found them to have very low urinary DHA levels when compared with normal non-pregnant and pregnant women. Further, there was a statistically significant inverse correlation between urinary DHA levels and the mean level of serum β-lipoproteins in normal non-pregnant, normal pregnant women and women with toxaemia of pregnancy. The elevated β-lipoprotein levels in toxaemia of pregnancy could not be attributed to reduced thyroid function since protein-bound-iodine levels were comparable with the normal pregnancy group. Kotasek et al (1967) proposed that DHA influenced the level of serum β-lipoprotein. They suggested lowered excretion of DHA stimulated G-6-P.D. which stimulates the pentose phosphate cycle, which in turn stimulates lipid synthesis. Indirect support of this DHA - lipid relationship is the hypocholesterolaemic effect of orally administered DHA, in rats rendered hypercholesterolaemic by propylthiouracil treatment (Ben-David, Dikstein, Bismuth & Sulman, 1967).

Further support for a relationship between lowered urinary

DHA levels and elevated G-6-P.D. comes from the studies of Lopez-S & Krehl (1967). They noted a positive correlation between percentage excess body weight and G-6-P.D. activity of red blood cells and at the same time a negative correlation between urinary DHA levels and percentage excess body weight, and, therefore, an inverse relationship between urinary DHA levels and G-6-P.D. activity of red blood cells. They further observed an increase in urinary DHA with decrease of body weight while patients were on weight reducing diets. Contrary to the findings of Hendrikx, Heyns, Steeno & De Moor (1965) who reported a marked drop in DHA, androsterone, aetiocholanolone in patients on a starvation diet. Though, it is possible the disparity in diets, one starvation and the other low in carbohydrates, may explain the difference. Lopez-S & Krehl (1967) speculated that DHA may possibly influence carbohydrate metabolism, which is in turn related to the development and perpetuation of obesity.

Patients with diabetes mellitus, a disease associated with atherosclerosis and gout (Whitehouse & Cleary, 1966; Berkowitz, 1966 and Pell & D'Alonzo, 1967), have recently been shown to excrete lower levels of androstenetriol, pregnanetriol and pregnenetriol than controls (Charro-Salgado, Clarke, Shackleton, Duncan & Mitchell, 1968). It is interesting that these steroids, pregnanetriol and pregnenetriol, were the ones which Genest et al (1961) and Kowaczynski et al (1964) found to be low in idiopathic, renal and malignant hypertension. Added to this is the recent renewed interest in

the association of diabetes mellitus and hypertension. Pell & D'Alonzo (1967) studied 662 diabetics and an equal number of controls and found the incidence of hypertension in two groups to be 36% and 24% respectively.

D. Nature of the Present Investigations.

The present investigations were mainly concerned with the individual urinary 11-deoxy-17-oxosteroid levels in vascular disease.

Kolbel et al (1964, 1965a) found zero urinary levels of DHA in hyperuricaemic, hypertensive patients. Since DHA and DHA.S are interconvertible in vivo, and DHA is excreted in the urine conjugated with either sulphuric acid or glucuronic acid, then the absence of DHA from the urine may be due to depressed secretion of DHA and DHA.S, and/or to altered metabolism. Secreted DHA and DHA.S are metabolised as in fig.1 (p. 85). It is, therefore possible that qualitative and/or quantitative differences in the metabolites of DHA and DHA.S might be found between hyperuricaemic hypertensive patients, normouricaemic hypertensive patients and normal controls. In the present study DHA, epiandrosterone, aetiocholanolone and androsterone in the sulphate and glucuronide fractions of urine were estimated. However, before measuring the urinary levels of these 11-deoxy-17-oxosteroids in normals and in patients with vascular disease a suitable method of estimating them was developed.

Steroid production is controlled by NADPH availibility, which in turn is controlled by the activity of G-6-P.D, an enzyme shown to be inhibited by DHA (Marks & Banks, 1960 and Tsutsui et al, 1962). Therefore, since hypertension may be a disease where adrenal

DHA production is abnormal the effect of DHA on adrenal steroid hydroxylation was studied.

Salt metabolism has long been intimately associated with hypertension, and many studies have attempted to verify the existence of a salt-losing steroid. Some of the results from previous studies have suggested that this hypothetical steroid may be an 11-deoxy-17-oxosteroid, therefore experiments were carried out to observe any changes in the urinary excretion of individual 11-deoxy-17-oxosteroid, which might be induced by varying the salt intake of normal and hypertensive subjects.

Kolbel et al (1965a) suggested that abnormally low urinary DHA levels found in hypertension were related to the elevated serum uric acid levels since they had previously demonstrated the same abnormality in gout, a disease characterised by elevated serum uric acid levels. Gout is now regarded as being intimately related with vascular disease. Therefore the individual 11-deoxy-17-oxosteroid levels were studied in this disease. On the basis of the low results found from this study of gouty individuals a comparison of the urinary levels of the same steroids in normolipidaemia and hyperlipidaemia was made and to obtain confirmatory evidence of the low urinary 11-deoxy-17-oxosteroid found in hyperlipidaemia and gout, myocardial infarct patients were then studied, since this is a condition characterised by elevated plasma lipids.

IV. EXPERIMENTAL PART I.

A. Routine Biochemical Methods.

1. Urinary electrolytes.

(a) Sodium and potassium. Urinary sodium and potassium were estimated on the Eel Flame Photometer (Evans Electroselenium Ltd., Harlow, Essex.) by the method employed at the Biochemistry Dept., Glasgow Royal Infirmary (1957).

(b) Chloride. Urinary chloride was estimated on an Eel Chloride Meter which employs a coulometric titration and gives a digital read out.

2. Kidney function tests.

(a) Serum urea. Serum urea nitrogen was estimated on the Autoanalyzer (Technicon, London) using a procedure based on the methods of Skeggs (1957) and March, Fingerhut & Kirsch (1957). The final colour reaction is based on the direct reaction of urea with diacetyl monoxime in the presence of strong acid and the intensity of the colour measured at 480 mμ in a flow cuvette.

(b) Serum creatinine. Serum creatinine was estimated by the method of Folin & Wu (1919) as modified by Stevens & Skeggs (1964) for automation on the Autoanalyzer. The final colour reaction, which is essentially the Jaffé reaction, is formed by creatinine in the presence of alkaline picrate.

(c) Urinary creatinine. Urinary creatinine was estimated by the method of Bonsnes & Taussky (1945) which also uses the Jaffé reaction for the final colour formation.

3. Plasma lipids.

(a) Cholesterol. Total serum cholesterol was estimated using the method of Pearson, Stern & McGavack (1953) with minor modifications. This method employs colour development by a sulphuric acid:acetic acid:water mixture in the presence of a colour stabiliser p-toluene-sulphonic acid and quantitation of colour formed is achieved by measuring the optical density at 625 mμ.

(b) Phospholipids. Lipids were extracted into an alcohol:ether mixture and digested with sulphuric acid and hydrogen peroxide according to the method of Youngburg & Youngburg (1930). The phosphorus thus converted to phosphate was estimated by the method of Fiske & Subbarrow (1925) as modified by Barlett (1959).

(c) Glycerides. Total serum glycerides were estimated by the method of Van Handel & Zilversmith (1957). In this procedure the extracted glycerides are hydrolysed in alkali and the liberated glycerol oxidised with periodate to formaldehyde, which is then measured colorimetrically after reaction with chromotropic acid.

4. Serum uric acid.

Serum uric acid was estimated on the Autoanalyzer by an automated procedure adapted from the method of Folin (1933).

This method involves the quantitative reduction of a phosphotungstate complex to a phosphotungstite complex by uric acid and the colour formed intensified by the presence of cyanide.

5. Blood and urine collection.

The blood samples for the estimation of serum urea, serum uric acid, serum creatinine, serum cholesterol, serum phospholipids and serum glycerides were collected in plain bottles, and after clotting the serum removed and stored at 0° till processing. Where serum glycerides were estimated the subject fasted overnight prior to blood collection.

Twenty-four hour urines for the estimation of electrolytes, creatinine, total 17-OS, total 17-OHCS and individual 11-deoxy-17-oxosteroid sulphates and glucuronides were collected in plain polythene bottles, volumes measured and suitable aliquots frozen until processing. To avoid any hydrolysis of steroid conjugates, which has been found to occur with repetitive thawing and freezing (Patti & Stein, 1964), separate aliquots of urine, for estimation of individual 11-deoxy-17-oxosteroid sulphates and glucuronides, were deep frozen.

6. Group urinary steroid analyses.

(a) 17-Oxosteroids (17-OS). Total urinary 17-OS were estimated by the method recommended in M.R.C. Committee on Clinical Endocrinology Memorandum (1963) as modified by James & Caie (1964). This method employs hot acid hydrolysis, methylene chloride extraction of free

steroids, Zimmerman reaction on the dry steroid residue followed by ether extraction of the Zimmerman chromogens and finally quantitation using DHA as standard.

(b) 17-Hydroxycorticosteroids (17-OHCS). Total urinary 17-OHCS were estimated by the method of Few (1961) as modified by James & Caie (1964) which employs borohydride reduction of the 17-hydroxy-corticosteroids at the C-20 position, removal of excess reducing reagent, metaperiodate oxidation of the reduced 17-OHCS to the corresponding 17-OS, alkaline hydrolsis of oxidation induced formates, methylene chloride extraction, Zimmerman reaction on the dry steroid residue followed by ether extraction of the Zimmerman chromogens and finally quantitation using DHA as standard.

B. Incubation Techniques.

1. Source of adrenal tissue.

Human adrenal tissue was obtained at operation on a patient undergoing treatment for breast cancer. The gland from this patient was assumed to be normal (Grant, Forrest & Symington, 1957). The tissue was chilled to about 0° after removal from the subject and transferred at this temperature to the laboratory. The time interval from extirpation to incubation was 1 hour.

2. Preparation of tissue.

The adrenal tissue (1.25 g) was scraped from the capsule and homogenised at about 0° in 13 ml of 0.5M-2-amino-2-hydroxymethyl-1, 3-propanediol (TRIS) buffer of pH 7.6 containing 0.02M-magnesium chloride

in a Potter-Elvehjem homogenizer. Three ml of the homogenate were added to 1.5 ml of a solution containing 5×10^{-2}M-adensosine-5-triphosphate (ATP), 1.7×10^{-3}M-$NADP^+$ and 3.9×10^{-2}M-glucose-6-phosphate (G-6-P).

3. Method of incubation.

Radioactive steroids in benzene methanol (9:1 v/v), were added to the incubation vessels along with 100 µl of propylene glycol. The benzene and methanol were evaporated in a stream of air at about 40° leaving the steroids dissolved in a film of propylene glycol. The adrenal tissue preparation in their incubation media were added to the flask and placed in a water bath at 37° and incubated with shaking for 1.5 hours.

At the end of the incubation period the reactions were stopped by the addition of 20 ml of acetone and refrigerating at -15°. The unlabelled carrier steroids were added shortly after this stage in a small volume of alcohol.

Radioactive steroids were purchased from the Radiochemical Centre, Amersham, Bucks., and were stored at -15° at an approximate concentration of 1µc/ml (^{14}C-steroids) or 10µc/ml (^{3}H-steroids) in a mixture of methanol:benzene (1:9 v/v). The purity of these compounds as claimed by the makers was checked by thin layer chromatography and scanning with a Packard Radiochromatogram Scanner, Model 7200.

Non-labelled steroids were obtained from Koch-Light Laboratories, Ltd., Colmbrook, Bucks., Steraloids Ltd., Croydon, Surrey and Ikapharm, Ramat-Gan, Israel.

4. Extraction of steroids.

The incubation mixtures were homogenised in a Silverson mixer with 3x50 ml of acetone and again with 50 ml of ethanol. After filtering through a sintered glass funnel, the acetone:ethanol mixture was taken to dryness under reduced pressure and the residue partitioned between 70% aqueous methanol and light petroleum (60-80°). The light petroleum fraction was back extracted with 70% aqueous methanol. The methanol:water mixture was taken to dryness under reduced pressure, adding ethanol to facilitate the evaporation of residual water. Solvents purifications was as described in Section IV.C.2.b.

5. Preparation of thin-layer chromatograms.

Glass plates (20 cm x 20 cm) were coated by a Desaga applicator (Camlab Glass Co., Cambridge) with a slurry of Merck silica gel $HF_{254/366}$ (25 g in 65 ml water). After 20 minutes drying at room temperature, the thin layers were activated at 110° for 60 minutes. The steroids were applied in chloroform and the chromatograms developed in appropriate solventsystems.

6. Detection and elution of steroids from thin-layer chromatograms.

The Δ^4-3-oxosteroids were visualised in light of wavelength 254 mμ (Universal U.V. Lamp, Camag, Muttenz, Switzerland.) as dark spots on a green background, and Δ^5-3β-hydroxysteroids were located in light of wavelength 350 mμ.

All steroids were eluted by scraping the silica gel containing the steroid on to black glazed paper, transferring it to a tube containing 1 ml of water plus 5 ml of ethyl acetate and thoroughly mixing the contents using a Whirlmixer (Scientific Industries, International Inc. (U.K.) Ltd., England). After centrifugation the upper layer was removed and the aqueous layer re-extracted with a further 5 ml of ethyl acetate. The combined extracts were evaporated in a stream of air at a temperature not exceeding 50^{o}.

Recovery of steroids by this method is normally 90-100%.

7. Preparation of derivatives.

(a) Acetylation. Steroids were acetylated by the procedure of Zaffaroni & Burton (1951). Re-distilled acetic anhydride and pyridine (3 drops of each) were added to the dried steroid and the reaction allowed to proceed overnight at room temperature in a tightly stoppered test-tube. The reaction mixture was then diluted with 1 ml methanol and the solvents evaporated completely in a stream of air.

(b) Saponification. Steroid acetates were saponified by the method of Neher, Désaulles, Vischer, Wieland & Wettstein (1958) as modified by Ward & Grant (1963). A solution (250 µl) of 2% aqueous K_2CO_3 (w/v) was added to the steroid dissolved in 1 ml methanol and the mixture allowed to stand overnight at room temperature. Water (2 ml) was then added and the steroids extracted with ethyl acetate.

(c) Reduction. The reduction mixture was made up by dissolving 5 mg $NaBH_4$ in 10 ml methanol at 0°. An aliquot (200 µl) of this solution was added to the dried steroid residue in a tube standing in crushed ice and the mixture maintained at 0° for 45 minutes. This is a modification of the method of Southscott, Bandy, Newson & Darrach (1956). The reaction was stopped by the addition of one drop of glacial acetic acid and the steroids extracted with ethyl acetate after the addition of 2 ml of water.

8. Quantitation of steroids.

Following purification by chromatography, the Δ^4-3-oxosteroids were dissolved in 5 ml or 2 ml of ethanol, and their extinctions measured at 240 mµ against ethanol in 1 cm cells of a Unicam SP 500 spectrophotometer. Quantitation was achieved by use of extinction coefficients and by comparison with standard solutions. Similar amounts of the chromatographic silica gel, taken through the elution procedure, served as blanks for extinction measurements.

9. Measurement of radioactivity in extracts.

Portions of steroid residues to be counted were placed in glass vials of low potassium content (Wheaton Glass Co., Millville, N.J.) and dissolved in 10 ml toluene containing 3g/l of 2,5-diphenyl-oxazole (PPO) and 0.1g/l of 1,4-bis-2(4-methyl-5-phenyl-oxazolyl)-benzene (dimethyl POPOP). Radioactivity was determined using a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3214

(Packard Instrument Co., Inc., La Grane, Illinois). Tritium and ^{14}C were determined simultaneously at voltage tap 3.797 with channel I voltage discriminator gate of 50-210 and amplifier gain of 25%, giving efficiencies of counting of approximately 3.8% for ^{14}C and 18% for ^{3}H. Channel II was set with a voltage discriminator gate of 190-1,000 and amplifier gain of 5%, giving efficiencies of counting of approximately 33% for ^{14}C and 0% for ^{3}H. Absolute quantities of tritium were calculated using a slight modification (Packard, 1962) of the standard equations of Okita, Kabara, Richardson & LeRoy (1957) and of ^{14}C from direct readings on channel II since, as stated above, no tritium counts appear on this channel under the conditions specified.

Standard equations.

$$R_r = A_1 E_{1r} + A_2 E_{2r}$$

$$R_g = A_1 E_{1g} + A_2 E_{2g}$$

Where R_r = counts/min. in red channel or channel I.

R_g = counts/min. in green channel or channel II.

A_1 = activity of isotope I (^{3}H).

A_2 = activity of isotope II (^{14}C).

E_{1r} = efficiency of counting for isotope I in channel I.

E_{1g} = " " " " " I " " II.

E_{2r} = " " " " " II " " I.

E_{2g} = " " " " " II " " II.

C. Quantitation of Urinary 11-Deoxy-17-oxosteroid (D.17-OS) Sulphates and Glucuronides.

1. Introduction.

Since the first practical application of gas chromatography by James & Martin (1952) its utilisation in the biomedical field has grown rapidly. One of the most important uses of gas-liquid chromatography is in the field of steroid biochemistry.

The first practical demonstration of the separation of steroids by gas chromatography was described by VandenHeuvel, Sweeley & Horning (1960) who were able to separate several androstane and pregnane derivatives on SE-30 columns at 200-220°. A short time later Wotiz & Martin (1960) reported the separation, identification and measurement of the three classical oestrogens. Although earlier workers, among them Eglington, Hamilton, Hodges & Raphael (1959) using apiezon columns, Sweeley & Horning (1960) using a thermostable polyester phase, and Beerthuis & Recourt (1960) using a silicone phase, were all able to separate steroids by gas chromatography, they were unable to prevent breakdown of the steroids caused by the high temperatures and long retention times required for their separation. VandenHeuvel, Sweeley & Horning (1960) were the first to succeed in demonstrating that by using thinner films of liquid phase in the column, it was possible to reduce both the retention time and the temperature, and thus achieve separation of steroids without their breakdown. Then in 1961 Luukkainen, VandenHeuvel, Haahti & Horning demonstrated that the 17-OS could be completely separated

by gas-liquid chromatography as their trimethylsilyl ether (TMSE) derivatives.

Several investigators have reported on the gas chromatographic separation of urinary D.17-OS using the free steroids, trifluoroacetates and TMSE derivatives. Haahti, VandenHeuvel & Horning (1961) determined the percentage composition of synthetic mixtures containing androsterone (A), aetiocholanolone (E) and DHA, using two stationary phases SE-30 and NGS. Later VandenHeuvel, Creech & Horning (1962) resolved a mixture of the same D.17-OS as TMSE derivatives requiring an NGS column only. Garret, Creech, Horning & DeBakey (1962), employing the latter technique, studied the urinary excretion of A, E and DHA in patients with atherosclerosis. Kirschner & Lipsett (1963), also using TMSE derivatives, published a quantitative procedure for the determination of the same D.17-OS. Since then there have been many methods published on the quantitation of urinary 17-OS and related steroids as their TMSE, among them are Chamberlain, Knights & Thomas (1963); France, Rivera, McNiven & Dorfman (1965); Jungmann, Calvary & Schweppe (1967); Cawley, Musser, & Tretbar (1967) and Rivera, Dorfman & Forchielli (1967). However, none of these investigators have published values for the urinary levels of the D.17-OS 3β-hydroxy-androstan-17-one (epiandrosterone, epiA). Yet the TMSE derivatives of DHA and epiA can be satisfactorily separated on stationary phase QF-1.

Sparagana, Keutmann & Mason (1963) reported a gas chromatographic method for the estimation of three $C_{19}O_2$ (A,E & DHA) and three $C_{19}O_3$ as the free steroids, and incorporated the use of an internal standard (11β-hydroxyandrost-4-en-3, 17-dione). It was introduced at the start of the analysis, thus correcting for loss throughout the entire extraction and purification procedures, as well as removing the difficulty of injecting exact microlitre quantities of organic solvents into the gas chromatograph.

Quantitation of urinary steroids by the unspecific detection of a gas chromatograph requires adequate and efficient prepurification of the urinary extracts before gas-liquid chromatography. The method of Kirschner & Lipsett (1964a) employed a single thin-layer chromatographic step which provided a good separation of 17-OS from non-steroidal contaminants as well as from 17-OHCS. The presence of 17-OHCS would otherwise have interfered with further analysis, since they undergo pyrolysis in the gas chromatographic column, yielding the corresponding 17-OS (VandenHeuvel & Horning, 1960). A more rapid method than thin-layer chromatography, if a reasonably large number of urines have to be assayed, with equally effective purification was employed by France et al (1965). They used silica gel columns. Similarly, Jungmann et al (1967) and Heyns & De Moor (1965) employed alumina columns for their purification step prior to gas chromatography.

The urinary D.17-OS are metabolites of the androgens DHA, DHAS, androstenedione and testosterone, which in men are secreted by the testis and adrenal gland and in the female by the adrenal gland. DHA, DHAS, androstenedione and testosterone are metabolised to D.17-OS as in fig.1 (Vande Viele, MacDonald, Gurpide & Lieberman, 1963). With slight modification this may be illustrated thus:

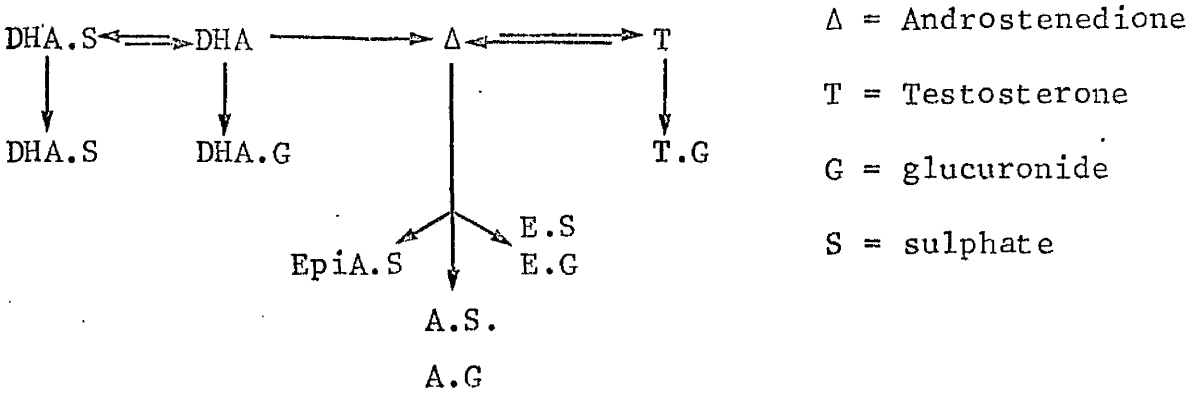


Fig. 1. Metabolism of Androgens.

Thus, the 17-OS appearing in urine are present conjugated to glucuronic acid and sulphuric acid. Yet, out of plethora of publications on urinary levels of 17-OS only three groups of investigators have reported the levels of both the 17-OS glucuronide and sulphate fractions (Kellie & Wade, 1957; Mauvais-Jarvis & Baulieu, 1962 and Menini, 1966). Of these investigators Menini (1966) employed gas chromatography for the separation and quantitation of the 17-OS.

In the present method for the quantitation of individual D.17-OS an attempt has been made to avoid the incorporation of

obvious disadvantages illuminated by previous work, (a) the separation of D.17-OS as free alcohols involves long retention times and poorer separation, (b) the use of an internal standard added just prior to injection into the gas chromatograph does not allow for losses of material occurring during extraction and purification. Further, the use of radioactive steroids added prior to hydrolysis as internal standard necessitates the provision of expensive radioactive counting equipment and a further internal standard must still be added prior to the gas chromatographic step, (d) many of the methods, through unsuitable selection of stationary liquid phase, measure only A, E and DHA and in many cases probably the estimated DHA is an estimation of both DHA and epiA, (e) inadequate purification of steroids prior to gas chromatography leads to unacceptable gas chromatographic tracings.

The following method estimates the four urinary D.17-OS, A,E,DHA and epiA in urinary sulphate and glucuronide fractions. Pregnenolone sulphate and pregnenolone were employed as internal standards for the urinary D.17-OS sulphate and glucuronide fractions respectively. The internal standards were introduced at the start of the analysis. Alumina column chromatography was used for pre-gas chromatography purification. The D.17-OS were chromatographed as their TMSE derivatives on a 3% OF-1 column.

2. Materials and Methods.

(a) Reagents. Trimethylchlorosilane, dimethylchlorosilane and

gas-chrom P (100-120 mesh) were obtained from Applied Science Laboratories, P.O. Box 140, State College, Pennsylvania, U.S.A. The diethylamine (B.D.H.Analar grade) was stored over KOH pellets. QF-1 stationary phase - Perkin Elmer Ltd., Beaconsfield, Bucks. Chromatographic grade alumina - Martindale Samoore Ltd., London. Non-labelled steroids. See Section IV.B.3.

Radioactive compounds. 7α-^{3}H-pregnenolone (specific activity 2.5 c/mM) was obtained from the Radiochemical Centre, Amersham, Bucks. The 7α-^{3}H-pregnenolone sulphate (specific activity 16c/mM) was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. (for storage and purity of radioactive steroids see Section IV.B.3.) Tritiated toluene was obtained from the Pakcard Instrument Co., Downers Grove, Ill., U.S.A.

β-Glucuronidase, preparation from limpet visceral sacs. Limpets were collected, the visceral sacs were removed and homogenized in water. Four volumes of ice-cold acetone were added with continuous stirring. The mixture was allowed to stand for 30 minutes and then Buchner filtered using Whatman No.1 filter paper. The precipitate was resuspended in ice-cold acetone, allowed to stand for a further 30 minutes and again filtered. The precipitate was washed with acetone until the filtrate was colourless. The resulting acetone powder was air dried overnight and placed in a desiccator to remove the last traces of acetone.

The acetone powder was assayed for β-glucuronidase activity by the method of Fishman (1963), using phenolphthalein glucuronide

as substrate. The β-glucuronidase activity was expressed in Fishman units, where 1 unit is the weight of acetone powder necessary to liberate 1 µg of phenolphthalein from phenolphthalein glucuronide.

(b) Solvents.

Ethyl acetate (B.D.H. AnalaR grade) was purified by washing with 3 x 0.1 volume of water, drying over anhydrous calcium chloride and glass distilling.

Ethanol and methanol (Burroughs AnalaR grade) were used without further purification.

Hexane (B.D.H. AnalaR grade) was washed with 3 x 100 ml of concentrated H_2SO_4/litre for 1 hour on a mechanical shaker, followed by two washes with 100 ml of 2N.NaOH/litre and finally with water until neutral. It was then dried over calcium chloride and glass distilled.

Acetone, ether and toluene (B.D.H. AnalaR grade) were used without further purification.

Benzene (B.D.H. AnalaR grade). Thiophene and other impurities were removed by shaking with 3 x 0.1 volume of concentrated H_2SO_4 for one hour, followed by water washes until neutral. The benzene was then dried over calcium chloride and twice glass distilled.

Methylene chloride (B.D.H. AnalaR grade) was purified by shaking for one hour with 3 x 0.1 volume of concentrated H_2SO_4, washing with water until neutral, drying over calcium chloride and twice glass distilling.

(c) Choice of internal standard.

The choice of an internal standard is subject to the following considerations:

1) It must not occur in the urine.
2) It must be as similar as possible in chemical structure to the compounds under investigation.
3) It must have a similar elution volume on column chromatography.
4) It must be separable on gas-liquid chromatography from the other compounds to be investigated.
5) It must have a detector response which is linearly related to the amounts present.
6) The position at which it emerges on the gas chromatographic trace must be free of other contaminating peaks.

Pregnenolone appears to fulfill these requirements. It is not present in urine in detectable amounts; on column chromatography it has an elution volume similar to that of the D.17-OS; it is easily separable from the D.17-OS by gas-liquid chromatography on a variety of columns including OF-1 and its peak free of extraneous contamination. Its structure is very similar to DHA, which is the most labile of the D.17-OS. However, under the standardised conditions employed, no significant degree of breakdown has been observed for any of the steroids investigated.

(d) Solvolysis of 11-Deoxy-17-oxosteroid sulphates (D.17-OS.S).

To 25 ml of a 24 hour urine specimen were added 10-75 µg of

internal standard pregnenolone (as pregnenolone sulphate), the exact amount depending on the level of the total urinary 17-OS which had been previously measured. The steroid sulphate conjugates were then solvolysed by the method of Burstein & Lieberman (1958). In this method urine is acidified to pH 1 by the addition of 4N-H_2SO_4 after the addition of 20% w/v of NaCl and twice extracted with equal volumes of ethyl acetate. The combined ethyl acetate extracts are incubated for 18 - 20 hours at 37°.

(e) Hydrolysis of 11-Deoxy-17-oxosteroid glucuronides (D.17-OS.G).

To 25 ml of a 24-hour urine specimen were added 20-75 µg of pregnenolone, depending on the level of total urinary 17-OS previously measured. Pregnenolone was used as the internal standard as the glucuronide was not available. The steroid conjugates and the standard pregnenolone were extracted by the method of Edwards, Kellie & Wade (1953), which involves saturation of the urine with ammonium sulphate and 3 extractions with equal volumes of ether:ethanol (3:1). The combined extracts were taken to dryness in a rotary evaporator. Steroids were leached from the dry residue with 10 ml of ethanol, and the ethanol evaporated to dryness. The conjugates were then hydrolysed with limpet β-glucuronidase. Five ml of 0.5M-acetate buffer (pH 4.7) containing 10^5 units of enzyme and 0.25×10^{-3} moles of phosphate to inhibit steroid sulphatases (Roy, 1956), were added to the dry steroid residue and incubated at 37° for 2 days. After incubation, 4 volumes of water were added and free steroids extracted twice with equal volumes

of ether:ethyl acetate (1:1).

From this stage D.17-OS.S and D.17-OS.G fractions were treated alike. The extract was washed with one-tenth volume of IN-NaOH 2 to 3 times, until no further pigments were removed. Alkali was removed by washing three times with one-tenth volume of water. The washed extract was dried by passing through anhydrous sodium sulphate and evaporated under a stream of air in a water bath.

(f) Alumina column chromatography.

Alumina was deactivated by the addition of 4% water and its suitability for the chromatography step was checked periodically by estimating the recovery of DHA standard taken through the following procedure. The column (0.6 cm 1.D.) was prepared by the addition of 1g alumina suspended in dry benzene with a topping of sand. The dry steroid extract was quantitatively transferred to the column using two 3 ml volumes of benzene. The chromatogram was developed with a further 10 ml of benzene and 20 ml of 0.5% ethanol in benzene. The 0.5 % ethanol in benzene eluate containing the internal standard pregnenolone, together with any A, E, DHA or epiA was taken to dryness (Heyns & De Moor, 1965).

(g) Trimethylsilyl ether (TMSE) formation.

Since moisture can prevent quantitative formation of TMSE (Lau, 1966), the steroid containing residues were dried in a vacuum desiccator. TMSE were prepared according to the technique of Thomas & Walton (1966). To tube A were added 5 ml of hexane and

0.4 ml of diethylamine and to tube B, 5 ml of hexane and 0.7 ml of trimethylchlorosilane. The contents of tube A were added to tube B, stoppered, mixed, centrifuged to deposit the thick white precipitate and 0.5 ml of supernatant added to dry residues. The tubes were stoppered, mixed and left overnight at room temperature. The reaction mixture was taken to dryness in vacuo and the residue leached with hexane to dissolve the TMSE. The amount of hexane added was such that the pregnenolone concentration was about 0.2 µg/µl. This was determined after preliminary experiments had indicated the recovery at this stage. The mixture was centrifuged, since breakdown of TMSE occurs on the gas chromatographic column, if any particles of the ammonium chloride precipitate are simultaneously injected.

Each day that samples were etherified, a mixture of equal weights of pregnenolone, A, E, DHA and epiA was treated similarly, thus giving a check of etherification and peak height ratio of the steroids under analysis relative to pregnenolone.

(h) Gas liquid chromatography.

1. Preparation of column packing. The support, gas chrom P (100-120 mesh) was treated with concentrated HCl for 12 hours with occasional stirring. The acid was removed by suction and this procedure repeated three times. After treatment with acid the support was washed in distilled water with gentle stirring. After 2 minutes finely divided particles were removed by decanting them off. The support was repeatedly washed and decanted in this manner

until the final wash was neutral. The support was dried by treating with acetone, filtering and preliminary drying at room temperature overnight before final drying in an oven at 80°.

Approximately 20 g of the dry support was silanized by treatment with 100 ml of 5% dichlorodimethylsilane in toluene in a Buchner filter flask. Air bubbles were removed from the surface of the support by gentle shaking under reduced pressure. The support was then filtered, washed repeatedly with methanol and again dried at room temperature before heating in an oven at 80°.

The acid-washed deactivated support was well mixed with the rquired amount of liquid phase dissolved in methylene chloride and the methylene chloride completely evaporated on a rotary evaporator. The coated support was then air dried and finally oven dried at 80°.

ii. Packing of column. Glass columns and glass wool used for packing were treated with 5% (v/v) dichlorodimethylsilane in toluene overnight and repeatedly washed with toluene and methanol and dried before use.

Columns were packed by plugging one end with glass wool and the coated support was then introduced gradually with the help of suction. While the support was being added the column was repeatedly tapped to ensure even packing. Before use, newly packed columns were conditioned to 230° in a stream of nitrogen for 24 hours. This pre-treatment ensured removal of volatile products which may contaminate the detector and prevent a stable recorder baseline.

iii. Gas chromatography. A Pye series 104, model 14, gas chromatograph equipped with a flame ionisation detector was employed. Seven foot (4mm 1.D.) glass columns packed with pretreated gas chrom P and coated with 3% OF-1 were used. The nitrogen carrier gas purified by Molecular Sieve 5a (W.G.Pye & Co. Ltd.) was maintained at an inlet pressure of 30 p.s.i. which gave a flow rate of 50 ml/ minute respectively. All flow rates were measured at the outlet of the detector using a soap bubble flow meter. The Leeds & Northrup recorder was used at a chart speed of 10"/hour.

All injections were made with a 3.5" needle, 10 µl Hamilton syringe (Hamilton Co. Inc., Whittier, California, U.S.A. The Pye 104 gas chromatograph is not equipped with a flash heater zone, and injections were made on to the column. For this reason, it was important to use a long needle syringe to avoid introducing the sample near the top of the column where the temperature was lower than the analyser oven. Volumes of hexane solution injected ranged from 0.5 - 3 µl.

(i) Calculation of results.

Quantitative evaluation of the recorder tracings was carried out by measuring the height of peaks appearing at the retention times of reference steroid TMSE derivatives and the peak height of the internal standard TMSE derivative. This, together with the known peak height ratio (f) for each steroid derivative (TMSE-S) relative to pregnenolone derivative (TMSE-P)

and the amount of internal standard added to the original 25 ml of urine, was used for the calculation of steroid urinary concentration. Thus -

$$\frac{\text{Peak height TMSE-S}}{\text{Peak height TMSE-P}} \times \frac{\mu\text{g internal std.}}{\text{f.1000}} \times \frac{\text{24 hr urine vol.}}{\text{25 ml}} = \text{mg/24hr.}$$

(j) Measurement of radioactivity.

Radioactivity was measured as described in Section IV.B.a. Steroid sulphates were dissolved in 1 ml of methanol and 9 ml of scintillator solution. This system gives rise to a significant amount of quenching, which was corrected by the use of internal standardisation i.e., a known amount of radioactivity (^{3}H-toluene) was added to the quenched samples after an initial radioactivity count and the degree of quenching of standard ^{3}H-toluene calculated after a second radioactivity count.

3. Results.

Evaluation of reliability of the method for determination of D.17-OS.

Complete TMSE formation of D.17-OS can be demonstrated on stationary phase QF-1 where the TMSE have shorter retention times than their parent alcohols. Using standard D.17-OS no free steroids could be detected after derivative formation.

The response of the detector to the TMSE derivatives of the D.17-OS relative to the response of the pregnenolone TMSE derivative was satisfactory. A straight line was obtained when the ratios of masses of individual D.17-OS to pregnenolone (0.1 to 10) were plotted against the ratios of corresponding TMSE derivative peak heights (fig. 2a & b).

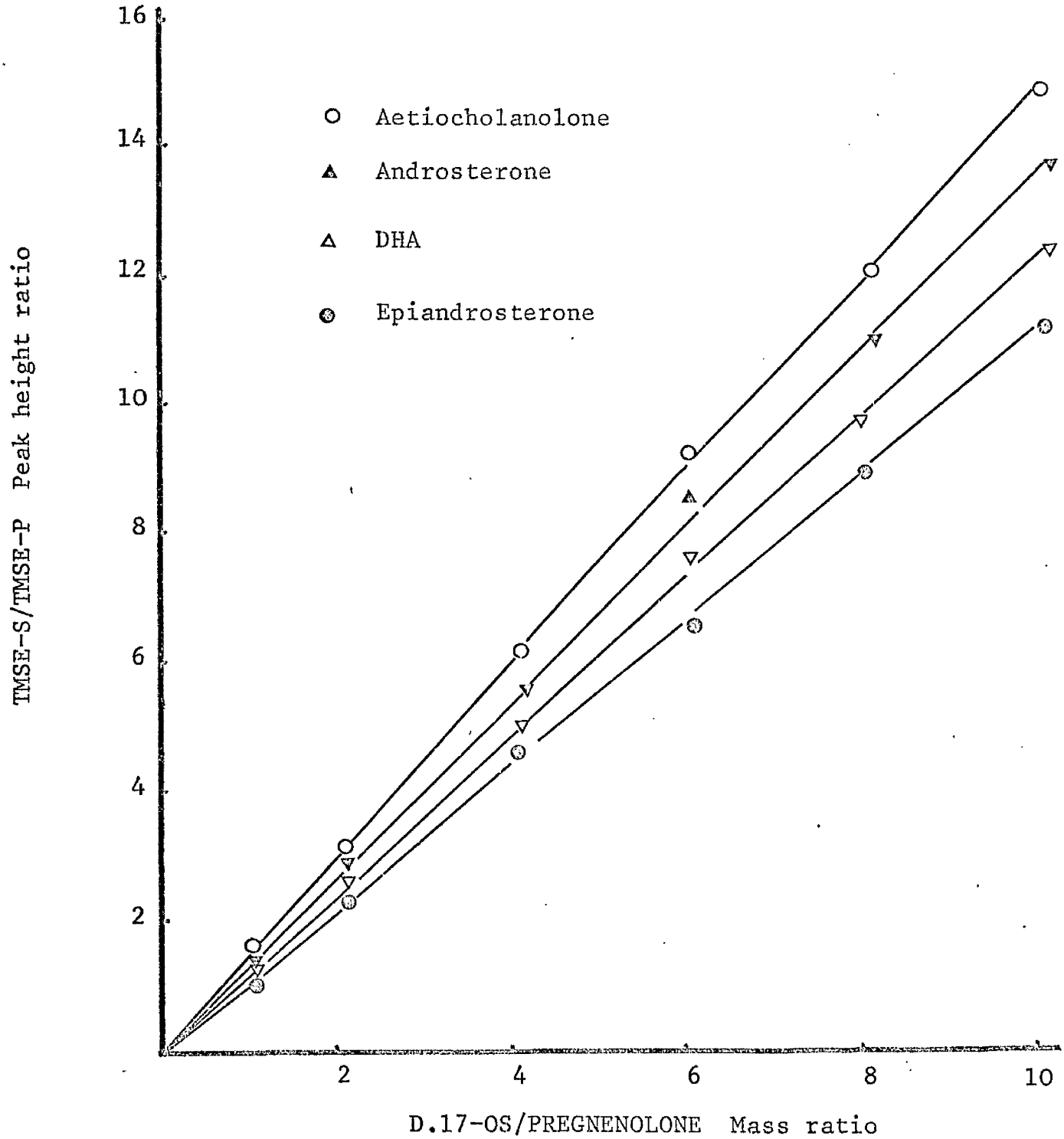


Fig. 2a. Linear response of gas chromatographic detector (TMSE-S/TMSE-P peak height ratio) to varying D.17-OS/pregnenolone mass ratios.

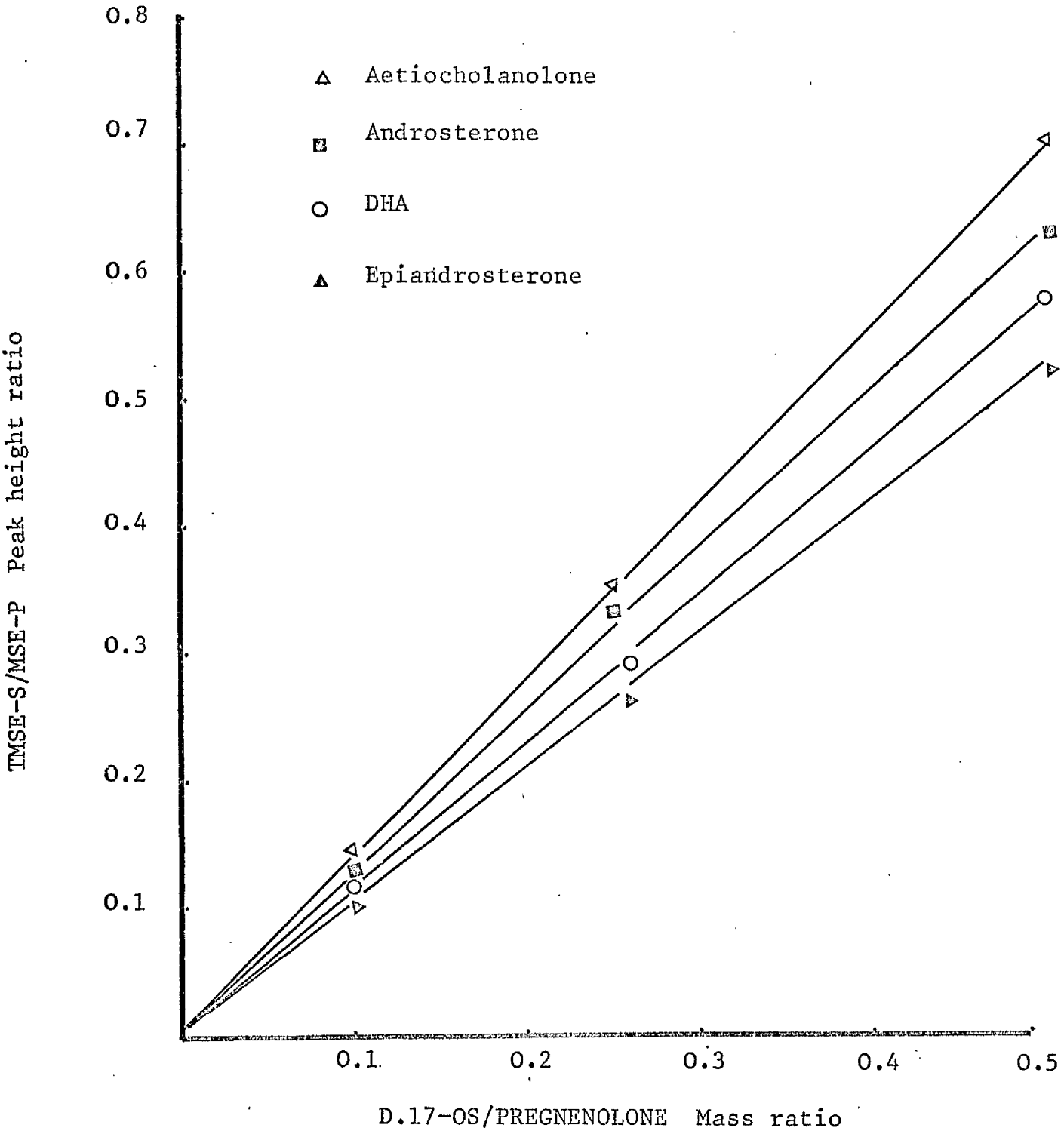


Fig. 2b. Linear response of gas chromatographic detector (TMSE-S/TMSE-P peak height ratio) to varying D.17-OS/pregnenolone mass ratios.

The retention time of individual TMSE derivatives relative to internal standard pregnenolone TMSE was found to be constant throughout the study (Table 1).

TABLE 1. Retention times of A, E, DHA and epiA relative to pregnenolone on 3% OF-1. Conditions as in text.

STEROIDS	RELATIVE RETENTION TIMES					
	AUTHENTIC STEROIDS			URINARY STEROIDS		
	n	x	S.D.	n	x	S.D.
A	25	0.572	0.006	25	0.572	0.006
E	25	0.633	0.007	25	0.633	0.007
DHA	25	0.716	0.006	25	0.714	0.008
epiA	25	0.798	0.018	25	0.798	0.008

n - Number determinations.
x - Mean relative retention time.
S.D - Standard deviation.

The TMSE derivative peak height ratio factor (f) used in the calculation of individual D.17-OS urinary concentration was checked regularly and re-estimated if necessary (Table 2).

TABLE 2. The ratio of the peak heights of individual D.17-OS TMSE to pregnenolone TMSE.

STEROIDS	D.17-OS/PREGNENOLONE			
	n	Mass Ratio	Peak Height Ratio x	S.D
A	10	1	1.975	0.037
E	10	1	1.771	0.048
DHA	10	1	1.631	0.034
epiA	10	1	1.468	0.039

n - Number of determinations.
x - Mean.
S.D - Standard deviation.

(a) Specificity.

The specificity of the method was based on:

1) The use of alumina column which ensured preferential isolation of the steroids in question.

2) The urinary steroid TSME derivatives were identified by their retention times relative to pregnenolone TMSE which were identical with authentic standard TMSE derivatives (Table 1).

3) Authentic steroids added to the urine for recovery studies only increased the respective peak areas without formation of new peaks.

4) The shape of the peak in both analytical and standards were identical, with no evidence of contamination from other unknown peaks.

(b) Sensitivity.

The sensitivity of the method was determined firstly by the

sensitivity of the gas chromatograph detector and secondly by the linear response relationship of the TMSE derivative peak height ratio to the mass ratio (figs. 2a & b). The limit of sensitivity set by these criteria was 0.1µg steroid/25 ml of urine.

(c) Precision.

The reproducibility of the method was checked by replicate analyses of urine pools from males between the age of 18 and 35, taken through the entire procedure. The results are summarised in Table 3 and demonstrate a satisfactory degree of reproducibility for each steroid under analysis. This is evident from the very low variation coefficients, E.S having the highest, 8.6 and 6.5% for urine pools 1 and 2 respectively.

(d) Accuracy.

The accuracy of the method was assessed by measuring the relative recoveries of D.17-OS added to water and to urine with pregnenolone sulphate and pregnenolone as internal standards, and by measuring the absolute recoveries of internal standards pregnenolone and pregnenolone sulphate.

i. Relative recoveries.

Recovery of D.17-OS added to water. Androsterone, E, DHA (50µg of each) and epiA (25µg) were added to twenty, 25 ml volumes of water and 10 taken through the complete procedure for estimation of urinary steroid sulphates and 10 through the procedure for urinary

TABLE 3. REPRODUCIBILITY OF THE METHOD FOR ESTIMATION OF INDIVIDUAL URINARY D.17-OS.S AND D.17-OS.G.

STEROID ESTIMATED		Urine Pool 1.				Urine Pool 2.			
		n	x	S.D.	V.C.	n	x	S.D.	V.C.
SULPHATES	A	10	1.05	0.066	6.2	10	1.27	0.063	5.0
	E	10	0.32	0.028	8.6	10	0.34	0.022	6.5
	DHA	10	2.95	0.126	4.3	10	3.87	0.161	4.2
	epiA	10	0.39	0.026	6.6	10	0.425	0.016	3.8
GLUCURONIDES	A	10	3.48	0.215	6.2	10	3.95	0.157	4.0
	E	10	2.87	0.101	3.5	10	3.14	0.149	4.7
	DHA	10	0.49	0.017	3.5	10	0.47	0.018	3.8

n. Number of determinations.

x. Mean. mgm/24 hours.

S.D. Standard deviation. mg/free steroid/24 hours.

V.C. Variation coefficient.%.

steroid glucuronides. The results are recorded in Table 4.

TABLE 4. Recovery of A,E, DHA and epiA added to water.

STEROID ADDED	µg ADDED	RECOVERY BY SULPHATE PROCEDURE n	x	S.D	RECOVERY BY GLUCURONIDE PROCEDURE n	x	S.D
A	50	10	99	3.2	10	96.8	9.3
E	50	10	102.2	2.5	10	100.2	3.6
DHA	50	10	103.3	1.7	10	96.8	4.1
epiA	25	10	102.2	2.0	10	101.4	4.9

n - Number of determinations.
x - Mean %.
S.D - Standard deviation %.

The mean % recovery in all the 8 cases was very good. It was within ±4% of the ideal recovery 100%, in every case. The standard deviation which indicated satisfactory reproducibility was less than 5% in every case excluding A.G where it was 9.3%.

Recoveries of D.17-OS added to urine. The D.17-OS sulphates and glucuronides in 25ml aliquots of a normal male urine pool were first measured by method already described and results present in Table 3. A, E, DHA (50µg of each) and epiA (25µg) were added to another twenty, 25 ml aliquots of the same pooled urine and processed in an identical manner. The results of the relative recoveries of the added D.17-OS are recorded in Table 5. The results were not just as accurate as those for recoveries from water, but are still satisfactory.

TABLE 5. Recovery of A, E, DHA and epiA added to urine.

STEROID ADDED	µg ADDED	RECOVERY BY SULPHATE PROCEDURE n	x	S.D	RECOVERY BY GLUCURONIDE PROCEDURE n	x	S.D
A	50	10	97	5.1	10	108.1	6.1
E	50	10	88.4	3.2	10	105.2	2.6
DHA	50	10	89.0	3.6	10	99.8	2.2
epiA	25	10	102.4	6.9	10	85.6	3.3

n - Number of determinations
x - Mean %.
S.D - Standard deviation%.

The mean % recovery was best for the glucuronides, where the only low values recorded were for epiA.C, and as this glucuronide has as yet to be demonstrated in significant amounts in normal urine, its low recovery relative to pregnenolone is of little interest. The mean % recoveries of A, E and DHA were low compared to the recoveries from water. However, the standard deviation in all cases was satisfactory. Thus, although the relative recoveries were not 100% the reproducibility of the recoveries was good.

ii. Absolute recoveries. The absolute recoveries of the internal standards was achieved by adding tritium labelled pregnenolone sulphate or tritium labelled pregnenolone to urine samples and estimating the recovery of radioactivity at various stages in the method.

7α-^{3}H-pregnenolone sulphate was diluted with non-radioactive pregnenolone sulphate and 50µg (containing 148,080 dpm) were added to each of ten, 25 ml aliquots of pooled urine and processed according to the method described for steroid sulphates. Recoveries were estimated after solvolysis (step 1) and after column chromatography (step 2). The results are shown in Table 6. The overall mean recovery of 85.0% for added ^{3}H-pregnenolone sulphate was very satisfactory, especially as the precision was very good. The variation coefficient was 3.5%.

7α ^{3}H-pregnenolone was diluted with non-radioactive pregnenolone and 50µg (containing 64,375 dpm) were added to each of ten, 25 ml aliquots of pooled urine and taken through the method described for steroid glucuronides. Recoveries were measured after extraction of β-glucuronidase incubate (step 1) and after column chromatography (step 2). The results are presented in Table 6. The mean recovery of the added ^{3}H-pregnenolone (72.5%) was not as high as for the sulphate procedure. This is natural when the additional steps involved in the glucuronide procedure are taken into consideration. The precision was again very good with a variation coefficient of 4.2%.

4. Discussion.

The Zimmerman colour procedure for the estimation of 17-OS is an inexpensive, simple and readily available assay (M.R.C. Committee on Clinical Endocrinology Memorandum, 1963). However, total

TABLE 6. RECOVERY OF 7α-^{3}H-PREGNENOLONE SULPHATE (64,375 dpm) AND 7α-^{3}H-PREGNENOLONE (148,080 dpm) ADDED TO URINE.

METHOD		Recovery Step 1.			Recovery from Step 1. to Step 2.			Overall Recovery	
	n	dpm	x	S.D	dpm	x	S.D	x	S.D
^{3}H-preg.SO4	10	137,927	93.3	4.1	125,521	91.2	3.7	85.0	3.5
^{3}H-preg.	10	49,712	77.3	3.5	46,728	94.1	5.4	72.5	4.2

n. Number of determination.

dpm. Mean disintegrations/min.

x Mean recovery %.

S.D Standard deviation %.

Zimmerman chromogens are of limited physiological significance, and often clinically are completely inadequate. When it is considered that Sonka et al (1964) found DHA totally absent from every case of gout studied and yet the values of their total urinary 17-OS were still within the normal range, it becomes apparent that fractionation into individual 17-OS is necessary.. And obviously further fractionation into individual 17-OS sulphates and glucuronides can only be an advantage.

Urinary D.17-OS are found conjugated with glucuronic acid and sulphuric acid. The hydrolytic methods used to cleave these ester linkages are very important, since isolation of the steroids without structural alteration is essential. Hot acid hydrolysis will cleave both 17-OS sulphates and glucuronides and although suitable for total Zimmerman routine assays, this hydrolysis is believed too vigorous for more delicate analyses. It causes pigmentation, non-steroidal chromgen formation, and degradation of certain steroids (Peterson & Zettner, 1963) in particular DHA is known to undergo decomposition (Cawley, Musser, Faucette, Beckloff & Learned, 1965). Vestergaard (1962) carefully re-investigated the use of mineral acids hydrolysis of glucuronides and concluded "hydrolysis with β-glucuronidase is at the present time a necessary step in analyses for individual 17-ketosteroids if maximal yields are sought", though other studies concerning steroid sulphates as well as glucuronides suggest that the use of low concentrations of perchloric acid in organic solvents free of water will prove

satisfactory (Burnstein & Lieberman, 1958a; Jacobsohn, & Lieberman, 1962 and DePaoli, Nishizawa & Eik-Nes, 1963).

β-glucuronidase is specific for only the glucuronide moiety of glucuronate conjugates, therefore the extent of hydrolysis by the β-glucuronidase is dependent on the enzyme to glucuronate ratio irrespective of the nature of the non-glucuronide portion of the conjugate. It is, therefore, of prime importance that patients be taken off medication, the by-products of which may be metabolised to glucuronates and, therefore, compete with steroid glucuronides for a site on the β-glucuronidase, e.g. salicylates (Stempfel, Sidbury & Migeon, 1960). The β-glucuronidase preparation obtained from limpets contains an appreciable amount of steroid sulphatase, an enzyme which shows preference for sulphate conjugates which have a 3β-hydroxy,Δ^5- or a 3β-hydroxy-5α- structure (Roy, 1956). DHA and epiA are, therefore, substrates for the enzyme. However, phosphate, a potent inhibitor of steroid sulphatase, was included in all the β-glucuronidase incubates.

The 17-OS sulphates were extracted and then solvolysed by a procedure specific for sulphate conjugates and without effect on glucuronides (Jacobsohn & Lieberman, 1962) and phosphates (Burnstein & Lieberman, 1958a). This procedure also proved to be efficient for the extraction and cleavage of the internal standard pregnenolone sulphate. It gave 93.3% mean recovery for ^{3}H-pregnenolone after the addition of ^{3}H-pregnenolone sulphate to the urine (Table 6). The

93.3% recovery was assumed to be for the free steroid and not sulphate since subsequent alkali and water washes contained no radioactivity.

Although there have been many applications of gas-liquid-chromatography to the measurement of steroids in biological fluids, the limitations of the technique have not always been fully appreciated. Unlike colorimetry, where a specific steroid or group of steroids can be quantitated, without interference in presence of other dissimilar steroids, in gas chromatography all organic substances in the eluting gas are detected and measured by the flame ionisation detector. Because of the sensitive detectors employed in gas-liquid-chromatography, the presence of interfering substances is of great concern. For this reason, pre-purification of biological extracts before gas chromatography is of the utmost importance. Where urinary steroids are present in high concentrations, e.g. urinary pregnanediol in pregnant subjects, the pregnanediol concentrations may be so much larger than any extracted urinary contaminants having retention times similar to pregnanediol, that it may be analysed without further purification. Thus, Seegar-Jones, Turner, Sarlos, Barnes & Cohen (1962) and Cox (1963) have measured pregnanediol in pregnancy urine after hydrolysis and extraction only. However, when these relatively simple methods were applied to non-pregnant subjects' urine, the chromatograms

obtained showed poorly separated, ill defined peaks and the resulting precision of the method was very bad.

This example clearly confirms the necessity for pre-purification of urinary extracts before gas chromatography. In the method presented, alumina column chromatography was used as the purification step, as it was simple, rapid and allowed the D.17-OS to be eluted together in a small distinct polarity group. This, permits subsequent analysis of the steroids to be carried out under optimum conditions. Recovery experiments using tritium labelled steroids show that losses incurred by introducing alumina column purification were small, 8.8% and 5.8% for steroid sulphate and glucuronide procedures respectively (see Table 6). The gas chromatographic tracings of normal human urine processed by the present method are devoid of interfering substances as is amply demonstrated by fig. 3 which is comparable even with a gas chromatographic tracing of pure steroids (fig. 4). The precision of the method is to a certain extent determined by the quality of the gas chromatographic tracings. They should show clear sharp peaks free from extraneous detector responses which cause "shoulders" and unsettled baselines. The clear tracings obtained by the present procedure confirm the satisfactory precision data recorded in Table 3. This reproducibility was maintained in the analysis of normal male and female urine specimens, where in general duplicate estimations of D.17-OS.S and D.17-OS.G still retained a variation coefficient

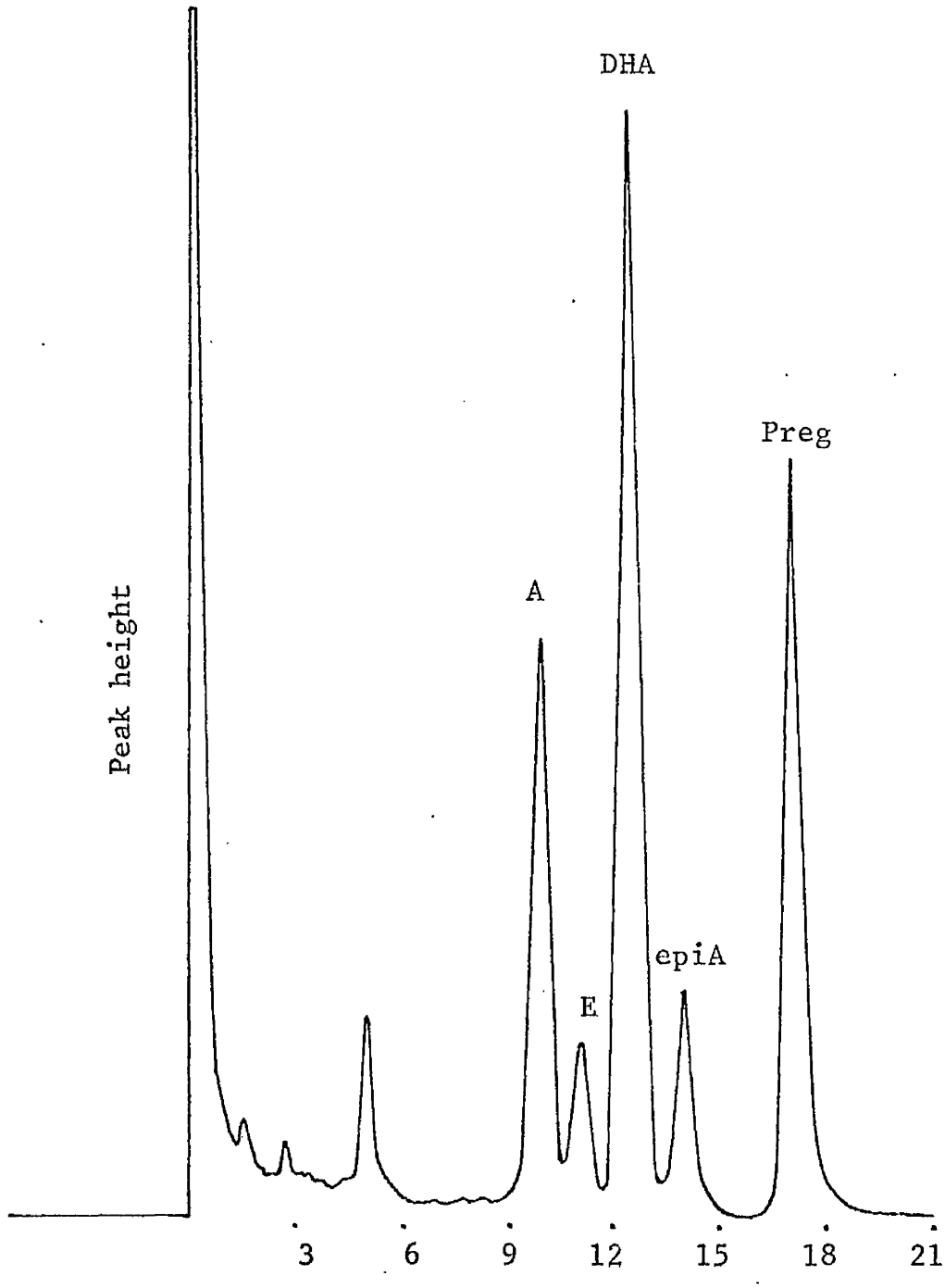


Fig. 3. Gas chromatographic tracing of the D.17-OS sulphate fraction obtained from a normal male urine. 3% QF-1 column at 200°. A) Androsterone, E) Aetiocholanolone, DHA) Dehydroepiandrosterone, epiA) Epiandrosterone and Preg) Pregnenolone all as TMSE derivatives.

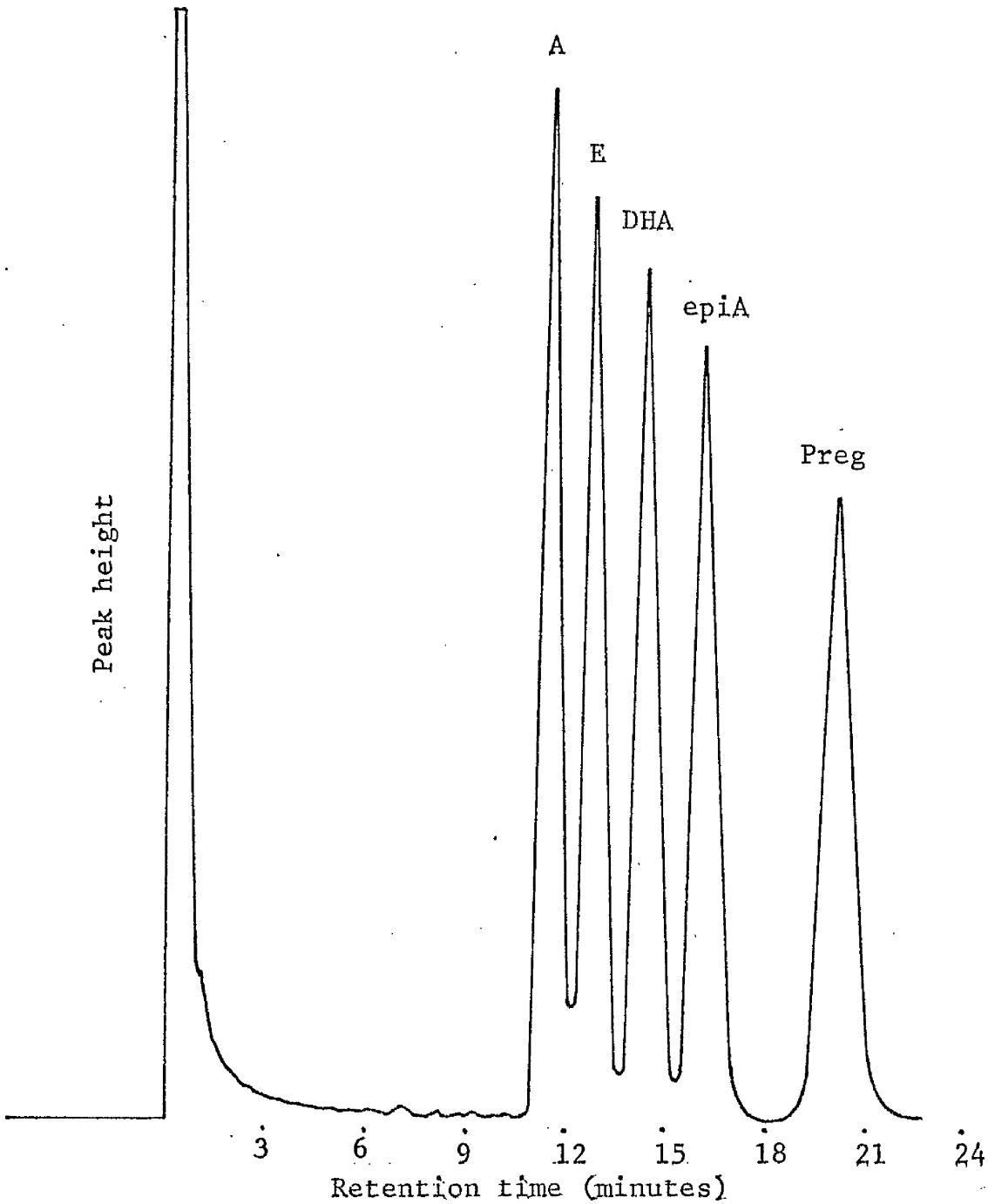


Fig. 4. Gas chromatographic tracing of a mixture of equal weights of standard D.17-OS and internal standard pregnenolone. 3% QF-1 column at 200°. A) Androsterone, E) Aetiocholanolone, DHA) Dehydroepiandrosterone, epiA) Epiandrosterone and Preg) Pregnenolone all as TMSE derivatives.

within 9.0% for all the steroids investigated.

When the present method was applied to 24 hour urines collected from normal males and females, and from patients suffering from a variety of different complaints, total urinary 17-OS were first estimated in all cases. This acted as guide as to the amount of internal standard to be introduced in each 25 ml urine sample prior to analysis. This procedure was found to be essential as the peak heights of the individual D.17-OS TMSE relative to the pregnenolone TMSE peak height had to be within prescribed limits (see figs. 2a &b). The importance of maintaining the proper ratios in all quantitative work utilising gas chromatography was pointed out by Horning, Maddock, Anthony & VandenHeuvel (1963). The linearity of the detector response for varying amounts of pregnenolone TMSE relative to constant amounts of individual D.17-OS TMSE and vica versa covered a range of 0.1 to 10. This proportionality of internal standard to the D.17-OS was determined regularly and re-estimated if necessary (Table 2).

The necessities for an internal standard reside in the difficulty of injecting exact microliter amounts of solvent into a gas chromatograph and in the importance of correcting for losses which occur during isolation steps. It was for these reasons that pregnenolone and pregnenolone sulphate were introduced as internal standards at the beginning of the glucuronide and sulphate procedures. Pregnenolone sulphate was a very satisfactory internal standard correcting not only for losses, but also checking on the

solvolysis of the steroid sulphates. Unfortunately, up to the present time pregnenolone glucuronide is not commercially available, and free pregnenolone was used as the internal standard for the glucuronide fraction. In order to check on a routine basis the β-glucuronidase hydrolysis, a pooled urine sample of known individual D.17-OS concentrations was repeated with each batch of analysis. The validity of using a single internal standard for the measurement of four D.17-OS was demonstrated by the recovery experiments (Tables 4 & 5). The recoveries of steroids added to water were very good, the relative recoveries in all cases being very close to 100% and precision with which that exercise was carried out was also very satisfactory, only in the case of added A, and its recovery as A.G, was the precision data (recovery 96.8 + 9.3%) not just as satisfactory. The recoveries of the same steroids added to urine were not just as efficient, however in all cases the precision with which the recoveries were carried out was very satisfactory. It could be concluded that the results of the recovery experiments justified the use of pregnenolone and pregnenolone sulphate as internal standards.

D. Statistical Procedures.

1. Tests of significance.

(a) Student's t-test. The student's t-test is used to compare the means of two independent samples and from the result conclude if the samples are from the same or different populations.

Given a sample of n values with $x_1, x_2, x_3, \ldots\ldots\ldots x_n$ denoting the sample values, with a sample mean $\bar{x} = \frac{1}{n}\Sigma(x)$ and sample estimate of variance $S^2 = \frac{1}{n-1}\Sigma\left[(x-\bar{x})^2\right]$.

To test the null hypothesis $H_0 : \mu_1 = \mu_2$ at α% significance against the alternative $H_1 : \mu_1 \neq \mu_2$. Therefore reject H_0 if $|t_{n-1}| > t_{(\alpha/2)}$ where $t_{n-1} = \frac{(\bar{x}-\mu)\sqrt{n}}{S}$ and α is obtained from the "t-distribution" tables. The conditions which must be satisfied to make the Student t-test a powerful one, and in fact before any confidence can be placed in any probability statement obtained by the use of the t-test, are at least the following.

1. The values must be independent.

2. The values must be drawn from normally distributed populations.

3. These populations must have the same variance or in special cases, they must have a known ratio of variances.

(b) The Mann-Whitney U test. The Mann-Whitney U test is used to test whether two independent samples have been drawn from the same population. This is one of the most powerful nonparametric tests and is used as an alternative to the parametric t-test when it is wished to avoid the assumptions of the t-test.

Method. Let n_1 = the number of values in the smaller of the two independent samples, i.e. sample 1.

n_2 = the number of values in the larger samples, i.e. sample 2.

Assign the rank of 1 to the lowest value in the combined $(n_1 + n_2)$ sample of scores. Calculate

$$U = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1$$

where R_1 = sum of the ranks assigned to sample 1.

It has been shown (Mann & Whitney, 1947) that as $n_1 n_2$ increases in size, the sampling distribution of U rapidly approaches the normal distribution with

$$\text{mean} = \frac{n_1 n_2}{2}$$

and standard deviation $= \sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{12}}$

That is when $n_2 \sim 20$ we may determine the significance of an observed value of U by

$$Z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{12}}}$$

which is practically normally distributed with zero mean and unit variance.

To test the null hypothesis $H_0 : \mu_1 = \mu_2$ at α% significance against the alternative hypothesis $H_1 : \mu_1 \neq \mu_2$. Therefore reject H_0 if $|Z| > Z_{(\alpha/2)}$, where α is obtained from tables of "The Normal Distribution Function".

2. Correlation and regression.

In measuring the association between two normal variables, it is convenient first to generalize the variance estimate used for measuring the variability of a single variable. Suppose then we have observed the variables x and y on n individuals, obtaining values x_1, y_2; x_1, y_2; .....; x_n, y_n. The variance of x and y are estimated by

$$S^2_x = \frac{\Sigma(x-\bar{x})^2}{n-1}$$

$$S^2_y = \frac{\Sigma(y-\bar{y})^2}{n-1}$$

A simple first measure of the association between x and y is the covariance, which is estimated by

$$C_{xy} = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{n-1}$$

The covariance estimate is a simple measure of association, but it is not easy to interpret because it depends on the scale on which the variables are measured. A more useful quantity is the coefficient of product - moment correlation (correlation coefficient) estimated by

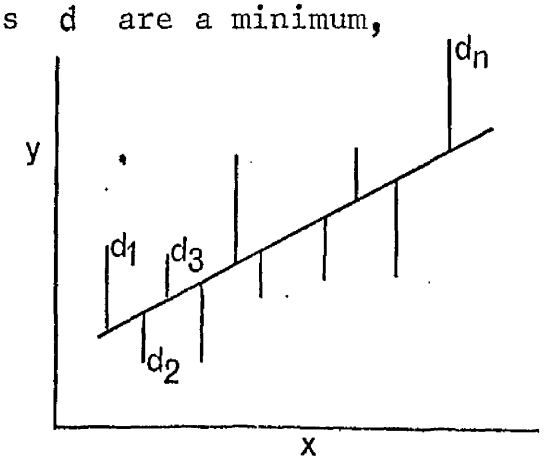
$$r = \frac{\text{Covariance}}{\text{S.D of x} \times \text{S.D of y}} \qquad \frac{C_{xy}}{S_x S_y}$$

It may be shown that the correlation coefficient never exceeds 1 in numerical value, i.e., $-1 \leq r \leq 1$. When r = 1 the sample relationship is a straight line with x and y increasing together and when r = -1

the relationship is again a straight line with, y now decreasing when x increases. Intermediate values of r are less easy to interpret, however, in general it may be said that values of r near to +1 or -1 indicate a close relationship with little departure from linearity.

The correlation coefficient tells us how close the relationship is, but does not indicate which straight line best represents the relationship. This is determined by linear regression analysis.

Linear regression is applicable to data in the form of pairs of observations y_1,x_1; y_2,x_2;.....; y_n,x_n, where y (the dependent variable) is not random. Let the line best fitted to pass through these n points be $y = \beta x + \alpha$. The parameters of the line β and α cannot be estimated simply from the plotted n points as these will deviate randomly from the above line. The "best" estimates of these parameters are those obtained such that the sum of the squares of quantities d are a minimum,



that is, such that $S = \Sigma(y-\alpha-\beta x)^2$ is a minimum. The least

squares estimators of α and β are

$$\hat{\alpha} = \bar{y} - \hat{\beta}\bar{x}$$

$$\hat{\beta} = \frac{\Sigma x.y - n.\bar{x}.\bar{y}}{\Sigma x^2 - n\bar{x}^2}$$

If we make the further assumption that the y's are each distributed normally and independently with common variance σ^2, we may go on to test the null hypothesis.

$$H_0 : \beta = \beta_0 \quad \text{with } \beta_0 = 0.$$

This gives an anser to the question ' is there any linear relationship between the variables x and y. The estimate $\hat{\beta}$ is a linear combination of the y's, and hence a normal random variable. In fact

$$\hat{\beta} \sim N\left(\beta, \frac{\sigma^2}{\Sigma(x-\bar{x})^2}\right)$$

i.e. $$\frac{(\hat{\beta}-\beta)\sqrt{\Sigma(x-\bar{x})^2}}{\sigma} \sim N(0,1)$$

In order to test $\beta = \beta_0$, an estimate of σ^2 is required. The statistic S^2 is used were

$$(n-2)S^2 = \Sigma y^2 - n.\bar{y}^2 - \hat{\beta}^2 (\Sigma x^2 - n.\bar{x}^2)$$

This expression, which is the sum of squares of deviations from the least squares regression line, is called the residual sum of squares, and it has expected value of $(n-2)\sigma^2$.

We now have that $t = \dfrac{(\hat{\beta} - \beta_0)\sqrt{(\Sigma x^2 - n.\bar{x}^2)}}{S}$

is a t-variate with n-2 degress of freedom under the null hypothesis, and so we may test H_0 by the critical region

$|t| \geq t_\rho$ where t_ρ is the appropriate percentage point of the t-distrobution.

In the studies presented in this report the data for correlation, linear regression and t-test analysis were all processed on the ICL KDF9 computer at Glasgow University. Since a computer procedure was employed it was decided to analyse the data thoroughly. Therefore, in each study all the available variables on subjects studied were processed in all the possible combinations of two. In each pair of variables there should be one a dependent and the other an independent variable. This criterion was not strictly observed in order that any hitherto unrecognised correlations would be revealed.

Some of the results presented show no obvious reason why one variable should be dependent on the other, but the fact there was lack of information on this matter does not mean that relationship does not hold. Also, when it is considered that many of correlations revealed were numerically very high ($r > \pm 0.9$), then it seemed expedient to present them.

3. Multivariate analysis.

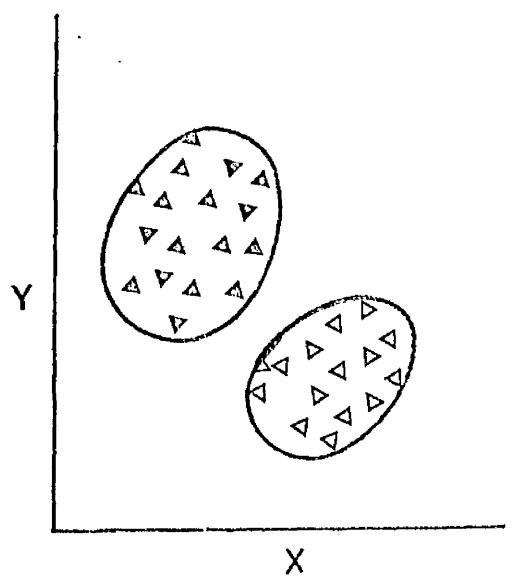
Univariate or bivariate observations are readily visualised

by 1 dimensional (1.D) and 2.D scatter diagrams. However, with trivariate observations this is very difficult and with hexavariate, septavariate, etc., observations it is impossible. Therefore, the problem is that of multivariate discrimination:- given a sample of members from each of k populations, and the values of h variables for each member, then if presented with a further member together with the appropriate values for h variables, a method is necessary for correct assignment of the new member to one of the k populations.

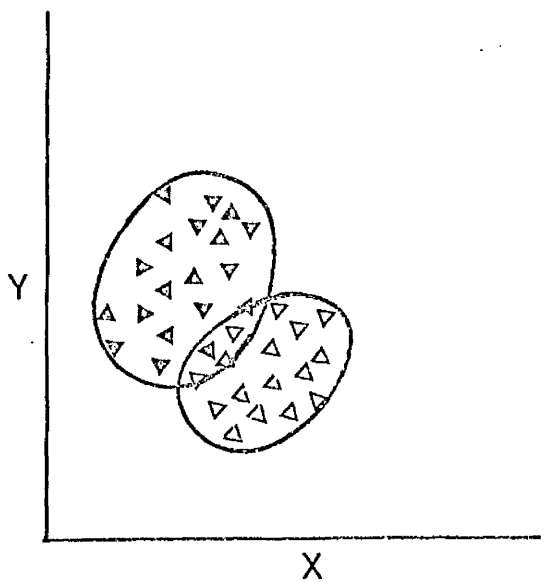
In the present study multivariate discrimination analysis will be used purely in an attempt to discriminate between normal individuals and patients suffering from various vascular diseases on the basis of individual D.17-OS urinary levels, with expectation in future of classifying new individuals using the same variables.

The method is that of O'Muircheartaigh (1968) and essentially consists of drawing circumscribing ellipsoids around each cluster of points obtained for each group of subjects and examining the degree of overlap. The individual points are obtained by manipulation of the values of the variables on that subject and within reason there is no limit on the number of variables per subject. The ellipsoids are special in that they are centered at the mean of the cluster and their orientation is determined by the orientation of the data. They are "drawn"

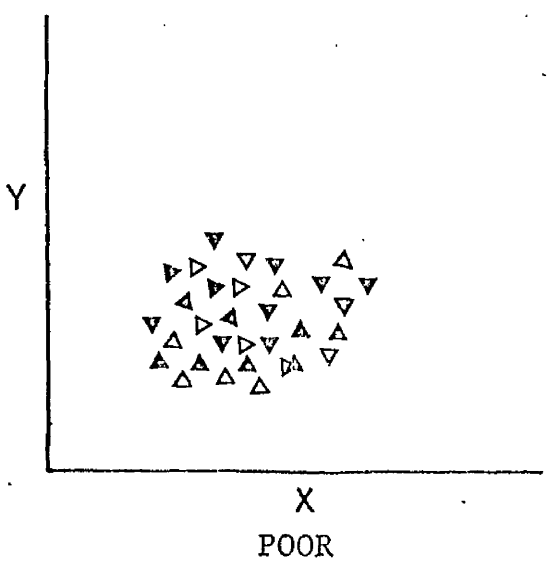
to go through the outermost points of the cluster. The examples below show in 2.D. what would be classed as good, fair and poor discrimination.



GOOD



FAIR



POOR

V. EXPERIMENTAL PART II.

A. Individual Urinary D.17-OS Sulphate and Glucuronides: Values in Normal Males and Females.

1. Subjects studied.

Thirty-seven subjects were studied. There were 22 males with a mean age of 44 ± 9 years (range 29-69) and 15 females with a mean age of 41 ± 9 years (range 25-58). All were free from disorders known to influence urinary 17-OS levels such as thyroid disease (Hellman et al, 1959), gout (Sonka et al, 1965 and Beck et al, 1967), fever (Kappas & Palmer, 1965), obesity (Hendrikx et al, 1965 and Lopez-S & Krehl, 1967), diabetes mellitus (Charro-Salgado et al, 1968 and Sonka & Gregorova, 1964), and other endocrine abnormalities (Loraine & Bell, 1966). Obesity was calculated in terms of % overweight from the tables compiled by Kemsley, Billewicz & Thomson (1962).

All subjects had normal renal function as estimated by blood urea and serum creatinine, and all had normal blood pressure. They were mainly members of staff or ambulant patients awaiting minor surgical procedures. All subjects were on normal diets and none were taking medication of any sort for one week prior to the study.

2. Analytical techniques.

Collection of blood and urine	-	Section IV A.5.
Blood urea & serum creatinine	-	Section IV A.2.a & b.
Serum uric acid	-	Section IV A.4.

17-OS & 17-OHCS. - Section IV A.6.a & b.

D.17-OS.S & D.17-OS.G. - Section IV C.2.

All analysis were duplicated.

3. Results.

The values for the individual urinary D.17-OS sulphates and glucuronides levels in males and females are shown in Tables 7 & 8. There was a wide range of values in both sexes for all the steroids studied, especially for the two 3β-hydroxysteroids (epiA and DHA). Only in males could this wide range be correlated with age. The inclusion of the four youngest males ages 29-34 greatly elevated the mean levels of the individual D.17-OS. For example, they had a mean DHA.(S+G) level of 10.38 mg/day compared with an overall mean for the 22 males of 3.12 mg/day. In order to see the influence of these four males on the urinary D.17-OS levels Table 7 gives mean ± S.D. for D.17-OS with and without the values for the 4 youngest males.

(a) Sulphate conjugates. DHA.S was quantitatively the most important D.17-OS.S in 87% of the males and 40% of the females. In one of the other 13% (3) male subjects, E.S and A.S were both greater than DHA.S and in a further 2 subjects A.S alone was greater. The typical D.17-OS.S urinary excretion pattern for males was DHA >A>epiA>E, and is ullustrated in the gas chromatographic tracing in fig.5. Among the females 4 subjects had E.S and A.S both greater than DHA.S and 5 subjects with either E.S or A.S larger than or equal to DHA.S. Thus, the females showed no typical steroid sulphate excretion pattern other

TABLE 7. EXCRETION OF INDIVIDUAL URINARY D.17-OS BY NORMAL MALES (mg. FREE STEROID/24 hr.), AND SERUM URIC ACID LEVELS.

	AGE	DEHYDROEPIANDROSTERONE			EpiA	AETIOCHOLANOLONE			ANDROSTERONE			S.U.A.
		S	G	S+G	S	S	G	S+G	S	G	S+G	mg%
1	29	11.63	1.38	13.01	1.29	0.80	4.47	5.27	3.74	4.02	7.76	5.2
2	31	3.74	0.77	4.51	0.29	0.39	2.63	3.02	1.08	3.27	4.35	4.6
3	33	10.03	1.04	11.07	0.94	0.68	3.68	4.6	2.18	5.51	7.69	4.7
4	34	11.93	0.98	12.91	0.84	0.24	5.86	6.10	1.84	7.42	9.26	4.6
5	37	0.19	0.14	0.32	0.11	0.08	4.15	4.23	0.60	5.70	6.30	4.8
6	40	1.44	0.53	1.97	0.22	0.09	3.74	3.83	0.62	5.36	5.98	5.2
7	40	6.54	0.54	7.08	0.35	0.06	2.89	2.95	0.61	3.51	4.12	5.9
8	40	1.07	0.20	1.27	0.08	0.06	1.30	1.36	0.12	1.61	1.73	4.8
9	41	2.08	0.29	2.37	0.10	0.08	1.52	1.60	0.28	1.61	1.89	3.9
10	42	0.39	0.09	0.48	0.19	0.20	3.85	4.05	0.67	2.19	2.86	4.0
11	43	2.32	0.29	2.61	0.30	0.21	2.01	2.22	0.67	2.42	3.09	4.5
12	44	0.71	0.11	0.82	0.23	0.61	2.54	3.15	0.70	1.75	2.45	5.7
13	44	0.66	0.40	1.06	0.08	0.25	2.07	2.32	0.39	1.26	1.65	3.0
14	44	1.75	0.36	2.11	0.12	0.08	1.66	1.74	0.65	2.12	2.77	5.2
15	46	2.69	0.31	3.00	0.43	0.21	4.02	4.23	1.16	6.46	7.62	7.3
16	48	1.2	0.17	1.37	0.43	0.25	2.25	2.50	1.02	3.15	4.17	7.1
17	48	0.07	0.06	0.13	0.01	0.03	0.36	0.39	0.05	1.48	1.53	5.3
18	52	0.58	0.17	0.75	0.07	0.05	1.02	1.07	0.28	1.85	2.13	5.3
19	55	0.06	0.04	0.10	0.06	0.21	1.92	2.13	0.30	1.69	1.99	5.8
20	57	0.46	0.13	0.59	0.13	0.07	2.97	3.04	0.33	2.63	2.96	5.6
21	61	0.14	0.03	0.17	0.06	0.24	1.03	1.27	0.13	0.86	0.99	3.8
22	69	0.82	0.12	0.94	0.22	0.21	1.60	1.81	0.56	1.25	1.81	4.9
MEAN	44	2.75	0.37	3.12	0.30	0.23	2.61	2.89	0.82	3.05	3.87	5.1
S.D.	9.8	3.75	0.36	4.07	0.33	0.21	1.36	1.36	0.84	1.89	2.46	1.0
RANGE	29-69	0.07-11.93	0.04-1.38	0.13-13.01	0.01-1.29	0.05-0.80	0.36-5.86	0.39-6.10	0.05-3.74	0.86-7.42	0.99-9.26	3.0 - 7.3
*MEAN	47.3	1.29	0.22	1.51	0.18	0.17	2.22	2.44	0.51	2.69	3.11	5.1
S.D.	8.5	1.53	0.15	1.65	0.129	0.136	1.11	1.15	0.300	1.73	1.85	0.9
RANGE	37-69	0.07-6.54	0.04-0.54	0.13-7.08	0.01-0.43	0.05-0.61	0.36-4.15	0.29-4.23	0.05-1.16	0.86-6.46	0.99-7.62	3.0 - 7.3

* Gives mean, S.D. and range for subjects 5 - 22.

TABLE 8. EXCRETION OF INDIVIDUAL URINARY D.17-OS BY NORMAL FEMALES (mg.FREE STEROID/24 hr.), AND SERUM URIC ACID LEVELS.

SUBJECT	AGE	DEHYDROEPIANDROSTERONE			EpiA	AETIOCHOLANOLONE			ANDROSTERONE			S.U.A.
		S	G	S+G	S	S	G	S+G	S	G	S+G	mg%
1	25	0.29	0.08	0.37	0.07	0.23	1.33	1.56	0.51	1.77	2.28	8.3
2	30	0.12	0.08	0.20	0.02	0.12	0.72	0.84	0.09	0.85	0.94	3.4
3	33	0.04	0.3	0.34	0.01	0.03	0.16	0.16	<0.01	0.10	0.10	5.4
4	33	1.03	0.29	1.32	0.04	0.11	0.88	0.99	0.11	1.10	1.21	3.5
5	36	0.03	0.02	0.05	0.01	0.28	1.15	1.43	0.05	0.4	0.45	3.2
6	40	0.04	0.02	0.06	0.01	0.03	0.37	0.40	0.04	0.20	0.24	3.0
7	40	0.04	0.02	0.06	0.02	0.02	0.35	0.37	0.01	0.17	0.18	3.8
8	42	0.14	0.07	0.21	0.07	0.62	1.70	2.32	0.36	1.22	1.58	4.3
9	42	0.05	0.06	0.11	0.04	0.05	0.39	0.44	0.07	0.30	0.37	4.0
10	44	3.85	0.92	4.77	0.12	0.16	1.76	1.92	0.20	1.42	1.62	5.9
11	45	0.85	1.98	2.83	0.18	0.21	3.23	3.44	0.30	2.36	2.66	3.2
12	46	0.09	0.04	0.13	0.05	0.19	1.64	1.83	0.28	1.41	1.69	4.7
13	46	0.06	0.05	0.11	0.02	0.02	0.29	0.31	0.02	0.11	0.13	3.1
14	53	0.24	<0.01	0.24	0.05	0.23	1.74	1.97	0.24	1.21	1.45	3.9
15	58	0.11	0.19	0.30	0.03	0.18	1.18	1.36	0.19	0.41	0.60	3.3
MEAN	41	0.45	0.27	0.67	0.05	0.16	1.12	1.28	0.16	0.87	1.03	4.2
S.D.	9	0.99	0.52	1.33	0.05	0.15	0.82	0.91	0.15	0.69	0.82	1.4
RANGE	25-58	0.03-3.85	<0.01-1.98	0.06-4.77	0.01-0.18	0.02-0.62	0.16-3.23	0.16-3.44	<0.01-0.51	0.10-2.36	0.10-2.66	3.1-8.3

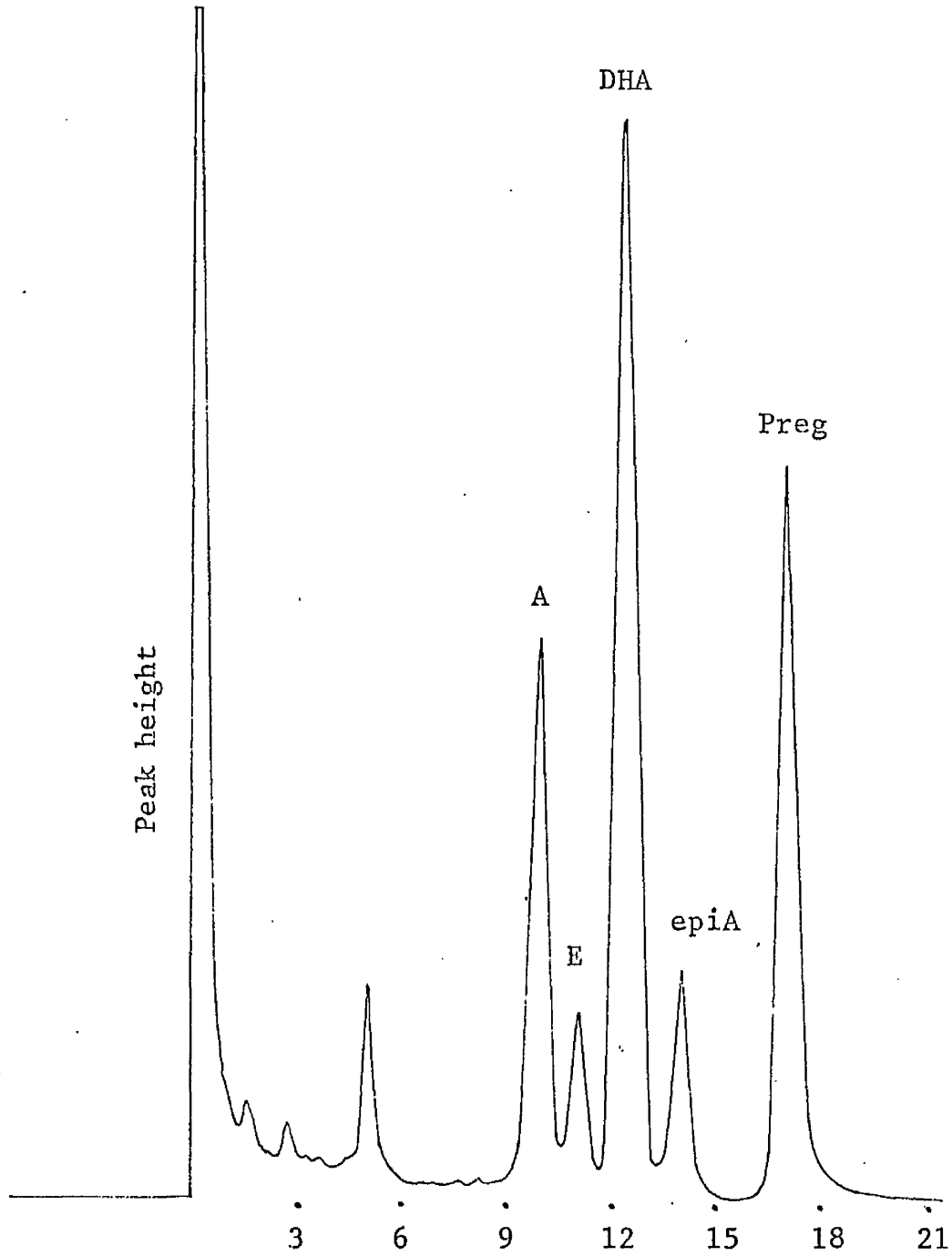


Fig. 5. Gas chromatographic tracing of the D.17-OS sulphate fraction obtained from a normal male urine. 3% QF-1 column at 200°. A) Androsterone, E) Aetiocholanolone, DHA) Dehydroepiandrosterone, epiA) Epiandrosterone and Preg) Pregnenolone all as TMSE derivatives.

than that epiA.S was the minor steroid sulphate. Males had consistently much higher urinary D.17-OS levels than females, with the exception of E.S where the mean values for males and females were 0.23 and 0.16 mg/day respectively (Table 9).

(b) Glucuronide conjugates. The urinary levels of A.G and E.G were much higher for males than for females, with less significant statistical difference for DHA.G. The predominant glucuronide in 66% of the males was A.G and E.G in 75% of the females. The typical steroid glucuronide excretion patterns in males (A>E>DHA) and in females (E>A>DHA) are illustrated by the gas chromatographic tracings shown in figs 6 & 7. One subject, a female was unique in having DHA as her major urinary glucuronide. It may be relevant that this female had a history of rheumatic heart disease. No values for epiA.G are given since in all instances peak heights were negligible and gross variation in retention time occurred.

(c) Sulphate plus glucuronide conjugates. In all cases, the D.17-OS.(S+G) urinary levels for males were much higher than the respective levels for females (Table 9). Seventy-seven % (17) of the 22 males had urinary levels of A.(S+G) greater than those for E.(S+G), whereas 80% (12) of the 15 females had E.(S+G) greater than A.(S+G). In both sexes the urinary levels of DHA(S+G) tended to be much lower than those for either E.(S+G) or A.(S+G), except among the younger males and females, where 6 of the 9 youngest males and 2 of the 4 youngest females had DHA.(S+G) as their major urinary D.17-OS conjugate.

TABLE 9. COMPARISON OF THE INDIVIDUAL D.17-OS URINARY LEVELS OF NORMAL ADULT MALES AND FEMALES.

	MALES MEAN ± S.D.	FEMALES MEAN ± S.D.	P VALUE
epiA.S	0.30 ± 0.326	0.05 ± 0.047	0.0008
DHA.S	2.75 ± 3.75	0.45 ± 0.99	0.0008
E.S	0.23 ± 0.212	0.16 ± 0.150	0.2
A.S	0.82 ± 0.838	0.16 ± 0.149	0.00006
DHA.G	0.37 ± 0.365	0.27 ± 0.520	0.044
E.G	2.61 ± 1.36	1.12 ± 0.82	0.0008
A.G	3.05 ± 1.89	0.87 ± 0.691	0.00002
DHA.(S+G)	3.12 ± 4.07	0.67 ± 1.330	0.0028
E.(S+G)	2.89 ± 1.36	1.28 ± 0.912	0.0012
A.(S+G)	3.87 ± 2.46	1.03 ± 0.823	0.00002
17-OS	12.3 ± 5.3	6.1 ± 3.7	<0.001
17-OHCS	12.7 ± 3.7	7.6 ± 3.4	<0.001

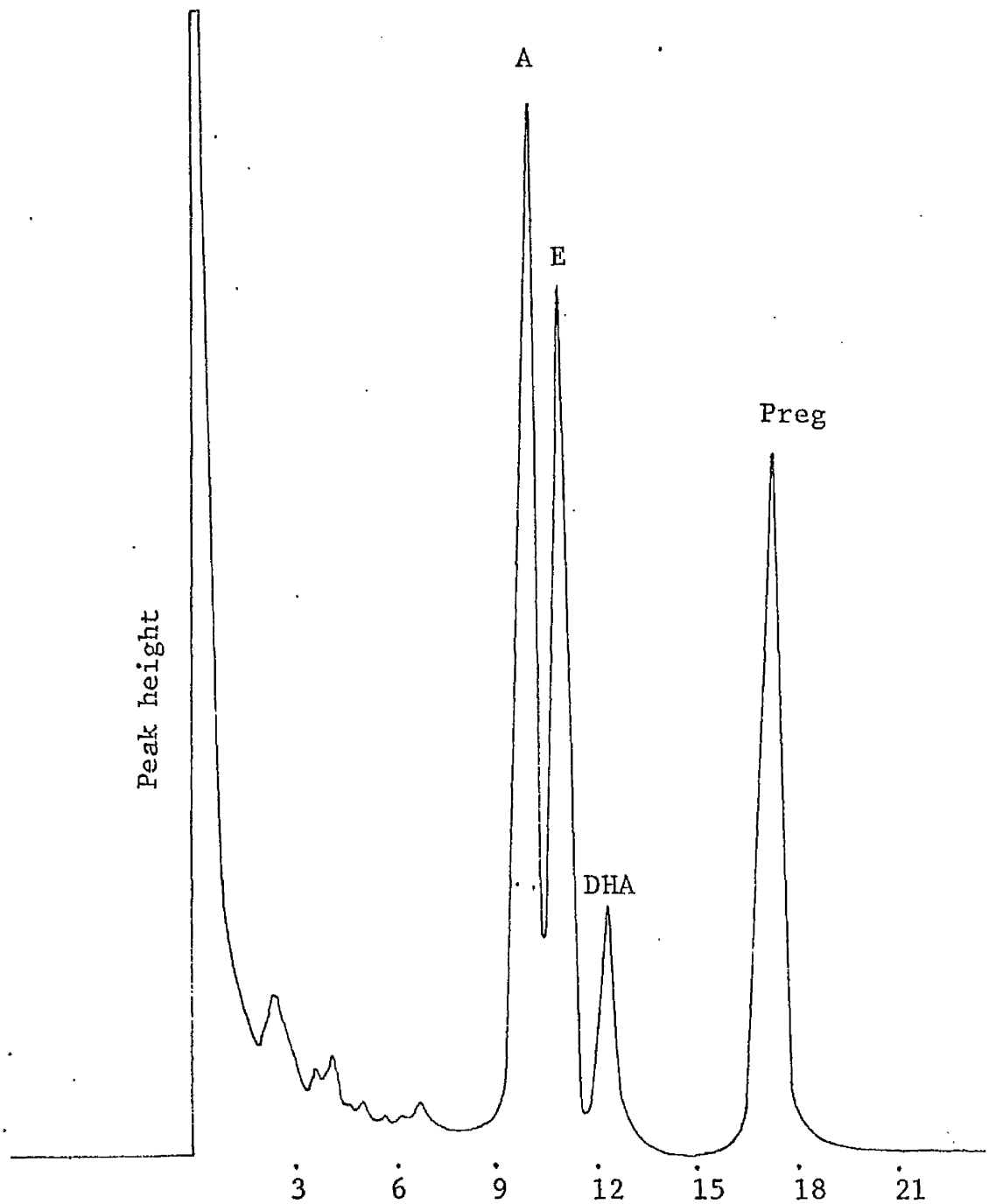


Fig. 6. Gas chromatographic tracing of the D.17-OS glucuronide fraction obtained from a normal male urine. 3% QF-1 column at 200°. A) Androsterone, E) Aetiocholanolone, DHA) Dehydro-epiandrosterone, epiA) Epiandrosterone and Preg) Pregnenolone all as TMSE derivatives.

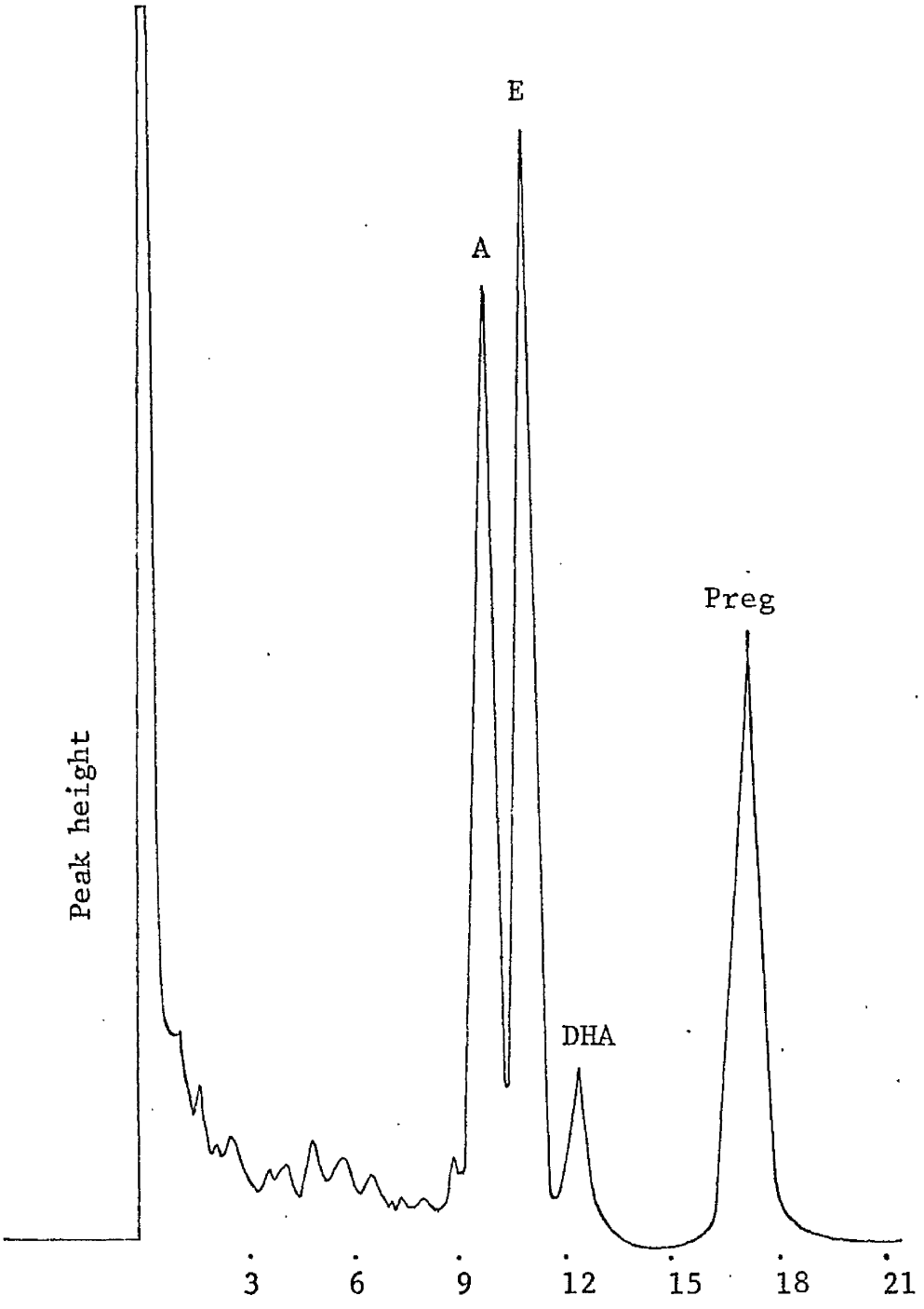


Fig. 7. Gas chromatographic tracing of the D.17-OS glucuronide fraction obtained from a normal female urine. 3% QF-1 column at 200°. A) Androsterone, E) Aetiocholanolone, DHA) Dehydro-epiandrosterone, epiA) Epiandrosterone and Preg) Pregnenolone all as TMSE derivatives.

(d) Correlations. Correlation coefficients (Section IV.D.2.) were calculated by computer for any combination of 2 of the 19 variables collected for all the subjects. The variables involved were age; serum uric acid; serum creatinine (S.C.); 24 hour urine volume; surface area (S.A.); % overweight; mean blood pressure $\left[\text{diastolic} + {}^{1}/_{3}\ (\text{systolic} - \text{diastolic})\right]$; 17-OS; 17-OHCS; epiA.S; DHA.S; E.S; A.S; DHA.G; E.G; A.G; DHA(S+G) and A.(S+G). Correlation coefficients for the most highly correlated of the 19 variables are shown for normal males and females in Tables 10 & 11 respectively. Correlation coefficients significant at the 2% level or less are underlined.

Excretion of all the individual D.17-OS, excluding E.S, by male subjects showed statistical negative correlation with age, especially DHA.G were $r = -0.72 (P<0.001)$.

Some hitherto unrecognised correlations between individual D.17-OS were demonstrated. EpiA.S was highly correlated with all other D.17-OS sulphates and glucuronides and particularly so with DHA.S ($r = 0.92$) and A.S ($r = 0.97$). Fig. 8 illustrates the regression of epiA.S on A.S. The equation for the regression line is

$$\text{epiA.S} = 0.38\ \text{A.S} - 0.01 \qquad r = 0.97 \quad P<0.001.$$

The two steroids E.(S+G) and A.(S+G), whose ratio is continually referred to in the literature (Rubin, Dofrman & Pincus, 1954; Kellie & Wade, 1957; James, 1961 and Hamman & Martin, 1964, to mention a few)

TABLE 10. MATRIX OF CORRELATION COEFFICIENTS FOR NORMAL FEMALES.

	S.C	Urine Vol.	17-OS	17-OHCS	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	DHA.(S+G)	E.(S+G)	A.(S+G)
S.C	1.0													
Urine V.	0.44	1.0												
17-OS	0.31	0.62	1.0											
17-OHCS	0.40	0.37	0.75	1.0										
epiA.S	0.71	0.67	0.74	0.58	1.0									
DHA.S	-0.07	0.50	0.84	0.61	0.56	1.0								
E.S	0.49	0.14	0.28	0.34	0.32	0.00	1.0							
A.S	0.49	0.50	0.40	0.36	0.59	0.16	0.66	1.0						
DHA.G	0.62	0.55	0.62	0.47	0.88	0.51	0.03	0.24	1.0					
E.G	0.72	0.46	0.71	0.55	0.88	0.36	0.56	0.70	0.72	1.0				
A.G	0.61	0.56	0.68	0.58	0.85	0.40	0.46	0.82	0.63	0.89	1.0			
DHA.(S+G)	0.19	0.58	0.86	0.63	0.76	0.94	0.01	0.21	0.77	0.55	0.54	1.0		
E.(S+G)	0.73	0.44	0.68	0.55	0.84	0.32	0.67	0.74	0.65	0.99	0.88	0.50	1.0	
A.(S+G)	0.61	0.56	0.65	0.56	0.82	0.37	0.51	0.88	0.57	0.88	0.99	0.50	0.87	1.0

Correlation coefficients significant at 2% level or less are underlined.

TABLE 11. MATRIX OF CORRELATION COEFFICIENTS FOR NORMAL MALES.

	AGE	S.A.	17-OS	17-OHCS	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	DHA.(S+G)	E.(S+G)	A.(S+G)
AGE	1.0													
S.A	-0.60	1.0												
17-OS	-0.51	0.43	1.0											
17-OHCS	-0.33	0.36	0.54	1.0										
epiA.S	-0.55	0.29	0.73	0.24	1.0									
DHA.S	-0.62	0.35	0.71	0.18	0.92	1.0								
E.S	-0.39	0.05	0.44	-0.02	0.74	0.57	1.0							
A.S	-0.58	0.25	0.68	0.21	0.97	0.85	0.79	1.0						
DHA.G	-0.72	0.30	0.69	0.29	0.88	0.93	0.63	0.88	1.0					
E.G	-0.56	0.52	0.74	0.46	0.68	0.65	0.37	0.67	0.62	1.0				
A.G	-0.55	0.44	0.66	0.57	0.61	0.62	0.18	0.56	0.58	0.86	1.0			
DHA.(S+G)	-0.63	0.35	0.72	0.19	0.92	0.99	0.58	0.86	0.94	0.65	0.62	1.0		
E.(S+G)	-0.58	0.49	0.76	0.43	0.74	0.69	0.49	0.74	0.67	0.99	0.83	0.71	1.0	
A.(S+G)	-0.62	0.42	0.74	0.51	0.80	0.77	0.41	0.77	0.74	0.89	0.96	0.95	0.89	1.0

Correlation coefficients significant at 2% level or less are underlined.

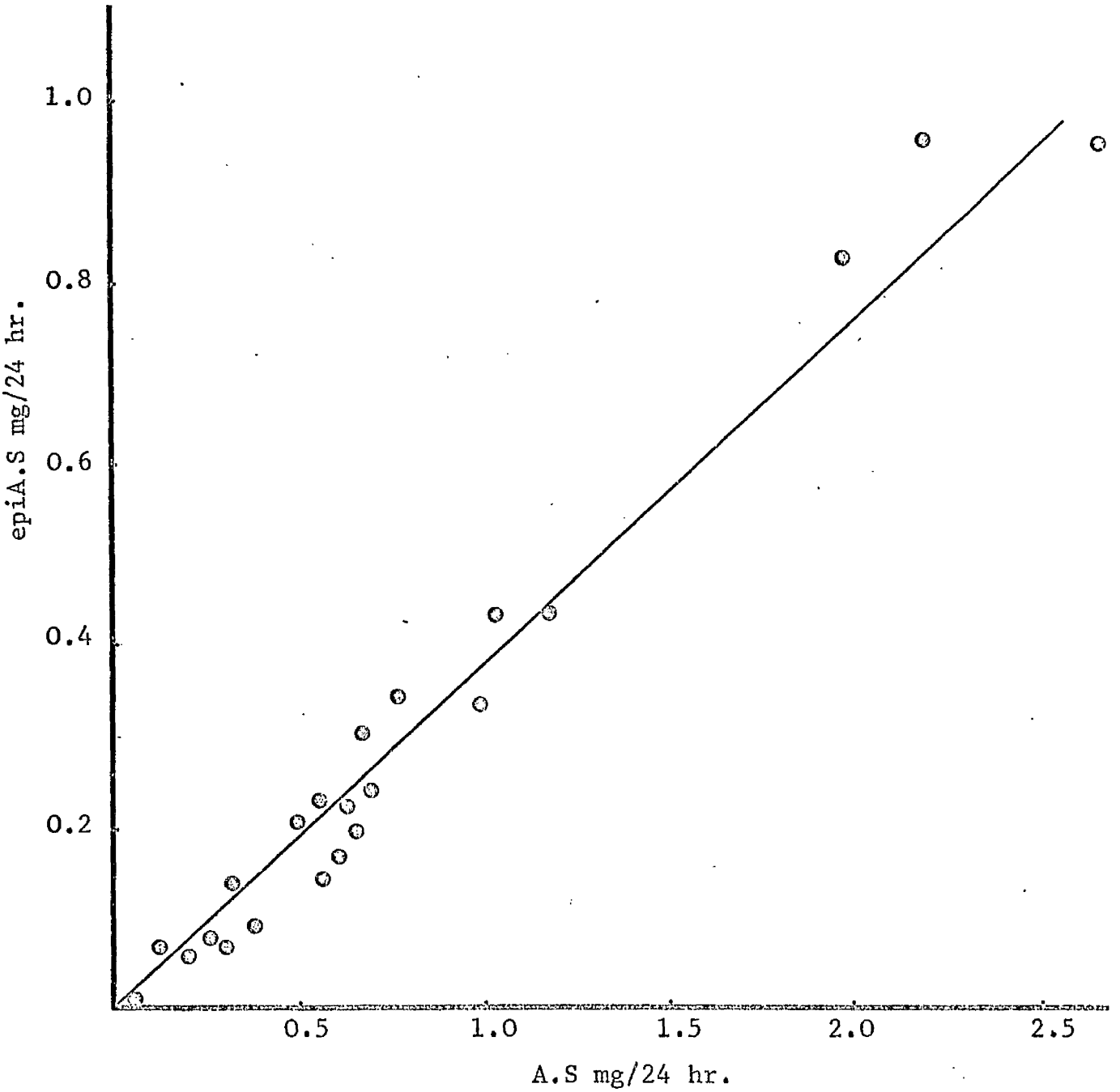


Fig. 8. Regression of epiA.S on A.S for normal males. The equation of the regression line is epiA.S = 0.38A.S - 0.01, r = 0.97, P<0.001.

showed a high degree of correlation ($r = 0.89$, $P<0.001$), as did their fractionation products, that is, E.S versus A.S ($r = 0.79$, $P<0.001$) and E.G versus A.G ($r = 0.86$, P 0.001).

Female urinary D.17-OS levels showed little correlation with age but showed surprisingly consistent and significant correlation with serum creatinine and urine volume. They also correlated well with total urinary 17-OHCS levels, unlike the males. The interrelationships of the individual D.17-OS were numerous though the degree of correlation was not just as high as it was for males. This, confirms and extends the observations that the gas chromatographic tracings for males tended to follow a more definite pattern than did those for females especially the female D.17-OS sulphate urinary patterns.

The simplest way of estimating the degree of interdependence of two variables from a set of data is by the constancy of the ratio of the two variables. The regression equation calculated from the same data demonstrates more accurately the degree of interdependence than does the constancy of the ratio of the two variables. Further, it demonstrates it in a more statistically sound fashion by means of r and P values. If the slope of the regression line passes through or close to the origin, arithmetically the value of the slope will be very similar to the above mentioned ratio.

Inspection of the male and female regression equations for E.S on A.S,

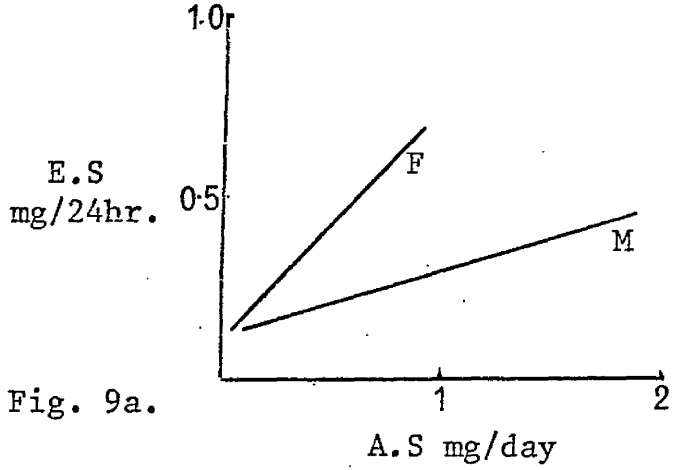


Fig. 9a.

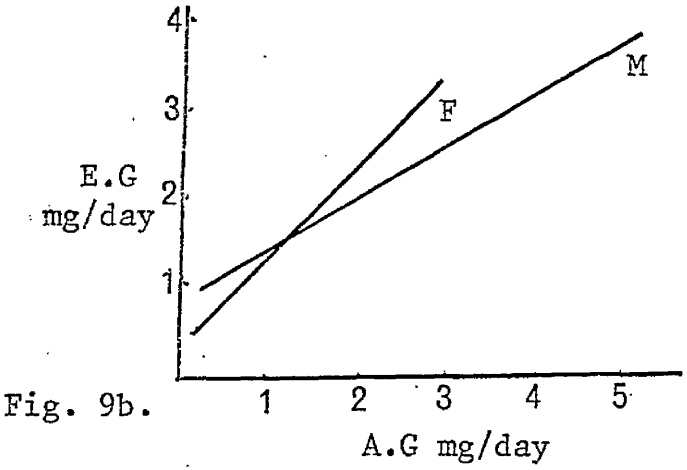


Fig. 9b.

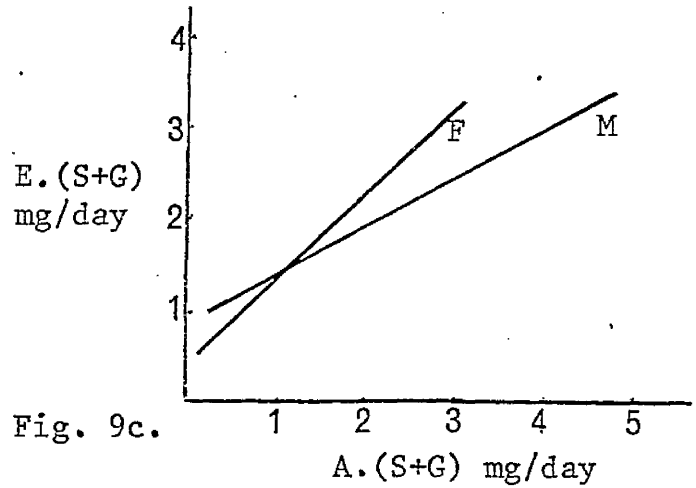


Fig. 9c.

Figs 9a,b & c. Regression of E.S on A.S, E.G on A.G and E.(S+G) on A.(S+G) for normal males (M) and normal females (F).

female E.S = 0.66 A.S + 0.058 r = 0.66 P<0.01

male E.S = 0.20 A.S + 0.068 r = 0.79 P<0.001

(fig.9a), shows that in both cases there is a high degree of correlation. The difference in the slopes of the lines 0.66 and 0.20 which are to a certain extent equivalent to the ratio of E.S/A.S indicates that there is more E.S relative to A.S excreted by females compared to males. This difference in the excretion of E relative to A by females and males for their sulphate conjugates is repeated for their glucuronide conjugates,

female E.G = 1.05 A.G + 0.22 r = 0.89 P<0.001

male E.G = 0.62 A.G + 0.73 r = 0.86 P<0.001

(fig.9b), and for the sum of their sulphate and glucuronide conjugates (fig.9c),

female E.(S+G) = 0.97 A.(S+G) + 0.29 r = 0.87 P<0.001

male E.(S+G) = 0.53 A.(S+G) + 0.81 r = 0.89 P<0.001

4. Discussion.

(a) Comparison of data with those of other investigators. Apart from the authors referred to in Table 12, previous investigators have reported the total amounts of individual urinary D.17-OS (Table 13) present without distinguishing between the contribution made by sulphate and glucuronide fractions. Some of the variations in results shown in Tables 12 & 13 may be due to choice of subjects or methodology. Aspects of methodology have been covered in Section IV.C.4.

TABLE 12. COMPARISON OF INDIVIDUAL URINARY D.17-OS.S AND D.17-OS.G LEVELS FROM FOUR DIFFERENT AUTHORS

(mg. FREE STEROIDS/24 hr.)

		AGE	SULPHATES				GLUCURONIDES			
			EpiA	DHA	E	A	EpiA	DHA	E	A
MALE:										
1	MEAN	44.4	0.30	2.75	0.23	0.82	0	0.37	2.61	3.05
*(22)	RANGE	29 - 69	0.01-1.29	0.07-11.93	0.05-0.80	0.05-3.74		0.04-1.38	0.36-5.86	0.86-7.42
2	MEAN	-	-	2.29	0.48	1.33	-	0.13	2.11	2.8
(9)	RANGE			0.47-4.55	0.16-0.88	0.47-3.24		0.08-0.21	0.92-3.61	1.91-3.56
3	MEAN	24.6	-	0.98	0.32	0.78	-	-	2.9	2.0
(5)	RANGE	20 - 29								
4	MEAN	30	-	0.91	0.39	0.72	-	0.29	3.81	2.88
(6)	RANGE	21 - 43		0.25-2.07	0.04-0.65	0.34-1.16		0.00-0.50	2.90-5.06	1.72-4.54
FEMALE:										
1	MEAN	41	0.05	0.45	0.16	0.16	0	0.27	1.12	0.87
(15)	RANGE	29 - 69	0.01-0.18	0.03-3.85	0.02-0.62	<0.01-0.51		<0.01-I.98	0.16-3.23	0.10-2.36
2	MEAN	-	-	0.89	0.59	0.77	-	0.12	1.62	2.06
(6)	RANGE			0.28-1.98	0.29-1.45	0.40-1.31		0.09-0.18	0.89-2.36	0.63-4.06
3	MEAN	27	-	0.24	0.36	0.14	-	-	2.1	1.0
(7)	RANGE	20 - 35								
4	MEAN	24	-	1.04	0.50	0.73	-	0.35	4.7	2.77
(6)	RANGE	18 - 29		0.57-1.40	0.24-0.76	0.54-1.21		0.07-0.80	3.57-5.44	2.38-3.23

* Number of people studied. - = No values given. 0 = None found.

1. Present report, internal standard, alumina column chromatography, GLC.
2. Menini (1966), oxidation with t.butyl chromate, GLC.
3. Mauvais-Jarvis & Baulieu (1962), paper chromatography, Zimmerman reaction.
4. Kellie & Wade (1957), gradient elution chromatography, Zimmerman reaction.

TABLE 13. COMPARISON OF INDIVIDUAL URINARY D.17-OS.(S+G) LEVELS FROM EIGHT DIFFERENT AUTHORS (mg free steroid/24 hr).

	1	2	3	4	5	6	7	8
AGE	29 - 69	18 - 43	40 - 49	40 - 49	-	-	20 - 30	-
MALES:								
EpiA	* 0.30	-	-	-	-	-	-	-
	0.01 - 1.29	-	-	-	-	-	-	-
DHA	3.12	* 2.3	§ 1.7	-	* 0.58	* 0.72	* 3.5	* 1.1
	0.13 - 13.01	0.8 - 6.4	3.14	-	0.03 - 1.66	0.2 - 1.71	0.4 - 9.3	0.2 - 5.7
E	2.89	2.8	4.4	-	1.5	2.5	3.3	5.0
	0.39 - 6.10	1.4 - 5.1	1.89	-	0.41 - 2.86	1.4 - 5.4	1.5 - 5.3	1.3 - 11.4
A	3.87	3.4	4.53	-	2.16	2.6	4.4	4.4
	0.99 - 9.26	1.5 - 6.0	1.33	-	0.75 - 3.82	2.1 - 3.8	2.4 - 6.7	0.6 - 7.9
FEMALES:								
EpiA	0.05	-	-	-	-	-	-	-
	0.01 - 0.18	-	-	-	-	-	-	-
DHA	0.67	1.1	0.21	0.53	1.05	0.56	2.1	0.5
	0.06 - 4.77	0.10 - 2.6	0.27	0.0 - 1.7	0.11 - 4.59	0.2 - 1.13	0.5 - 5.7	0.2 - 1.4
E	1.28	2.7	2.27	2.15	1.18	1.8	3.1	1.7
	0.16 - 3.44	0.7 - 4.3	1.19	0.8 - 7.6	0.12 - 2.77	0.6 - 2.2	1.7 - 5.5	0.6 - 4.0
A	1.03	3.1	2.04	1.1	1.37	1.4	2.9	2.0
	0.10 - 2.66	0.9 - 4.8	1.12	0.36 - 2.5	0.10 - 3.31	0.9 - 2.6	1.6 - 2.9	0.6 - 5.0

* mean & range: § mean & standard deviation: - no values given:

1. Present report, internal standard, alumina column chromatography - gas-liquid chromatography (GLC).
2. Fehér (1966), paper chromatography, Zimmerman reaction.
3. Keutmann & Mason (1967), internal standard, GLC.
4. Solomon et al (1967), silica gel column chromatography, GLC.
5. Rivera et al (1967), silica gel column chromatography, GLC.
6. Kirschner & Lipsett (1964a), thin layer chromatography, GLC.
7. Vestergaard (1962), column chromatography, Zimmerman reaction.
8. Cawley et al (1967), GLC.

Age and sex are important variables (Feher, 1966 and Keutman & Mason, 1967). Normal diets are necessary since low calorific diets can markedly decrease urinary D.17-OS excretion (Hendrikx et al, 1965 and Cawley et al, 1967). Finally a number of diseases are known to influence D.17-OS excretion (Hellman et al, 1959a,b; Sonka et al, 1964; Kappas & Palmer, 1965; Charro-Salgado et al, 1968 and Loraine & Bell, 1966).

A wide range of values for the individual D.17-OS in normal adult males and females was found by all the investigators listed in Table 13, thus confirming the findings of the present study. Since this appears to be a common finding by a variety of methods in various countries, it could be said that this is a definite characteristic of urinary D.17-OS levels in adults. The mean values for the individual D.17-OS found by the various investigators, though obviously more consistent, still show variation especially in the case of DHA. One constant feature of the results in Table 13 is that the mean urinary D.17-OS levels for males are always higher than the respective levels for females. This held for all steroids studied by all investigators, except for the DHA levels reported by Rivera et al (1967) where the reverse held.

Inspection of the urinary E/A ratio may be a more valid method of comparison of values by the different investigators, since, though many variables may affect the total amount of each steroid estimated they may not affect the relative amounts of each steroid estimated. The E/A ratio for the 8 investigators listed in Table 13 are shown below.

		1	2	3	4	5	6	7	8
male	E/A	0.75	0.82	0.97	-	0.70	0.96	0.75	1.14
female	E/A	1.24	0.87	1.11	1.95	0.86	1.29	1.07	0.85

In all cases excluding Cawley et al (1967) number 8 the E/A ratio for females is greater than the respective values for males. Generalising it might be said that the E/A ratio for females is less than one and greater than one for males. Although comparison of the results in this fashion is more favourable, there is still definite indication that factors other than those documented are to some degree controlling the excretion of D.17-OS.

Some of the comments made above are relevant for the values listed in Table 12. There is again a wide range of values for all the steroids reported. Males as before have higher values for the individual urinary D.17-OS sulphates and glucuronides than females, though not in all cases. Kellie & Wade (1957) reported higher individual D.17-OS values for females than for males, except in the case of A.G where the values for males and females were almost equal. Also, in all the studies, except the present one, the values for E.S in females are higher than in males. Apart from these two differences, all other steroid levels reported for males are greater than the respective levels for females.

The results of the present study show that the percentage conjugation of A and E with sulphuric acid and glucuronic acid are in close agreement with those reported by Kellie & Wade (1957). They

found 79% of total A and 90% of total E excreted as glucuronide conjugates. On the other hand Menini (1966) reported corresponding values of 70% and 79%, which were also lower than the values of 72% and 87% calculated from the results of Mauvais-Jarvis & Baulieu (1962). A similar trend was found in female subjects where Kellie & Wade (1957) found 79% of A and 90% of E were excreted as glucuronide conjugates. In the present study our results are in close agreement with Mauvais-Jarvis & Baulieu (1962), who found 87% of both A and E were excreted as glucuronides. Menini (1966) again reported lower values of 74% and 73%. He also found that 5% of the DHA in males and 12% in females is conjugated with glucuronic acid. The values calculated from the data of Kellie & Wade (1957) are 24% and 34% for males and females respectively and we found 12% and 38% respectively.

It may be relevant that Menini (1966) in the procedure for estimating sulphates, extracted the conjugates from urine in the presence of pyridinium sulphate and solvolysed the sulphate conjugates in dioxan without introducing acid, following the method of McKenna & Rippon (1965), whereas the other groups listed in Table 12 used methods which are essentially solvent extraction of salt saturated urine at pH 1, followed by solvolysis over a given time period.

Because of the difficulty of separating epiA from structurally similar steroids data on the excretion of this steroid are limited. Pesonen (1962), reported urinary epiA values as high as those of DHA. Okano, Matsumoto, Akehi, Mizutani, Kikkawa & Seki (1963) obtained epiA values of less than 0.1 mg/24 hour in urine of men by the ion-exchange chromatographic procedure of Sōdi & Matsumoto (1963).

Lim, Fesler & Dingman (1964) found no sex difference in the excretion of epiA. Feher (1967) estimated values of epiA in males and females and found mean values of 0.4 and 0.1 mg/day respectively. However, none of these authors attempted to separate conjugated forms. The results now shown in Tables 7 & 8, which agree fairly well with those of Feher (1967), would appear to be the first available for urinary levels of epiA in sulphate and glucuronide fractions.

Examination of the values for the excretion of A.S and E.S shows that men excrete much lower amounts of E.S relative to A.S than do women. The E.S/A.S ratios for males calculated from Table 12 are 0.28, 0.36, 0.42 and 0.55 and for females 1.0, 0.77, 2.57 and 0.69. Similarly for the E.G/A.G ratios observed by all the investigators, however, with less difference between males (0.85, 0.75, 1.45 and 1.32) and females (1.28, 0.79, 2.09 and 1.70).

The urinary values of the individual D.17-OS sulphates and glucuronides for females by the present report tend in general to be lower than the values given by the other investigators in Table 12. The same conclusion appears to hold true for the D.17-OS.(S+G) levels shown in Table 13. However, the values for males by the present report are average and above average on comparison with the values of other investigators (Tables 12 & 13). Yet, the levels for males and females were estimated by the same method. Accepting that the individual urinary D.17-OS for females by the present report are low,

then this indicates that some factor or factors other than those described is, or are, in operation. Valid comparison with these levels will probably be indecisive and only in cases where there is a marked statistical difference could conclusions be formed. However, comparison of the ratios of various individual D.17-OS for normal females, as reported here, with similar ratios for other groups of individuals may not be invalidated.

(b) Comparison of males and females. In a considerable number of endocrine conditions the determination of urinary individual 17-OS has been helpful for both the understanding and diagnosis of these conditions. However, in many other problems as well as in many so called endocrine problems, the value of the test may be limited. At times this may be ascribed to a lack of appreciation of the large variation in the excretion of these steroids by normal individuals. This was first pointed out by Fraser, Forbes, Albright, Sulkowitch & Reifenstein (1941), who found that the daily urinary excretion of total 17-OS for 14 women (20 - 40 years) to range from 5.1 to 14.2 mg/24 hour and in 9 males of similar ages from 8.0 to 22.6 mg/24 hour.

The extent of the variation for all the steroids estimated in this report is shown below. In both sexes DHA of all the steroids estimated, showed the most striking variation, and this was most pronounced for females, who had variation coefficients for DHA.G and DGA.(S+G) of 193% and 199% respectively.

	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	DHA.(S+G)	E.(S+G)	A.(S+G)
male	108	132	92	107	97	52	62	130	47	63
female	94	122	94	94	193	73	79	199	71	79

These very high variation coefficients are in close agreement with those found by Keutmann & Mason (1967). These variations in the excretion of individual D.17-OS emphasise the difficulty encountered in obtaining suitable control data. It follows that conclusions concerning the relative excretion of these steroids by different groups of subjects should be made only after sound statistical evaluation.

Despite the wide variation in excretion of the D.17-OS steroids by both sexes, males excrete much greater amounts of each steroid than females (Table 9). This is to be expected since it is well known that total urinary 17-OS levels are much higher in males than in females. Further, the main precursors of the urinary D.17-OS, DHA.S, DHA, testosterone and androstenedione, excluding androstenedione, are secreted in larger amounts by males compared to females (MacDonald, Chapdelaine, Gonzalez, Gurpide, Vande Wiele & Lieberman, 1965). However, in the case of E.S there was no statistical significant difference between males and females, this finding tends to be in agreement with those of the other investigators whose results appear in Table 12, who found urinary E.S levels to be greater in females than in males. An adequate explanation for this finding does not appear to be available, though it has been shown in females that no matter the precursor, E.S excretion in the urine is greater than the A.S and the reverse in males (Baulieu, 1963 and Mauvais-Jarvis, Floch, Jung, Robel & Baulieu, 1968).

By the present report the amount of E.S excreted relative to A.S was greater for females than for males. The E.S/A.S ratios for females and males were 1 and 0.27 respectively (Table 9, fig.9a). The same pattern held for E.G and A.G with respect to relative excretion by male and female, the E.G/A.G ratios for females and males were 1.29 and 0.85 respectively (Table 9, figs. 6,7 & 9b). Similarly for E.(S+G) and A.(S+G) where the E.(S+G)/A.(S+G) ratios were 1.25 and 0.74 for females and males respectively (Table 9, fig.9c).

The ratio between the amount of E and A produced is partly under the influence of enzymes concerned with the reduction of the unsaturation involving the C-5 atom of the D.17-OS precursors, and partly a reflection of the relative amounts of the various D.17-OS precursors. The presence of two reducing enzymes, located in the liver parenchymal cells, has been demonstrated. A 5β-Δ^4 reductase located in soluble cytoplasm of the liver cells (Tomkins, 1956), and 5α-Δ^4 reductase in the endoplasmic reticulum, (Forchielli & Dorfman, 1956 and McGuire & Tomkins, 1958). With both groups of enzymes NADPH functions as the hydrogen donor.

It is believed, though now in some doubt, that all the A(5α) and E(5β) formed from DHA.S, DHA, androstenedione and testosterone is derived from androstenedione as a common intermediate (Ungar, Miller & Dorfman, 1956, and Dorfman, 1954). According to this concept, whatever the precursor(s) the ratio of the urinary androstanes would always be the same, that is 1/1. Obviously this cannot be completely

true since several reports, including the present one, have shown that the urinary E/A ratio for males is less than one and greater than one for females.

It is interesting that Johnsen (1968) in an extensive study of urinary E/A ratio, covering many disorders and involving the analysis of 2362 urines, demonstrated that the administration of large doses of testosterone to 45 individuals, including 12 normals, induced a significant decrease in E/A ratio in the majority of patients and in 11 out of the 12 normals. However, contrary to this Kirschner & Lipsett (1964b) showed minimal increases in E/A ratio after testosterone administration. The mean reported secretion rate for testosterone in females is 0.14 mg/day and in males 7.0 mg/day (Horton & Tait, 1966; MacDonald et al, 1965 and Baulieu & Mauvais-Jarvis, 1964a). Thus the difference in the secretion rates of testosterone for males and females might help explain the difference in the E/A ratio shown by males and females. However, care must be taken in extrapolating the results of the metabolism of exogenous steroids to that of endogenous steroids.

If the very high testosterone secretion rate in males, relative to females, does influence the E/A ratio, then eunuchs would be expected, since they are secreting minimal amounts of testosterone, to have E/A ratios lower than normal males and possibly equivalent to females. This was found to be true. Johnsen (1968) studied 10 castrated sex criminals and 13 cases of testicular insufficiency and

found the E/A ratio as a group to be substantially higher than normal. Similarly, Keutmann & Mason (1967) found two castrated males to have E/A ratios in the region of that for females. However, reasoning such as this does not explain the remarkable difference between British and Japanese women with respects to E/A ratios (Bulbrook, Thomas, Utsunomiya & Hamaguchi, 1967). They found Japanese women to have E/A ratios much lower than British women of the same age group, namely 40-60 years. Unfortunately, the E/A ratios for Japanese men were not available, thus the presence or absence of the sex difference in Japanese people could not be ascertained. The sex difference in E/A ratio could be racial or even environmental.

Metabolism of C_{21} steroids has been shown to influence urinary D.17-OS levels, though the extent of the influence may only be significant in pathological conditions where large amounts of C_{21} steroids are produced as in Cushing's syndrome and congenital adrenal hyperplasia. However, since the metabolism of C_{21} steroids have been shown to favour specific orientations of the hydrogen atom attached to the C-5 atom, consideration of their contribution though small may have some bearing on the E/A ratio. The oxygen atom at C-11 depending on whether it is present as ketone or hydroxyl has been shown to favour 5β and 5α orientation respectively (Slaunwhite, Neely and Sandberg, 1964). However, as C_{21} steroids with these

functional groups at C-11 retain them on metabolism to 17-OS, they, therefore, have no effect on the E/A ratio. The side chain of C_{21} steroids has been shown to strongly favour reduction of Δ^4 double bond to give 5β steroids, thus metabolism of 11-deoxy C_{21} steroids to urinary 17-OS will elevate the E/A ratio. The two C_{21} steroids lacking oxygen at C-11 are 17α-hydroxyprogesterone and 17α,21-dihydroxyprogesterone. Both these steroids were isolated from adrenal vein blood of women with cancer of the breast in amounts ranging from 5 - 22 μg/100 ml (Lombardo, McMorris & Hudson, 1959) and Fukushima et al (1961) reported the secretion rate of 17α-hydroxyprogesterone in normals as 3 mg/day. These steroids could, therefore, be metabolised peripherally to excretory products including D.17-OS. If the proportion of these steroids metabolised to D.17-OS in males and females is greater in females, then the D.17-OS derived from these two steroids could influence the E/A ratio. Specific investigations relating to this appear to be lacking though Richardson, Touchstone & Dohan (1955) reported appreciable increases in 17-OS levels after administration of 17α,21-dihydroxyprogesterone to a female with adrenal cortical insufficiency, whereas Brooks (1960) found the conversion of exogenous 17α-hydroxyprogesterone to A and E in normal man to be 0.2% Fukushima et al (1961) found the same conversion in another man to be 1.7%.

The results of Baulieu, Corpechot, Dray, Emiliozzi, Lebeau, Mauvais-Jarvis & Robel (1965) using tritiated DHA.S and carbon

labelled DHA indicate that in males the metabolism of DHA.S produces more E.G than A.G whereas DHA produces equal amounts of E.G and A.G. Therefore, the DHA.S/DHA secretion ratio could be important in determining the urinary E/A ratio. The secretion rates of DHA.S and DHA in normal man were 10.1 and 7.2 mg/day and in normal woman 7.7 and 0.7 mg/day respectively (MacDonald et al, 1965). Assuming the above findings to be typical then the higher DHA.S/DHA secretion ratios for females would contribute to an elevated urinary E/A ratio in females relative to males.

Recent studies on the metabolism of testosterone have uncovered a new catabolic pathway the "17β-hydroxy pathway" (Baulieu & Robel, 1963 and Baulieu & Mauvais-Jarvis, 1964b). The principal metabolites of this pathway, 5α and 5β-androstane-3α,17β-diols are formed, not through androstenedione, but through testosterone (Baulieu & Mauvais-Jarvis, 1964b). The "17β-hydroxy pathway" produces very high 5β/5α urinary androstanediol ratios, this together with the fact that androstane diols may be converted in vivo to their corresponding androstanes, would result in a tendency to elevate the E/A ratio. Therefore, since testosterone is secreted in much larger amounts by males, and since it is an important substrate in the "17β-hydroxy pathway", the net result would be the elevation of the urinary E/A ratio in males relative to females. However, the pathway is considered as being a very minor one for the formation of androstanes. Also contrary to Baulieu & Mauvais-Jarvis (1964b), Koreman & Wilson (1966) did not find a consistently high

urinary androstanediol 5β/5α ratio, but found it to vary from individual to individual with no particular trend in favour of a high 5β/5α ratio.

DHA.S was the dominant steroid sulphate in normal male urine, and the general pattern for excretion of steroid sulphates by males was DHA>A>epiA>E. Females on the other hand had no set pattern and DHA.S was not the major steroid sulphate. Baulieu et al (1965) have shown that only part of the circulating DHA.S is excreted as such in the urine, more in men (0.9 - 15.0%, mean 10%) than women (0.3 - 9.0%, mean 4%) according to a short series of results. The findings of Baulieu et al (1965) might, therefore help to explain why DHA.S is the dominant sulphate in male and not in females, though application of these figures to DHA.S urinary levels in younger males would result in very high secretion rates, in the region of 100 mg/day whereas in other males it would result in minimal secretion rates.

Tables 7 & 8 show no values for epiA.G, for either males or females. This is in agreement with earlier results of Jayle (1959) and Baulieu (1963) who both found that the administration of large doses of epiA resulted in no urinary epiA.G, but did result in the formation of appreciable amounts of epiA.S as well as some A.S. Further, administration of ^{3}H-epiA to a normal male resulted in only 0.2% conversion to epiA.G whereas the conversion to epiA.S was 5.6% (Baulieu, 1963).

The pattern of steroid sulphate excretion for normal males by the present report is also in agreement with the earlier findings of Jayle (1959) and Baulieu (1963). Both of them found that: the 3β-hydroxy steroids are exclusively sulphoconjugated, the one with equatorial C-3 hydroxyl group (epiA) as well as that with the axial (3β, 5β-androstane, not found in the urine); the sulphation of A (axial, 3α-hydroxyl) is more important than that of E (equatorial, 3α-hydroxyl); DHA as regards extent of sulphoconjugation falls between epiA and A. However, males do not show the glucuro-conjugation predicted by Jayle (1959), which is the reverse of sulphoconjugation, that is E>A>DHA>epiA. Thus, although DHA.G was by the present report the minor glucuronide conjugate (disregarding epiA.G) for males and females, males excreted greater amounts of A conjugated to glucuronic acid than they did for E, whereas females showed the reverse pattern, which agreed with the pattern predicted by Jayle (1959).

The results in Tables 10 & 11 giving the matrix of correlation coefficients for individual D.17-OS shows the high degree of inter-correlations among these steroids. This extensive interrelationship of D.17-OS would suggest a simple pathway as the major catabolic route for the degradation of the D.17-OS precursors DHA.S, DHA, androstenedione and testosterone. The simplest pathway in keeping with a high degree of intercorrelation would be metabolism through a common pathway. Therefore, the old established pathway through

androstenedione would appear most suitable. However, metabolism by this route would be expected to result in a 1/1 ratio for 5α and 5β androstanes derived from androstenedione. The results presented here do not show this 1/1 ratio. However, when consideration is given to the variety of factors influencing the A/E ratio, plus the further metabolism of D.17-OS conjugates, A.G alone being excreted without further metabolism, the disparity in ratios from 1/1 can be better appreciated, and therefore, metabolism of the D.17-OS precursors through androstenedione is more acceptable.

B. Individual Urinary D.17-OS Sulphates and Glucuronides. Values in Male and Female Hypertensive Patients.

Urinary D.17-OS sulphate and glucuronide levels and serum uric acid levels were estimated in male and female hypertensive patients and compared with the corresponding levels obtained in Section V.A.3 for normal males and females.

1. Patients studied.

Thirty-two patients with idiopathic hypertension were studied. Idiopathic hypertension was determined by clinical history, physical examination and special investigations including infusion pyelogram, isotope renogram and aortography where indicated. There were 15 males with a mean age of 52 ± 9 years (range 30-63) and 17 females with a mean age of 49 ± 11 years (range 29-69). The mean diastolic and systolic blood pressure for males was 119 ± 16 mmHg (range 100-150) and 197 ± 30 mmHg (range 150-250) respectively and for females 121 ± 16 mmHg (range 95-160) and 200 ± 39 mmHg (range 140-260) respectively. The majority had satisfactory renal function as determined by serum creatinine and blood urea apart from 5 males with serum creatinine above 1.4 mg/100 ml (mean 1.5 mg/100 ml) and one female with a blood urea of 80 mg/100 ml. These patients were dealt with separately. No patient had malignant hypertension.

Six patients, all females (mean age 49 ± 8 years, range 35-60 years) had hypertension secondary to renal disease. The underlying

abnormality was chronic pyelonephritis in 3, renal artery stenosis in 2 and polycystic kidneys in one. The mean diastolic blood pressure for this group was 118 ± 18 mmHg (range 94-140) and the mean systolic blood pressure was 193 ± 20 mmHg (range 170-220).

Two males and one female had mild diabetes, the others had no obvious endocrine disturbances. One male had ischaemic heart disease, and one female had peripheral vascular disease. All patients were on normal diets and no medication was given for one week prior to the study.

2. Analytical techniques.

Collection of blood and urine	-	Section IV.A5.
Blood urea and serum creatine	-	Section IV.A.2.a & b.
Serum uric acid	-	Section IV.A.4.
17-OS & 17-OHCS	-	Section IV.A.6.a & b.
D.17-OS.S & D.17-OS.G	-	Section IV.C.2.

All analysis were duplicated.

3. Results.

(a) Male hypertensive patients. The urinary levels of the individual D.17-OS for idiopathic hypertensive males are shown in Table 14 and are in order of increasing age except for the last five sets of results which are the D.17-OS urinary levels for the patients with serum creatinines greater than 1.4 mg/100 ml. Compared with the values of the other hypertensive patients there is no obvious

TABLE 14. EXCRETION OF INDIVIDUAL URINARY D.17-OS BY IDIOPATHIC HYPERTENSIVE MALES (mg.FREE STEROID/24 hr.), AND SERUM CREATININE AND URIC ACID LEVELS.

	AGE	DEHYDROEPIANDROSTERONE			EpiA.S	AETIOCHOLANOLONE			ANDROSTERONE			S.U.A. mg%	SERUM CREATININE mg%
		S	G	S+G		S	G	S+G	S	G	S+G		
1	30	5.76	1.48	7.24	0.62	1.57	9.8	11.37	2.45	6.27	8.72	6.8	1.0
2	41	6.78	0.77	7.55	0.88	0.13	4.47	4.60	1.69	6.77	8.46	6.4	1.1
3	43	4.44	1.36	5.80	0.27	0.13	2.58	2.71	0.41	1.42	1.83	4.5	0.6
4	43	0.28	0.15	0.43	0.04	0.32	2.96	3.28	0.26	1.38	1.64	6.5	0.9
5	46	0.08	<0.004	0.08	0.14	0.59	3.50	4.09	1.06	3.78	4.84	4.5	1.2
6	50	0.44	0.14	0.58	0.07	0.14	1.53	1.67	0.10	1.15	1.25	4.6	0.8
7	50	0.09	0.02	0.11	0.01	0.05	0.94	0.99	0.05	1.68	1.73	5.0	1.1
8	53	0.22	0.04	0.26	0.07	0.31	1.22	1.53	0.16	0.86	1.02	5.9	1.0
9	57	0.22	0.13	0.35	0.09	0.12	4.35	4.47	0.35	2.49	2.84	5.1	1.2
10	62	0.25	0.19	0.44	0.06	0.08	1.10	1.18	0.04	0.38	0.42	5.6	1.2
11	63	0.94	0.16	1.10	0.18	0.70	2.58	3.28	0.78	1.34	2.12	5.0	1.0
12	64	0.18	0.10	0.28	0.04	0.12	1.72	1.84	0.15	0.82	0.97	4.7	0.8
13	54	0.19	0.29	0.48	0.07	0.31	0.84	1.15	0.08	0.06	0.14	4.6	1.4
14	56	0.06	0.01	0.07	0.05	0.06	1.98	2.04	0.15	1.33	1.48	6.6	1.5
15	58	0.15	0.04	0.19	0.01	0.14	1.65	1.79	0.36	0.58	0.94	4.7	1.5
16	61	0.62	0.14	0.76	0.19	0.92	4.40	5.32	0.52	1.65	2.17	7.4	1.7
17	70	0.03	0.02	0.05	0.02	0.14	1.03	1.17	0.08	0.53	0.61	5.4	1.8
MEAN	52	1.22	0.30	1.52	0.16	0.34	2.74	3.09	0.51	1.91	2.42	5.5	1.2
S.D.	10	2.171	0.459	2.586	0.235	0.401	2.207	2.540	0.661	1.938	2.559	0.9	0.3
RANGE	30-70	0.06-6.78	<0.004-1.48	0.07-7.55	0.01-0.88	0.05-1.57	0.84-9.80	0.99-11.37	0.05-2.45	0.06-6.27	0.14-8.72	4.5-7.4	0.6-1.8

difference shown by these five with renal damage secondary to hypertension.

A statistical comparison between the D.17-OS urinary levels of idiopathic hypertensive males and normal controls is shown in Table 15. The urinary epiA.S and DHA.S levels were significantly higher in the control group than in the hypertensive group, with P values of 0.056 and 0.067 respectively. A.G urinary levels were significantly higher in the control group (P = 0.028) than in the hypertensive group. The control group also showed higher values for DHA.(S+G) and A.(S+G). Thus in general the control group tended to show higher values than the hypertensive group except in the case of E.S where the reverse held and in case of E.G and E.(S+G) where there were no differences between the two groups. Total urinary 17-OS levels were also significantly higher in the control group (P = 0.02), this was probably due to an accumulation of the less significant differences shown by individual D.17-OS levels of the control group over the hypertensive group. Total urinary 17-OHCS were also excreted in moderately greater amounts by the control group (P =<0.1).

i. Serum uric acid - urinary DHA relationship. The relationship of a raised S.U.A. and an absence of urinary DHA could not be demonstrated. In fact there was no statistical difference between the S.U.A. levels of hypertensive patients (5.5 ± 0.9 mg/100 ml) and normal subjects (5.1 ± 1.0 mg/100 ml). Of the 15 hypertensive

TABLE 15. COMPARISON OF THE INDIVIDUAL D.17-OS URINARY LEVELS OF IDIOPATHIC HYPERTENSIVE MALE PATIENTS AND NORMAL MALE CONTROLS.

	NORMOTENSIVE MEAN ± S.D	HYPERTENSIVE MEAN ± S.D.	P VALUE
epiA.S	0.30 ± 0.326	0.16 ± 0.235	0.056
DHA.S	2.75 ± 3.75	1.22 ± 2.171	0.067
E.S	0.23 ± 0.212	0.34 ± 0.401	0.24
A.S	0.82 ± 0.838	0.51 ± 0.661	0.12
DHA.G	0.37 ± 0.365	0.30 ± 0.459	0.25
E.G	2.61 ± 1.36	2.74 ± 2.207	1.0
A.G	3.05 ± 1.89	1.91 ± 1.938	0.028
DHA.(S+G)	3.12 ± 4.09	1.52 ± 2.586	0.062
E.(S+G)	2.89 ± 1.36	3.09 ± 2.54	1.0
A.(S+G)	3.87 ± 2.46	2.42 ± 2.559	0.028
17-OS	12.3 ± 5.3	8.8 ± 3.2	0.02
17-OHCS	12.7 ± 3.7	10.5 ± 3.9	<0.1

patients there were 4 with S.U.A. levels greater than 6.1 mg/100 ml, none of whom showed an absence of urinary DHA. In fact, two of them had DHA.(S+G) levels of 7.24 and 9.55 mg/day. There was no statistical significant correlation between urinary DHA and S.U.A. levels. However, urinary E.S, E.G and E.(S+G) showed significant correlations with S.U.A. levels, E.S ($r = 0.45$ $P < 0.1$), E.G ($r = 0.51$ $P < 0.05$) and E.(S+G) ($r = 0.51$ $P < 0.05$).

ii. Sulphate conjugates. The majority of normotensive subjects showed the pattern DHA>A>epi>E for the relative amounts of each urinary steroid sulphate excreted. Yet only 2 (12%) of the hypertensive patients showed this pattern, in the other 15 (88%) E.S was present in amounts larger than at least one of the 3 other steroid sulphates. Figs.10a,b & c, show the E.S/DHA.S, E.S/epiA.S and E.S/A.S ratios for hypertensive patients and normal controls and statistical comparison of the two groups demonstrated a highly significant difference in all three sets of ratios $P = 0.0008$, $P = 0.0001$ & $P = 0.0004$ respectively. Study of fig.10a indicates that, in 82% of the normotensive subjects, the value of DHA.S was at least double that of E.S, whereas in the hypertensive patients this held for only 29%. In 88% of the hypertensive patients the value of E.S was equal to or greater than the respective value for epiA.S, while in the normotensive controls this was true only for 12% (fig.10b). One normotensive subject, only, had a value for

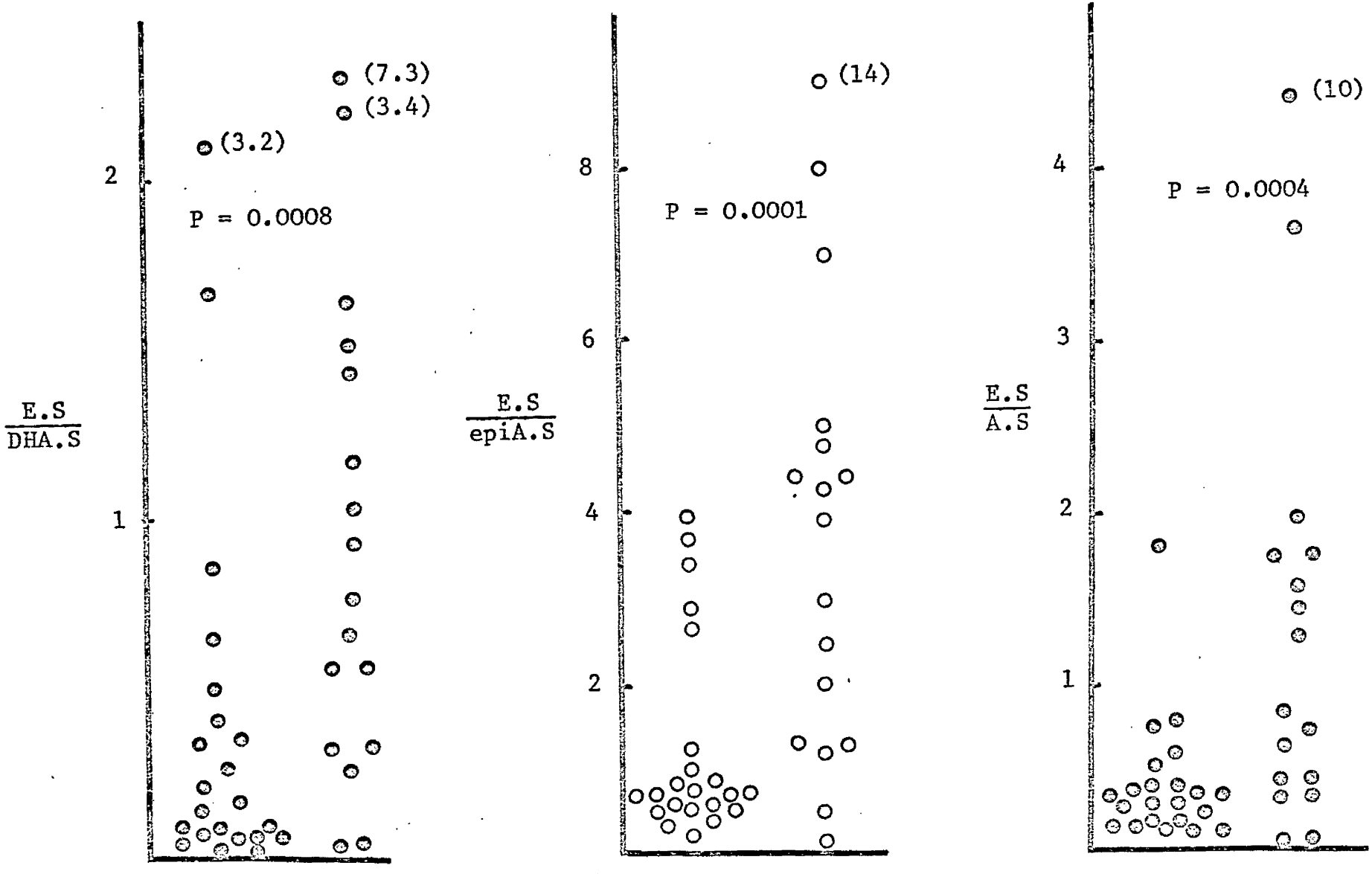


Fig. 10a. Fig. 10b. Fig. 10c.

Figs 10a,b,c. Comparison of the ratios of E.S/DHA.S, E.S/epiA.S and E.S/A.S for idiopathic hypertensive males (H) and normal males (N).

E.S>A.S whereas 47% of the hypertensive patients had values for E.S>A.S (fig. 10c).

iii. Glucuronide conjugates. The urinary excretory pattern of steroid glucuronide conjugates for hypertensive patients was E>A>DHA whereas in normal controls it was A>E>DHA. The E.G/A.G ratios of the hypertensive patients were significantly higher ($P = 0.00004$) than the same ratios for the normal controls (fig.11a). From fig. 11a it can be seen that 64% of the normal subjects have A as their major steroid glucuronide conjugate whereas 87% of the hypertensive patients have E. The 5 patients with renal impairment secondary to hypertension all showed E to be their major urinary steroid glucuronide and one of them, patient 13, was the only individual to show the steroid glucuronide excretory pattern of E>DHA>A.

iv. Sulphate plus glucuronide conjugates. In 77% of the hypertensive patients the major D.17-OS excreted was E, whereas in the control group E was the major steroid in only 27%. In the other 73%, A(46%) and DHA (27%) were the major D.17-OS excreted. The difference between the E.(S+G)/A.(S+G) ratios for the two groups was highly significant ($P = 0.00002$ see fig. 11b). Eighty-two percent of the control subjects had a value for A equal to or greater than E, whereas in the hypertensive group this was true for only 18%.

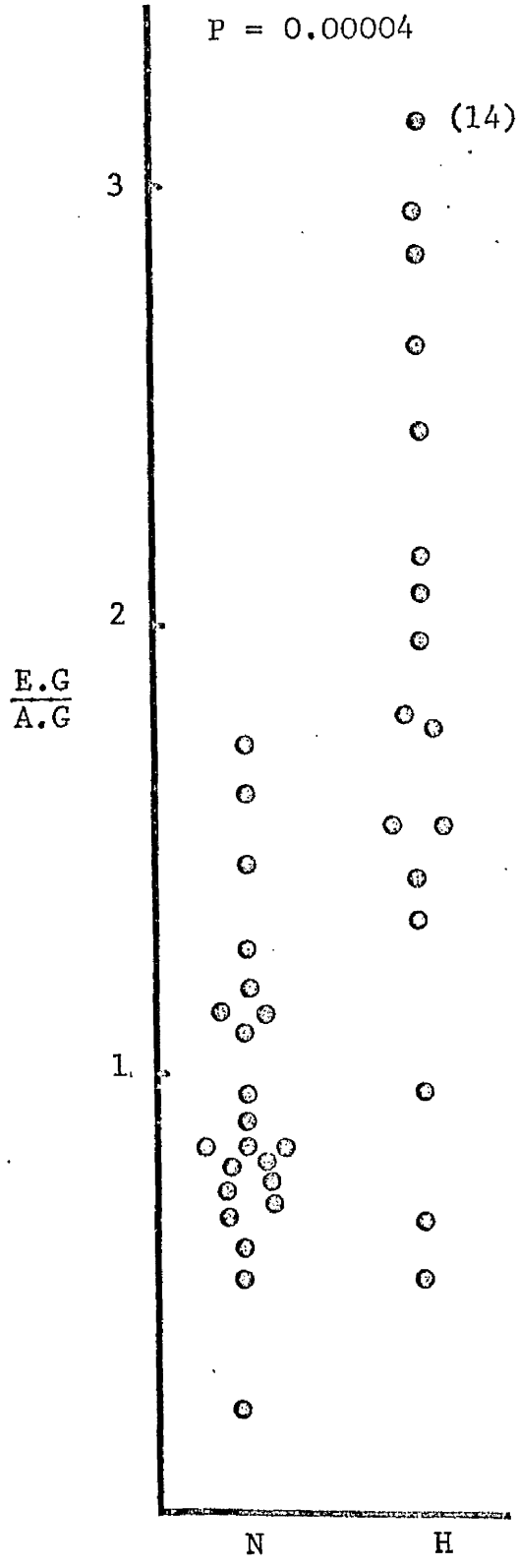


Fig. 11a.

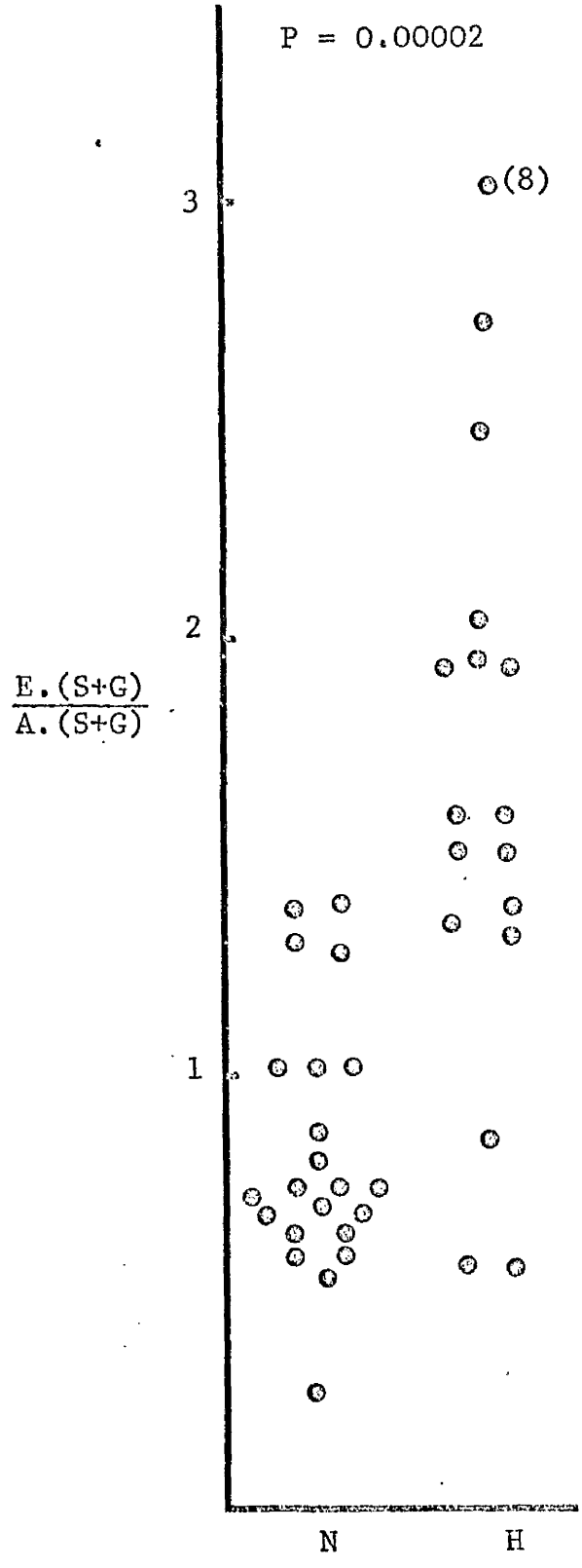


Fig. 11b.

Figs 11a & b. Comparison of the ratios of E.G/A.S and E.(S+G)/A.(S+G) for idiopathic hypertensive males (H) and normal males (N)

(b) Female hypertensive patients. The urinary levels of the individual D.17-OS for idiopathic and renal hypertensive females are shown in Tables 16 & 17. Excluding DHA.S all the urinary D.17-OS sulphate levels for female idiopathic hypertensives tended to be higher, though not significantly, than the corresponding values for normotensive individuals, (Table 18). For the individual D.17-OS glucuronide levels, the idiopathic hypertensives showed higher levels for both E.G and A.G, but lower levels for DHA.G, the differences however, were not significant. The results for the excretion of individual D.17-OS(S+G) showed the same pattern as the glucuronide conjugates, with the levels of A.(S+G) and E.(S+G) higher in the hypertensive group $P = 0.08$ and $P = 0.19$ respectively, and no difference shown for DHA.(S+G) levels.

Comparison of the urinary steroid levels found in renal hypertensive patients with normotensive individuals shows no significant differences, though the urinary levels of DHA.S, DHA.G and DHA.(S+G) tend to be higher in the control group. Table 19 gives a statistical comparison of urinary D.17-OS levels in renal and idiopathic hypertensive patients. Idiopathic hypertensive patients tended to have higher D.17-OS urinary levels than renal hypertensive patients. Both A.G and A.(S+G) urinary levels were significantly higher ($P = 0.03$ & $P = 0.03$) in the idiopathic hypertensive group than the renal hypertensive group.

TABLE 16. EXCRETION OF INDIVIDUAL URINARY D.17-OS BY IDIOPATHIC HYPERTENSIVE FEMALES (mg. FREE STEROID/24 hr.) AND SERUM URIC ACID LEVELS.

PATIENT	AGE	DEHYDROEPIANDROSTERONE			EpiA.S	AETIOCHOLANOLONE			ANDROSTERONE			S.U.A. mg%
		S	G	S+G		S	G	S+G	S	G	S+G	
1	29	0.31	0.04	0.35	0.08	0.11	0.72	0.83	0.31	1.26	1.57	3.2
2	36	0.01	<0.004	0.01	0.01	0.02	0.59	0.61	0.03	0.72	0.75	4.8
3	39	<0.004	0.007	0.007	<0.004	0.03	0.63	0.67	<0.004	0.37	0.37	10.5
4	40	0.50	0.06	0.56	0.07	0.12	4.65	4.77	0.17	3.34	3.51	6.0
5	40	0.26	0.08	0.34	0.07	0.27	1.97	2.24	0.36	1.67	2.03	3.9
6	40	1.34	0.40	1.74	0.10	0.22	0.93	1.15	0.23	0.50	0.73	6.4
7	47	0.08	0.13	0.21	0.03	0.12	2.29	2.41	0.14	2.13	2.27	9.7
8	48	0.10	0.05	0.15	0.07	0.78	2.63	3.41	0.20	2.48	2.68	7.9
9	49	2.78	0.80	3.58	0.39	0.89	3.97	4.86	1.41	2.19	3.60	3.6
10	51	0.09	0.05	0.14	0.05	0.19	0.24	0.43	0.06	0.21	0.27	4.2
11	52	0.40	0.07	0.47	0.35	0.47	1.48	1.95	0.51	1.54	2.05	3.9
12	53	0.23	0.06	0.29	0.11	0.30	1.35	1.65	0.41	1.21	1.62	2.3
13	54	1.11	0.56	1.67	0.23	0.45	2.42	2.87	1.28	2.26	3.54	4.1
14	58	0.27	0.06	0.33	0.17	0.28	1.84	2.12	0.68	0.58	1.26	5.3
15	59	0.12	0.06	0.18	0.05	0.27	0.59	0.86	0.27	0.74	1.01	2.4
16	65	0.25	0.05	0.30	0.04	0.19	0.70	0.89	0.10	0.37	0.47	3.9
17	69	0.03	0.02	0.05	0.01	0.10	0.70	0.80	0.03	0.64	0.67	5.5
MEAN	49	0.46	0.15	0.61	0.11	0.28	1.63	1.91	0.36	1.31	1.67	5.1
S.D.	11	0.702	0.223	0.919	0.115	0.243	1.251	1.393	0.412	0.912	1.138	2.3
RANGE	29-69	<.004-2.78	<.004-.56	0.01-3.58	<.004-0.39	0.02-0.89	0.24-4.65	0.43-4.77	<.004-1.41	0.21-3.74	0.67-3.60	2.3-10.5

TABLE 17. EXCRETION OF INDIVIDUAL URINARY D.17-OS BY RENAL HYPERTENSIVE FEMALES (mg. FREE STEROID/24 hr.)AND SERUM URIC ACID LEVELS.

PATIENT	AGE	DEHYDROEPIANDROSTERONE			EpiA.S	AETIOCHOLANOLONE			ANDROSTERONE			S.U.A. mg%
		S	G	S+G		S	G	S+G	S	G	S+G	
1	35	0.34	0.17	0.51	0.05	0.24	0.60	0.84	0.13	0.37	0.50	4.4
2	46	0.61	0.03	0.64	0.15	0.21	0.92	1.13	0.54	0.71	1.25	4.7
3	51	0.20	<0.004	0.20	0.08	0.36	1.62	1.98	0.40	0.67	1.07	4.6
4	53	0.04	0.02	0.06	<0.004	0.07	0.32	0.39	0.04	0.23	0.27	5.3
5	53	0.20	0.20	0.40	0.03	0.04	0.60	0.46	0.02	0.17	0.19	9.2
6	60	0.08	0.03	0.11	<0.004	0.12	1.05	1.17	0.06	0.60	0.66	5.9
MEAN	50	0.24	0.08	0.32	0.05	0.17	0.85	1.03	0.19	0.46	0.64	5.7
S.D.	8	0.208	0.085	0.231	0.057	0.120	0.450	0.554	0.218	0.233	0.436	1.8
RANGE	35-60	0.04-0.61	<.004-0.20	0.06-0.64	<.004-0.15	0.04-0.36	0.60-1.62	0.46-1.98	0.02-0.54	0.60-0.67	0.19-1.25	4.4-9.2

TABLE 18. COMPARISON OF THE INDIVIDUAL D.17-OS URINARY LEVELS OF IDIOPATHIC HYPERTENSIVE FEMALE PATIENTS AND NORMAL FEMALE CONTROLS.

	NORMOTENSIVE MEAN ± S.D.(N).	HYPERTENSIVE MEAN ± S.D.(H).	P VALUE
epiA.S	0.05 ± 0.047	0.11 ± 0.115	0.11
DHA.S	0.45 ± 0.990	0.46 ± 0.702	0.58
E.S	0.16 ± 0.150	0.28 ± 0.243	0.12
A.S	0.16 ± 0.149	0.36 ± 0.412	0.17
DHA.G	0.27 ± 0.520	0.15 ± 0.223	0.72
E.G	1.12 ± 0.820	1.63 ± 1.251	0.25
A.G	0.87 ± 0.691	1.31 ± 0.912	0.14
DHA.(S+G)	0.67 ± 1.33	0.61 ± 0.919	0.58
E.(S+G)	1.28 ± 0.912	1.91 ± 1.393	0.19
A.(S+G)	1.03 ± 0.823	1.67 ± 1.138	0.08
17-OS	6.1 ± 3.7	7.1 ± 2.8	0.40
17-OHCS	7.6 ± 3.4	9.6 ± 3.8	<0.20

TABLE 19. COMPARISON OF THE INDIVIDUAL D.17-OS URINARY LEVELS OF IDIOPATHIC AND RENAL HYPERTENSIVE FEMALE PATIENTS.

	IDIOPATHIC HYPERTENSIVES MEAN ± S.D.	RENAL HYPERTENSIVES MEAN ± S.D.	P VALUE
epiA.S	0.11 ± 0.115	0.05 ± 0.057	0.20
DHA.S	0.46 ± 0.702	0.24 ± 0.208	0.72
E.S	0.28 ± 0.243	0.17 ± 0.120	0.4
A.S	0.36 ± 0.412	0.19 ± 0.218	0.4
DHA.G	0.15 ± 0.224	0.08 ± 0.085	0.29
E.G	1.63 ± 1.250	0.85 ± 0.450	0.2
A.G	1.31 ± 0.912	0.46 ± 0.233	0.03
DHA.(S+G)	0.61 ± 0.919	0.32 ± 0.231	1
E.(S+G)	1.91 ± 1.390	1.03 ± 0.554	0.2
A.(S+G)	1.67 ± 1.137	0.64 ± 0.436	0.03
17-OS	7.1 ± 2.8	4.7 ± 1.7	<0.1
17-OHCS	9.6 ± 3.8	6.5 ± 1.6	<0.1

i. Serum uric acid - urinary DHA relationship. The idiopathic hypertensive patients showed marginally higher serum uric acid levels ($P < 0.25$) than the control group. In support of the findings of Kolbel et al (1965a) a 39 year old idiopathic hypertensive patient (Table 16, No.3) with a S.U.A. level of 10.5 mg/100 ml had a urinary DHA.(S+G) level of 0.007 mg/24 hr. However, in contrast to the findings of Kolbel et al (1965a) two other female hypertensives (Table 16, No.7 & 8) with S.U.A. levels of 9.7 and 7.9 mg/100 ml had urinary DHA.(S+G) levels of 0.21 and 0.15 mg/24 hr respectively. No correlation could be found between S.U.A. levels and urinary DHA levels or any other urinary D.17-OS for idiopathic or renal hypertensive patients, though urinary A.S levels for normal female subjects was positively correlated with S.U.A. levels ($r = 0.69$ $P = 0.02$).

ii. Glucuronide conjugates. The major urinary steroid glucuronide in the two hypertensive groups and the control group was in all cases E.G. The overall pattern of steroid glucuronide excretion by all 3 groups was E>A>DHA. This pattern held for all the renal hypertensive patients, 71% of the idiopathic hypertensive patients and 67% of the normal individuals. There was a significant difference ($P = 0.074$) between idiopathic hypertensive patients and normotensive controls for the A.G/DHA.G ratio.

iii. Sulphate conjugates. No set pattern of steroid sulphate excretion was shown by any of the three groups. The major steroid sulphate for normal subjects was DHA.S (40%), for idiopathic hyper-

tensive patients A.S (59%) and for renal hypertensive patients E.S (50%). The only definite feature shown by all the individuals of all three groups was the excretion of epiA.S in amounts less than that for DHA.S, A.E and E.S.

iv. Sulphate plus glucuronide conjugates. The predominant pattern shown by all three groups was E>A>DHA, which is the same as the pattern shown for the excretion of steroid glucuronides.

(c) Correlations. Correlation coefficients were calculated by computer for any combination of 2 of the 19 variables, collected for all the male and femal hypertensive patients. The variables were the same as those for the normal subjects. Correlation coefficients for the most highly correlated of the 19 variables are shown, for idiopathic hypertensive males in Table 20, for idiopathic hypertensive females in Table 21 and for renal hypertensive females in Table 22. Correlation coefficients at the 5% level and less are underlined.

i. Males. Excluding E.S, the excretion of all other D.17-OS conjugates by idiopathic hypertensive males showed statistically significant negative correlations with age, especially DHA.G where $r = -0.68$ ($P<0.01$). These are exactly the same results as were obtained for normal male subjects, that is, a generalised correlation of the D.17-OS steroids with age, with DHA.G showing the highest degree of correlation. The regression equations of urinary D.17-OS sulphate levels on age showed a set pattern when

TABLE 20. MATRIX OF CORRELATION COEFFICIENTS FOR IDIOPATHIC HYPERTENSIVE MALES.

	AGE	17-OS	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	DHA.(S+G)	E.(S+G)	A.(S+G)
AGE	1.0											
17-OS	-0.49	1.0										
epiA.S	-0.58	0.79	1.0									
DHA.S	-0.64	0.76	0.95	1.0								
E.S	-0.34	0.51	0.42	0.36	1.0							
A.S	-0.60	0.77	0.86	0.80	0.73	1.0						
DHA.G	-0.68	0.65	0.72	0.89	0.45	0.67	1.0					
E.G	-0.56	0.81	0.70	0.66	0.80	0.89	0.66	1.0				
A.G	-0.63	0.81	0.89	0.79	0.49	0.92	0.58	0.81	1.0			
DHA.(S+G)	-0.66	0.75	0.92	0.99	0.38	0.79	0.92	0.67	0.77	1.0		
E.(S+G)	-0.54	0.78	0.67	0.63	0.86	0.89	0.64	0.99	0.78	0.64	1.0	
A.(S+G)	-0.63	0.82	0.90	0.81	0.56	0.95	0.61	0.84	0.99	0.79	0.82	1.0

Correlation coefficients significant at 5% level or less are underlined.

they were compared for normal (N) and hypertensive (H) males.

epiA.S on age.

N. epiA.S = -0.018 age + 1.12 r =-0.55 P<0.01

H. epiA.S = -0.013 age + 0.86 r =-0.58 P<0.05

DHA.S on age.

N. DHA.S = -0.24 age + 13.3 r =-0.62 P<0.002

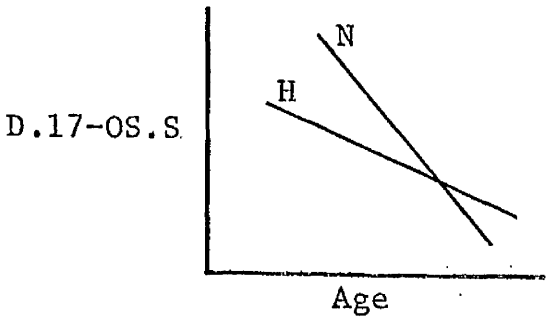
H. DHA.S = -0.14 age + 8.4 r =-0.64 P<0.01

A.S on age.

N. A.S = -0.05 age + 3.0 r =-0.58 P<0.01

H. A.S = -0.04 age + 2.56 r =-0.60 P<0.01

Generalising the pattern shown by these 3 sets of regression equations may be illustrated thus:-



At any age between 30-62 for epiA.S, between 30-50 for DHA.S and between 30-44 for A.S, the urinary D.17-OS.S levels for normal subjects tend to be higher than the respective value for the hypertensive patients, though there is little difference between the two groups for individuals older than 50 years.

The opposite pattern is shown by the regression equations of urinary D.17-OS glucuronide levels on age, when normal subjects are compared with hypertensive patients.

DHA.G on age.

N. DHA.G = -0.027 age + 1.56 r = -0.72 P<0.001

H. DHA.G = -0.031 age + 1.9 r = -0.68 P<0.01

E.G on age.

N. E.G. = -0.077 age + 6.07 r = -0.56 P<0.01

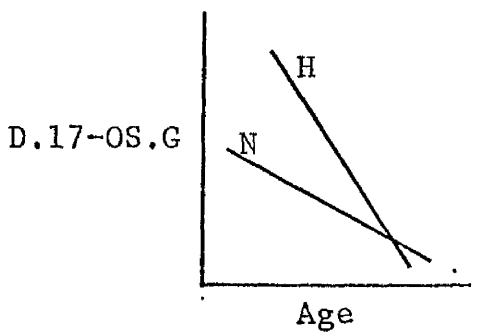
H. E.G = -0.122 age + 9.09 r = -0.56 P=0.02

A.G on age.

N. A.G = -0.107 age + 7.8 r = -0.55 P<0.01

H. A.G = -0.12 age + 8.2 r = -0.63 P<0.01

Generalising the pattern shown by the first 2 sets of regression equations may be illustrated thus:-



At any age between 30-85 for DHA.G and between 30-67 for E.G the urinary D.17-OS.G levels for hypertensive patients tend to be higher than the respective values for normal subjects. The regression equations for A.G on age are very similar for the two groups, suggesting that for this steroid glucuronide both groups behave the same.

Comparison of the regression equations of E.S on A.S for normal male subjects (N) and hypertensive patients (H),

N. E.S = 0.20 A.S + 0.07 r = 0.79 P<0.001

H. E.S = 0.44 A.S + 0.12 r = 0.73 P<0.001 (fig.12a)

shows a marked difference in the slope of the lines. A value of 0.2 and 0.44 for normal subjects and hypertensive patients respectively indicates that hypertensive patients excrete much more E.S relative to A.S than do normal male subjects. If these regression lines are compared with those in fig.9a it will be seen that the hypertensive males have altered their slope in the direction of that for a normal female. The equations for the regression lines of E.G and A.G

N. E.G = 0.62 A.G + 0.73 r = 0.86 P<0.001

H. E.G = 0.92 A.G + 0.98 r = 0.81 P<0.001 (fig.12b)

and for E.(S+G) on A.(S+G)

N. E.(S+G) = 0.53 A.(S+G) + 0.81 r = 0.89 P<0.001

H. E.(S+G) = 0.82 A.(S+G) + 1.11 r = 0.82 P<0.001 (fig.12c)

for normal male subjects and hypertensive patients show the same pattern as the E.S on A.S. Therefore, it would appear that hypertensive males excrete much more E relative to A than do normal males, and that hypertensive males show strong similarities to normal females with respects to their excretion of E relative to A (see figs.9a,b & c).

The regression equations of DHA.S on epiA.S

N. DHA.S = 10.60 epiA.S - 0.41 r = 0.92 P<0.001

H. DHA.S = 8.76 epiA.S - 0.23 r = 0.95 P<0.001

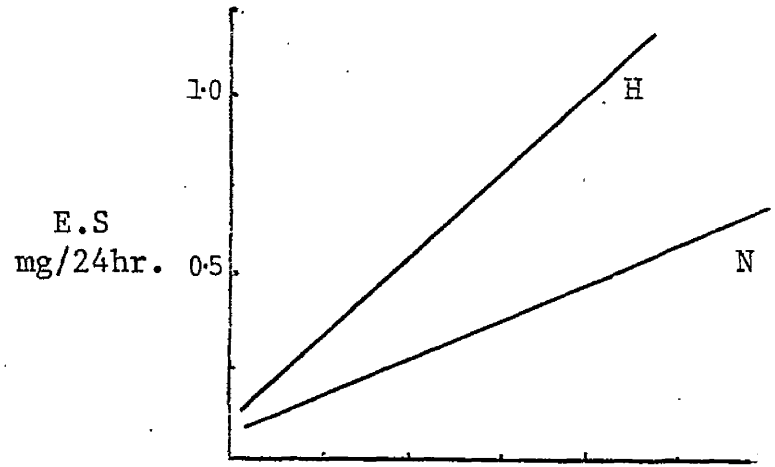


Fig. 12a.

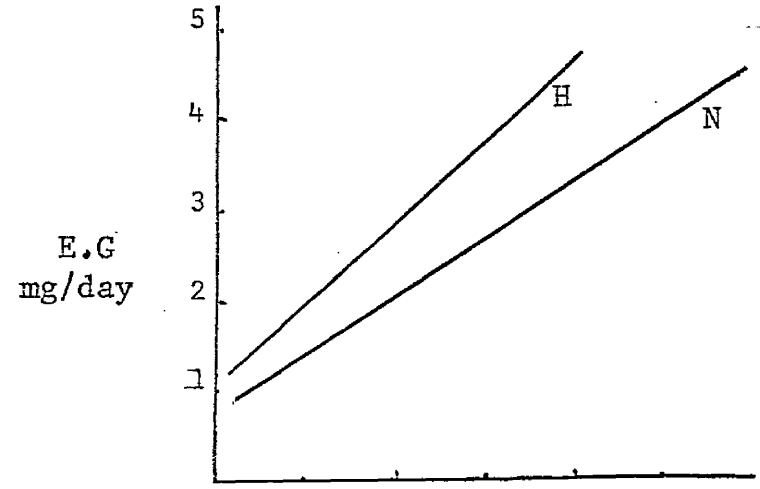


Fig. 12b.

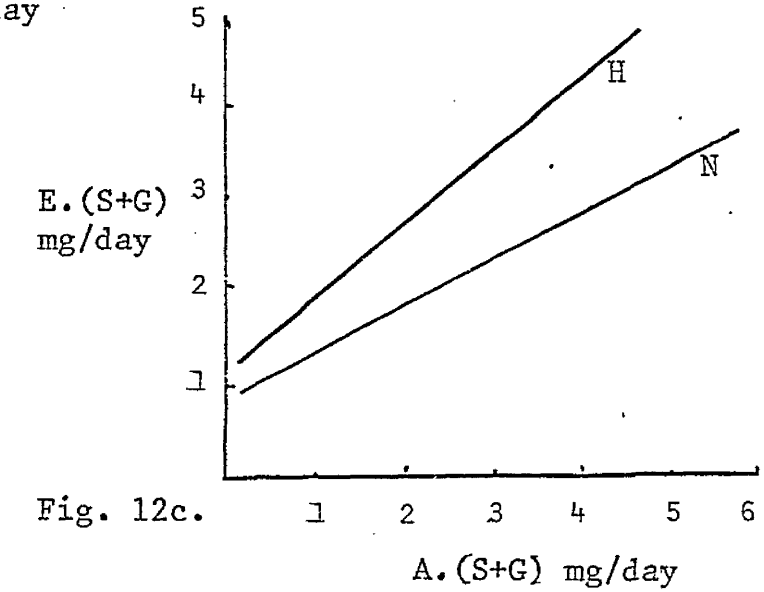


Fig. 12c.

Figs 12a,b & c. Regression of E.S on A.S, E.G on A.G and E.(S+G) on A.(S+G) for idiopathic hypertensive males (H) and normal males (N).

and A.S on epiA.S

N. A.S = 2.49 epiA.S - 0.07 r = 0.97 P<0.001

H. A.S = 2.43 epiA.S - 0.10 r = 0.86 P<0.001

although very highly correlated for both normal males and hypertensive patients, show that there is very little difference in the slope of the lines for either sets of equations, in fact, in the case of the regression of A.S on epiA.S the two groups have almost the same regression equations.

ii. Females. The urinary D.17-OS levels for female idiopathic hypertensive patients, like the normal females, showed no correlation with age. But, like the normal female subjects, they did show a correlation with urine volume (Table 21), though they did not reciprocate the correlations between serum creatinine and urinary D.17-OS levels shown by normal female subjects.

The regression equations of E.S on A.S for normal females (N), idiopathic hypertensive females (I.H.) and renal hypertensive females (R.H.) are:-

N. E.S = 0.66 A.S + 0.06 r = 0.66 P<0.01

I.H. E.S = 0.41 A.S + 0.13 r = 0.69 P=0.002

R.H. E.S = 0.39 A.S + 0.10 r = 0.71 P<0.25

The regression line for the idiopathic hypertensive females, though quite similar to the line for normal female subjects, is very interesting in that the slope of the line (0.41) has changed in the direction of that for normal males. Thus, the slopes for normal

TABLE 21. MATRIX OF CORRELATION COEFFICIENTS FOR IDIOPATHIC HYPERTENSIVE FEMALES.

	Urine vol.	17-OS	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	DHA.(S+G)	E.(S+G)	A.(S+G)
Urine vol.	1.0											
17-OS	0.60	1.0										
epiA.S	0.43	0.70	1.0									
DHA.S	0.44	0.68	0.73	1.0								
E.S	0.52	0.45	0.73	0.63	1.0							
A.S	0.52	0.59	0.85	0.78	0.69	1.0						
DHA.G	0.60	0.71	0.69	0.95	0.62	0.83	1.0					
E.G	0.23	0.51	0.46	0.52	0.52	0.51	0.48	1.0				
A.G	0.32	0.49	0.34	0.31	0.45	0.39	0.32	0.90	1.0			
DHA.(S+G)	0.49	0.69	0.73	0.99	0.63	0.80	0.97	0.51	0.31	1.0		
E.(S+G)	0.29	0.54	0.54	0.58	0.64	0.58	0.58	0.99	0.88	0.57	1.0	
A.(S+G)	0.45	0.61	0.58	0.53	0.62	0.67	0.56	0.91	0.94	0.54	0.92	1.0

Correlation coefficients significant at 5% level or less are underlined.

TABLE 22. MATRIX OF CORRELATION COEFFICIENTS FOR RENAL HYPERTENSIVE FEMALES.

	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	DHA.(S+G)	E.(S+G)	A.(S+G)
epiA.S	1.0									
DHA.S	0.91	1.0								
E.S	0.59	0.36	1.0							
A.S	0.95	0.77	0.71	1.0						
DHA.G	-0.18	0.08	-0.37	-0.48	1.0					
E.G	0.41	0.11	0.76	0.59	-0.50	1.0				
A.G	0.62	0.45	0.69	0.79	-0.66	0.79	1.0			
DHA.(S+G)	0.76	0.93	0.19	0.52	0.44	-0.08	0.16	1.0		
E.(S+G)	0.47	0.17	0.84	0.64	-0.49	0.99	0.80	-0.03	1.0	
A.(S+G)	0.82	0.64	0.73	0.94	-0.60	0.73	0.95	0.36	0.76	1.0

Correlation coefficients significant at 5% level or less are underlined.

males and females are 0.20 and 0.66 respectively and for idiopathic hypertensive males and females 0.44 and 0.41 respectively. Thus, the slopes of the regression lines of E.S on A.S for idiopathic hypertensive males and femaled would appear to be more or less the same, in fact the regression equations for male and female hypertensives are almost identical.

female I.H. $E.S = 0.41A.S + 0.13$

male I.H. $E.S = 0.44A.S + 0.12$

It might, therefore, be concluded that hypertension results in the two very different regression equations of E.S on A.S for normal males and females merging into one. The regression equations of E.S on A.S for renal hypertensive patients though very similar to the regression lines for idiopathic hypertension has very poor statistical validity ($P<0.25$), therefore any conclusions formed relating to it are equivocal.

The regression lines for E.G on A.G for normal female subjects and idiopathic hypertensive females are very similar.

N.	$E.G = 1.05\ A.G + 0.22$	$r = 0.89$	$P<0.001$
I.H.	$E.G = 1.23\ A.G + 0.02$	$r = 0.90$	$P<0.001$
R.H.	$E.G = 1.55\ A.G + 0.14$	$r = 0.79$	$P<0.1$

However, there is no similarity between the regression lines of E.G on A.G for male and female hypertensive patients, therefore, unlike E.S on A.S hypertension does not induce the different regression lines of E.G on A.G for normal males and females to merge into one. The renal

hypertensive though showing a high correlation coefficient for the regression of E.G on A.G still showed poor statistical significance ($P<0.1$), due to the small number of patients studied. Therefore, no valid conclusion can be made relating to regression line.

4. Discussion.

(a) Serum uric acid - urinary DHA relationship. If the S.U.A. level above which hyperuricaemia is recognised is defined as the mean normal + 2 S.D., then in the present report values of 7.1 and 7.0 mg/100ml are obtained for males and females respectively - a less severe criterion for defining hyperuricaemia than that imposed by Breckenridge (1966). By the present report 12%, 1 male and 3 females, of the idiopathic hypertensive patients and 8%, 2 males and 1 female from the normotensive control were therebye found to be hyperuricaemic. This is a much lower incidence of hyperuricaemia in hypertension than the 27% cited by Breckenridge (1966), the 46% by Kinsey et al (1961) or the 50% by Itskovitz & Sellers (1963). It is an incidence more in agreement with the results of Barlow & Fry (1966), who found, using the same criterion as above for defining hyperuricaemia, that 7% from 70 hypertensive patients, and 3% from 140 normotensive controls were hyperuricaemic. Tibblin (1967) found no difference between the S.U.A. levels in 468 normotensive subjects and 33 severe hypertensive subjects.

Kolbel et al (1965a) gave their normal S.U.A. levels as 3.42 ± 0.74 and 2.52 ± 0.57 mg/100 ml for males and females; therefore, values greater than 4.86 and 3.64 mg/100 ml for males and females respectively could be

classed as hyperuricaemic. In 16 hypertensive males and females with measurable amounts of urinary DHA Kolbel et al (1965a) reported the mean S.U.A. as 4.8 and 3.9 mg/100 ml respectively and for 30 hypertensive males and females with an absence of urinary DHA the reported mean S.U.A. levels were 5.7 and 5.5 mg/100 ml respectively. Since only 46 hypertensive patients were studied, it may be concluded that by the above criterion they were all probably hyperuricaemic, indicating selection and, therefore, making comparisons difficult. Further, the investigators gave no indication of withdrawal of anti-hypertensive chemotherapeutic drugs, several of which have been shown to elevate S.U.A. levels (Laragh, 1958 and Dollery et al, 1960).

A variation coefficient of 12.5% for urinary DHA levels was calculated for normal males mean age 52 (range 17 - 69 years) from the results of Kolbel et al (1965a). Yet the value found by Keutmann & Mason (1967) covering a similar age range and the value by the present report covering a narrower range, 29 - 69 years, were 180% and 130% respectively. The wide range for urinary DHA levels reported by other investigators confirms these large variation coefficients (Feher, 1966, Vestergaard, 1962 and Cawley et al, 1967, and others). Kolbel et al (1965a) used the method of Sonka, Gregorova, Slabochova & Rath (1963) for estimating urinary DHA. It entails hot acid hydrolysis, solvent extraction, paper chromatography and quantitation by comparing unknown spots with standard spots, after staining the chromatograms with Zimmerman reagent.

It would appear that the defect, that is the absence of urinary

DHA , obeys an "all or none" ruling, that is, there does not appear to be any gradation in urinary DHA levels with the increase in S.U.A. levels. This is confirmed by the present report where no correlation between urinary DHA and S.U.A. levels could be found in either normal subjects or idiopathic hypertensives. Only one idiopathic hypertensive female with a grossly elevated S.U.A. (10.5 mg/100 ml) had an abnormally low DHA.(S+G) level. This is possibly significant since almost all the hypertensive patients with an absence of urinary DHA investigated by Kolbel et al (1965a) had S.U.A. levels greater than mean normal + 3 S.D.

(b) Urinary D.17-OS levels in hypertension and a proposed theory for the explanation of steroid abnormalities in this disease.

Kolbel et al (1964) reported total urinary 17-OS levels in normal males and females as 12.8 ± 6.1 and 8.51 ±3.88 mg/24 hr respectively and in patients suffering from hypertension they reported levels of 6.33 ± 2.2 and 6.38 ± 3.12 mg/24 hr for males and females respectively. Thus the male hypertensive patients showed a highly significant drop in 17-OS excretion but the drop was not nearly so significant for the female hypertensive patients. By the present report hypertensive males tended to have lower 17-OS urinary levels than the controls ($P = 0.02$), whereas the femal hypertensive patients showed no difference compared to controls. Earlier reports are also equivocal, Bruger et al (1944), Selye (1947) and Wallace et al (1955) all reported lower urinary 17-OS levels in hypertension, whereas Hetzel & Hine (1952) and Warter et al (1960) found no difference in urinary 17-OS levels of hypertensive patients when compared with normal controls.

Of the earlier studies only Waters et al (1960) estimated urinary DHA levels. These,,they found to be normal even when the hypertensive group was subdivided into those with elevated urinary aldosterone levels and those with normal aldosterone levels and the DHA levels again compared. However, they often found urinary 17-OS and DHA levels to be subnormal in the hypertensive group, but the urinary aldosterone/DHA ratios were not significantly different on comparing normal controls with hypertensive patients. Their results are in disagreement with the very recent report of Nowaczynski, Genest, & Fragachan (1968). These investigators reported elevated plasma aldosterone levels in 11 out of 17 idiopathic hypertensive patients and markedly low levels of urinary DHA.S in 16 out of 16 idiopathic hypertensive patients. They also estimated urinary DHA.G levels but found no difference between hypertensive patients and normal controls. The results of the present report would tend to agree with those of Nowaczynski et al (1968). By the present report urinary DHA.S levels were found to be lower in the male hypertensive patients than in the normal controls, however, the difference was not highly significant ($P = 0.067$). Also as found by Nowaczynski et al, there was no difference between the urinary DHA.G levels for male hypertensive patients and normal controls. Finally, as previously mentioned, Kolbel et al (1965a)reported an absence of urinary DHA in 65% of the hypertensive patients they studied and normal urinary levels in the other 35%, - they further claimed that the same hypertensive patients could be classified into the same two groups of 65% and 35% by their S.U.A. levels.

Generalising it would appear that urinary DHA levels tend to be depressed in hypertension. Further the present report and that of Nowaczynski et al (1968) are in agreement that urinary DHA.G levels of idiopathic hypertensive patients are no different from normal controls, unfortunately this tends to disagree with the results of Kolbel at al (1965a) who reported zero levels for total DHA. However, when it is considered that the technique employed by Kolbel et al was incapable of accurately distinguishing between zero and very low urinary DHA levels, and that relative to DHA.S, urinary DHA.G levels are small, by the present report DHA.G was only 12% of total urinary DHA for males, then the disparity in the results seems less important. The difference between the urinary DHA.S levels of Nowaczynski et al (1968), all extremely low, and those of the present report, tending to be low, is not so easily explained, though the possible cause of this difference will be considered further in the next section on the effect of salt intake on urinary D.17-OS excretion.

The results of Nowaczynski et al (1968) and those of the present report are the only two studies on idiopathic hypertension where DHA has been estimated in both the sulphate and glucuronide fraction, therefore close comparison of values would be of interest. Unfortunately, the report of Nowaczynski et al is at present only available as an abstract. Therefore, no details of age and sex are available and for valid comparison to be made these two variables are indispensable.

Thyroid hormones have been found to favour the production of A over E and through this decrease of the urinary E/A ratio (Bradlow, Hellman, Zumoff & Gallagher, 1956, and Hellman et al, 1959a,b). Also Hellman et al (1959a,b) studying patients with myxoedema - a disease caused by marked hypothyroid function - found them to have elevated urinary E/A ratios, as was found by the present report for hypertensive males. It is not suggested that the hypertensive males studied were suffering from hypothyroidism. Besides any indication of thyroid disfunction would have been uncovered in each patient's clinical "work up". This aside, it is of interest to note that hypothyroidism is closely associated with hypercholesterolaemia and that a similar relationship between hypertension and hypercholesterolaemia was reported by Thomas (1966). Further, several authors have demonstrated that hypertension is more common in myxoedematous patients than in the normal population (Thompson, Dickie, Morris & Hilkevitch, 1933 and Fuller, Spittell, McConahey & Schirger, 1966). However, there the similarity ends for although the urinary E/A ratio is raised in both conditions the urinary levels of E and A are much lower in myxoedema, especially A, than in hypertension where only the level of A is reduced (see Table 15).

Lombardo et al (1959) found values ranging from 5 - 22 µg/100 ml for 11-deoxycortisol from adrenal vein blood of women with breast cancer whereas Touchstone et al (1959) estimated 11-deoxycortisol in adrenal venous blood of 9 hypertensive males and found values ranging from 2 to 200 µg/100 ml with a mean of 77 µg/100 ml. If these values of

Touchstone et al (1959), which are contrary to his own theory of a gradual diminution of 17α-hydroxylase activity in hypertension, are to be accepted then the preferential metabolism of 11-deoxycortisol to E might to some extent explain the elevated urinary E/A ratios of male hypertensive patients.

It is interesting that the values of the E/A ratio for male hypertensive patients are more comparable with values for normal females than they are with the values for normal males. Comparison of urinary steroid levels for male hypertensive patients and females shows another similarity in that normal pregnant females show elevated levels of the polar steroid 6β-hydroxycortisol (Burstein, Dorfman & Nadel, 1954), the same compound found to be elevated in blood and urine of the idiopathic hypertensive patients (Kornel & Motohashi, 1965 and Kornel & Takeda, 1967).

Since an elevated E/A ratio is a distinctive feature of male hypertensive patients, it might be expected that a similar feature would be displayed by female hypertensive patients, however, this does not appear to be the case. The elevated E/A ratio in male hypertensive patients is probably a long standing phenomenon since it was present in the majority of the patients, irrespective of the duration of the hypertension, which ranged from 2 to 10 years. The change in E/A ratio is probably due to changes in relative and absolute amount of D.17-OS precursors secreted, and to possible changes in the catabolic enzyme systems involved in D.17-OS precursors' degradation.

It was mentioned in Section V.A.4 that castration of males resulted in elevation of the E/A ratio to values similar to those of normal females. Thus in the eunuch DHA.S and DHA were the precursors of the elevated E/A ratio. The testes in man has been shown to secrete some DHA and much lesser amounts of DHA.S (Chapdelaine, MacDonald, Gurpide, Van de Wiele & Lieberman, 1965a,b). If in the eunuch the adrenals behave as in normal males then castration will result in the elevation of the DHA.S/DHA secretion ratio. Further Baulieu et al (1965) demonstrated that in normal male the metabolism of DHA.S produces more urinary E.G than A.G, whereas DHA produces equal amounts of urinary E.G and A.G. Thus it could be that the secretion ratio plays a major role in the control of the urinary E/A ratio, and that alteration of it may be involved in the production of elevated E/A ratio in male hypertensive patients.

Any theory attempting to explain the abnormal urinary D.17-OS patterns in hypertension, would have to take into consideration; the decreased urinary DHA.S levels which are concomitant with normal urinary DHA.G levels; the consistently elevated urinary E/A ratio in male hypertensives and the fact that there is no similar change in the E/A ratio in female hypertensive patients. Further if the theory could also explain the other abnormal steroid patterns presented in hypertension, such as the decreased urinary levels of pregnanediol, pregnanetriol and pregn-5-ene-3β, 17α, 20β-triol; the decreased pregnenolone production rates; the increased plasma and urinary levels of 6α and 6β-hydroxycortisol

sulphate; the elevated 20α and 20β-cortisol adrenal tissue levels; the theory might be coming close to resolving the role played by steroids in the aetiology of hypertension.

A theory fulfilling the above provisos might be a modification of that proposed by Cooper et al (1958) to explain the changes in cortisol/corticosterone production ratios they obtained on incubating sliced adrenal gland in autologous plasma from a series of patients. Cooper et al proposed a progressive diminution of "17α-hydroxylase" activity with increase in the severity of the hypertension. However, let this theory be simply that in hypertension there is a "17α-hydroxylase" block and that the block is restricted to the enzyme converting pregnenolone to 17α-hydroxypregnenolone as illustrated below.

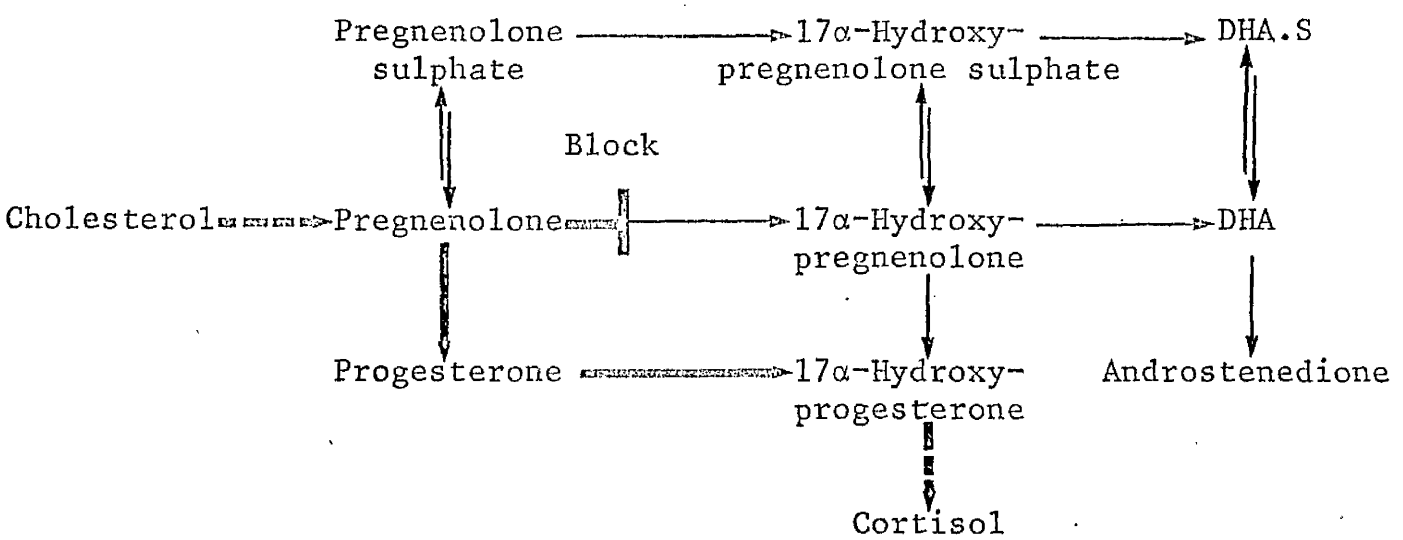


Fig. 13. A proposed mechanism to explain the steroid abnormalities present in hypertension - an enzymatic block.

A block in the conversion of pregnenolone to 17α-hydroxy-pregnenolone would result in the decreased secretion of DHA. Kolbel et al (1965a), Nowaczynski et al (1968) and the present report respectively found,

an absence of urinary DHA in 66% of all the hypertensives studied, very low urinary DHA.S levels in all the idiopathic hypertensives studied and a tendency to low urinary DHA.S levels in the majority of idiopathic hypertensive studied. Accepting the proposed mechanism for explaining the steroid abnormalities presented in hypertension (fig.13) then low urinary DHA.S levels probably indicate decreased secretion of DHA and not decreased secretion of DHA.S. Now DHA.S and DHA are interconvertible in vivo (Chapdelaine et al, 1965a, and MacDonald et al, 1965) and under conditions of depressed DHA secretion the DHA.S⇌DHA equilibrium will favour DHA formation. This is very necessary if normal DHA blood levels are to be maintained. However, since DHA blood levels relative to DHA.S are extremely small (Kirschner et al, 1965 and Plager, 1966) the conversion of more DHA.S to DHA than vice verca would not greatly alter the DHA.S blood levels. But when this is considered in relation to blood half-lives, which for DHA.S is only minutes compared with that of hours for DHA (Baulieu et al, 1965 and Wang, Bulbrook, Sneddon & Hamilton, 1967), then it is realised that any attempt to maintain normal DHA blood levels could result in appreciable conversion of DHA.S to DHA. Therefore, the amount of DHA.S normally excreted in hypertension will be diminished dependent on the conversion of DHA.S to DHA which is governed by the DHA secretion rate, which itself in turn is determined by the extent of the block in the conversion of pregnenolone to 17α-hydroxy-pregnenolone.

The differences in the urinary DHA levels found by Waters et al (1960), Kolbel et al (1965), Nowaczynski et al (1968) and the present report may be explained by a difference in the extent of the enzyme

block together with the effect of salt intake on D.17-OS metabolism (see Section V.C.) and probably other environmental differences as yet not known.

In hypertensive males a block in the conversion of pregnenolone to 17α-hydroxypregnenolone, through a decrease in the secretion of DHA, would result in an increased DHA.S/DHA secretion ratio, and therefore, elevate the urinary E/A ratio. In normal females, there is very little DHA secreted (MacDonald et al, 1965), therefore the presence of a "17α-hydroxylase" block in hypertensive females would probably have more influence on the androstenedione secretion. The net result of an increase in DHA.S/DHA secretion ratio plus a drop in androstenedione would be little or no change in the urinary E/A ratio.

Genest et al (1960) and Vermeulen & Van der Straeten (1963) both studying urinary steroid levels in hypertension reported a significant decrease in the excretion of pregnanetriol, the catabolic product of 17α-hydroxyprogesterone and to a smaller extent 17α-hydroxy-pregnenolone. Further Nowaczynski et al (1964) found the urinary levels of pregn-5-ene-3β, 17α, 20β-triol, the unique metabolite of 17α-hydroxy-pregnenolone (Roberts et al, 1961), to be significantly lower in idiopathic, renal and malignant hypertension when compared to normal subjects. These metabolic abnormalities may be explained also by the presence of a "17α-hydroxylase" block. A decrease in the conversion of pregnenolone to 17α-hydroxy-pregnenolone would presumably result in a decrease in the secretion of

17α-hydroxypregnenolone, and therefore a decrease in the urinary levels of it's unique metabolite pregn-5-en-3β, 17α, 20β-triol. Also since 17α-hydroxypregnenolone is also metabolised in part to pregnanetriol, the decrease in secretion of 17α-hydroxypregnenolone would contribute to the diminished levels of pregnanetriol found in the urine. However, a more complete explanation of the low urinary pregnanetriol levels would probably be better understood after consideration of pregnenolone production rates in this disease.

Additional support of the theory for a "17α-hydroxylase" block would be an explanation of it's function together with some confirmatory experimental proof of its existence. Figure 13 indicates that in the adrenal gland pregnenolone is the precursor of both D.17-OS and glucocorticoids including cortisol. Since it has long been known that cortisol is necessary for maintenance of life, a constant source of cortisol is, therefore, always necessary, and is presumably maintained if necessary at the cost of a decreased production of other steroids. Quantitatively there is normally a large production of D.17-OS steroids, therefore, by shunting the pregnenolone normally going to DHA through progesterone to cortisol by means of a "17α-hydroxylase" block, a constant source of cortisol would thus be available. Such a mechanism for maintaining cortisol output would be feasible in the event of circumstances where there was a reduced availability of pregnenolone.

Nowaczynski et al (1968) estimated pregnenolone production rates in 11 idiopathic hypertensive patients and 10 normal controls and found mean production rates 4.1 and 11.7 mg/24 hr respectively. Therefore, it would appear that hypertension, is a disease where there is a reduced availability of pregnenolone. Regrettably, concomitant estimates of cortisol secretion rates were not available. However, Vermeuleu & Van der Stracten (1963) estimated cortisol secretion rates in hypertensive patients and found them normal. Therefore, the function of the proposed "17α-hydroxylase" block in hypertension could be to maintain normal blood cortisol levels in presence of reduced biosynthetic precursors.

The marked difference between the mean production rates of pregnenolone for hypertensive patients and normal controls 11.7 - 4.1 = 8.6 mg/24 hr would demand a very extensive "17α-hydroxylase" block and even with an extensive block the secretion of cortisol might still be depressed. However, if the secretion rates, but not the production rates, of the glucocorticoid biosynthetic intermediates between pregnenolone and cortisol were markedly depressed then a normal cortisol output might be maintained. In support of this both Genest et al (1960) and Van der Stracten (1963) found decreased urinary levels of pregnanetriol the catabolic product of 17α-hydroxyprogesterone a biosynthetic intermediate of cortisol. Further, Nowaczynski et al (1964) reported decreased urinary levels of pregnanediol in hypertension. Pregnanediol is the metabolic product of progesterone which is also a biosynthetic intermediate of cortisol.

Besk et al (1962) demonstrated in dogs with experimental hypertension a 50% increase in the formation of 20β-hydroxycortisol. Touchstone et al (1965) have shown in vitro that the biosynthesis of 20α and 20β -hydroxycortisol are increased in the adrenal tissue of hypertensive patients. Kornel & Motohashi (1965) and Kornel & Takeda (1967) found increased urinary and plasma levels of 6α and 6β-hydroxy-cortisol sulphate and another still more polar steroid sulphate in patients with idiopathic hypertension. All these findings might be explained by the "17α-hydroxylase" block (fig. 13). Restriction of the conversion of pregnenolone to 17α-hydroxypregnenolone would result in a decrease in secretion of both DHA and androstenedione. Both these steroids are inhibitors of the enzyme G-6-P.D.(Marks & Banks, 1960). Therefore, their absence or their reduced level especially at the adrenal level could result in a stimulation of available G-6-P.D. The stimulation of G-6-P.D. would in turn lead to excessive NADPH production which could then potentiate further and possibly excessive steroid hydroxylation as has been demonstrated in hypertension.

C. An Investigation of the Effect of DHA on Steroid Hydroxylation in Human Adrenal Tissue.

1. Analytical techniques.

(a) Source of tissue. - Section IV.B.1.

(b) Preparation of tissue. - Section IV.B.2.

(c) Incubation procedure. - Section IV.B.3. Two incubations were set up, one with 500 µg of DHA (3.8×10^{-4}M-DHA) and one without any DHA. In each case 3 ml of adrenal tissue preparation plus 1.5 ml of incubation medium were incubated with 100 µmoles of 7α-^{3}H DOC (25 µc/µmole) and 4-^{14}C progesterone (21.7 µc/µmole) at 37° for 1.5 hours.

At the end of the incubation the reactions were stopped by the addition of 20 ml of acetone and refrigerating at -15°. The following unlabelled carrier steroids, 500 µg of each, were added to each of the two flasks, progesterone, DOC, 11β-hydroxyprogesterone, 17α-hydroxyprogesterone and corticosterone.

(d) Extraction of steroids. - Section IV.B.4.

(e) Purification and quantitation. - Section IV.B.5,6 & 7.

(f) Radioactive measurement of steroids and derivatives. - Section IV.B.8

2. Results.

The evidence for the identification of the steroids isolated is given in Tables 23 a & b. The results from these two tables are summarized in histogram fashion as illustrated in fig. 14.

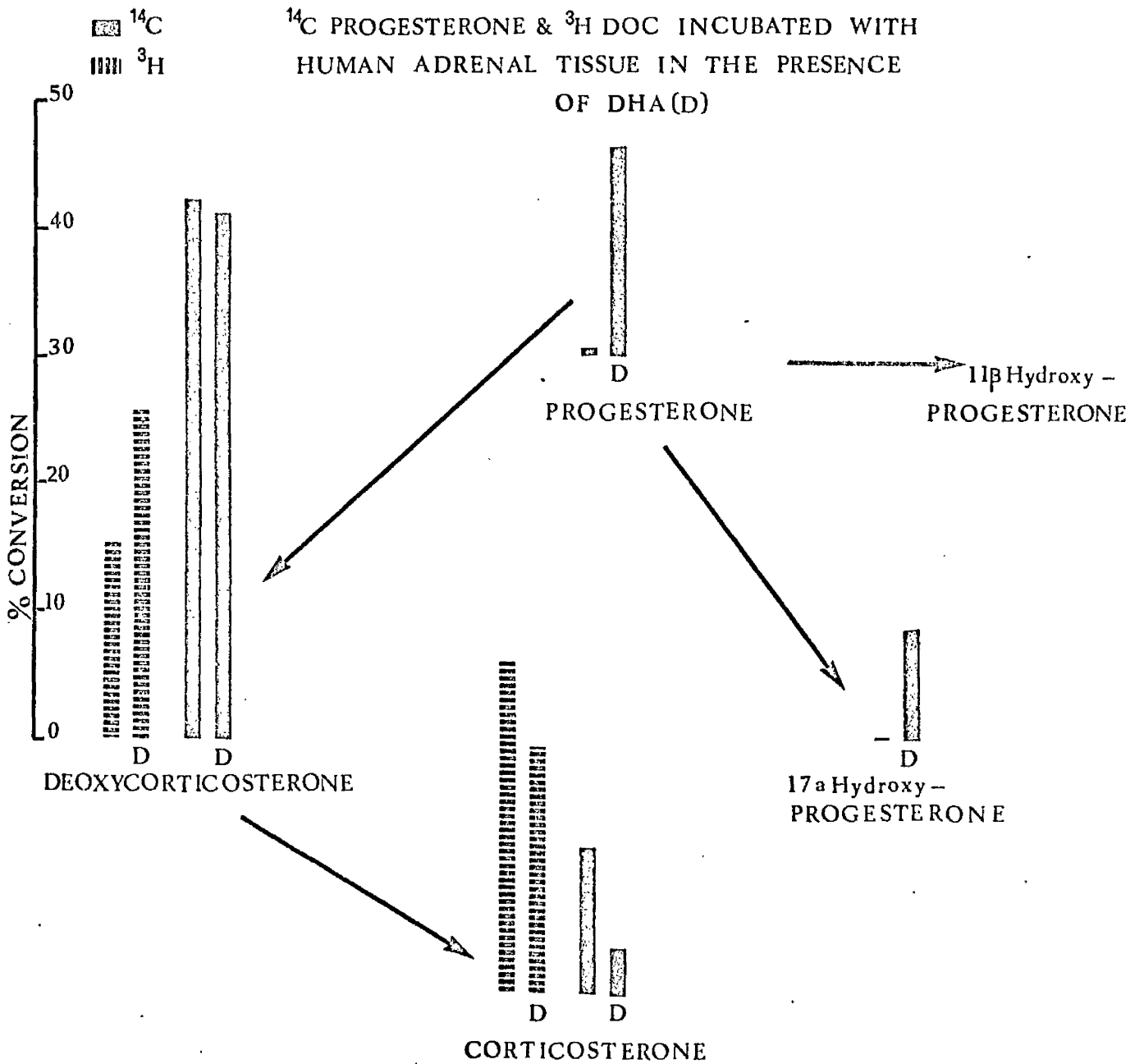
TABLE 23a. EVIDENCE FOR THE IDENTIFICATION OF STEROIDS FROM THE INCUBATION CONTAINING 3.8×10^{-4} M-DHA.

Steroids investigated and the chemical reaction.	Chromatographic mobility identical with that of :-	Specific activities (dpm/mμmole). H^3	C^{14}	% Conversion. H^3		C^{14}	
1. Progesterone							
-	progesterone	0	398	0		15.6	
reduction	20β-hydroxypregn-4-en-3-one	0	412	0		16.5	16.4
acetylation	20β-acetoxypregn-4-en-3-one	0	425	0		17.1	
2. DOC							
-	DOC	1060	1152	25.6		41.8	
acetylation	21-acetoxypregn-4-en-3,20 dione	1155	1100	27.9	25.5	40.0	41.3
reduction	20β,21-dihydroxypregn-4-en-3-one	935	1187	23.1		43.0	
3. 17α-Hydroxyprogesterone							
-	17α-hydroxyprogesterone	0	212	0		8.1	
reduction	17α,20β-dihydroxypregn-4-en-3-one	0	218	0		8.3	8.4
acetylation	17α-hydroxy-20β-acetoxypregn-4-en-3-one	0	231	0		8.8	
4. 11β-Hydroxyprogesterone							
-	11β-hydroxyprogesterone	0	7	0		0.27	
reduction	11β,20β-dihydroxypregn-4-en-3-one	0	1.4	0		0.05	0.05
acetylation	11β-hydroxy-20β-acetoxypregn-4-en-3-one	0	1.4	0		0.05	
5. Corticosterone							
-	corticosterone	785	82.7	19.0		3.0	
reduction	11β,20β,21-trihydroxypregn-4-en-3-one	830	89.3	20.0	19.5	3.2	3.1
acetylation	11β-hydroxy-20β;21-diacetoxypregn-4-en-3-one	795	84.2	19.4		3.1	

TABLE 23b. EVIDENCE FOR THE IDENTIFICATION OF STEROIDS FROM THE INCUBATION WITHOUT DHA.

Steroids investigated and the chemical reaction.	Chromatographic mobility identical with that of :-	Specific activities (dpm/mμmole) H^3	Specific activities (dpm/mμmole) C^{14}	% Conversion. H^3		% Conversion. C^{14}	
1. Progesterone							
-	progesterone	0	18.4	0		0.7	
reduction	20β-hydroxypregn-4-en-3-one	0	17.8	0		0.7	0.6
acetylation	20β-acetoxypregn-4-en-3-one	0	12.1	0		0.5	
2. DOC							
-	DOC	629	1155	15.2		42.0	
acetylation	21-acetoxypregn-4-en-3;20-dione	646	1180	15.6	15.4	42.8	42.2
reduction	20β,21-dihydroxypregn-4-en-3-one	635	1177	15.4		42.0	
3. 17α-Hydroxyprogesterone							
-	17α-hydroxyprogesterone	0	2.8	0		0.11	
reduction	17α,20β-dihydroxypregn-4-en-3-one	0	1.4	0		0.05	0.07
acetylation	17α-hydroxy-20β-acetoxypregn-4-en-3-one	0	1.1	0		0.04	
4. 11β-Hydroxyprogesterone							
-	11β-hydroxyprogesterone	0	38	0		1.45	
reduction	11β,20β-dihydroxypregn-4-en-3-one	0	2.7	0		0.10	0.07
acetylation	11β-hydroxy-20β-acetoxypregn-4-en-3-one	0	1.4	0		0.05	
5. Corticosterone							
-	corticosterone	983	306	23.7		11.1	
reduction	11β,20β,21-trihydroxypregn-4-en-3-one	1036	327	26.2	25.7	11.8	11.7
acetylation	11β-hydroxy-20β,21-diacetoxypregn-4-en-3-one	1122	337	27.1		12.2	

Fig. 14. EFFECT OF DEHYDROEPIANDROSTERONE ON STEROID HYDROXYLATION:



The residual amounts of ^{14}C-progesterone are markedly higher for the incubation containing DHA compared with the incubation without DHA, therefore indicating an inhibitory effect by DHA. Similar findings were found for ^{3}H-DOC where the residual ^{3}H-DOC was higher in the test incubation relative to the control.

Twenty-one hydroxylation of progesterone, that is conversion to DOC, does not appear to be affected by DHA. However, the conversion of DOC to corticosterone was higher in the control incubation for both ^{3}H-DOC and ^{14}C-DOC, therefore, again indicating inhibitory action by DHA. The reverse holds for the formation of 17α-hydroxyprogesterone where DHA would appear to have stimulated the 17α-hydroxylation of progesterone.

The formation of 11β-hydroxyprogesterone could be detected in only trace amounts in the test and control incubations.

3. Discussion.

The results of the present incubations support, within limits, the results of the in vitro studies of Tsutsui et al (1962). They demonstrated in rat adrenal tissue that under conditions of excess $NADP^{+}$, that NADPH formation was reduced by the addition of DHA. In the present studies it was found that the residual amounts of ^{14}C-progesterone and ^{3}H-DOC were much lower in the incubation without added DHA. This was especially true for ^{14}C-progesterone where the ratio of counts remaining as ^{14}C-progesterone in the incubation with DHA to the incubation without DHA was 27/1. From this it was

concluded that much more progesterone was metabolised in the control incubation over that time period than was metabolised in the test incubation and therefore presumably, more NADPH dependent hydroxylation reactions occurred.

In the present incubations it was expected that DHA would influence NADPH dependent hydroxylation steps through inhibition of G-6-P.D. Therefore, it would have been expected that all the hydroxylative steps utilising NADPH as a coenzyme would have been affected equally. This expectation was not realized, for example, the difference between the ratios of the residual counts of the incubation with DHA to the incubation without DHA for ^{14}C-progesterone was 27/1 and for ^{3}H-DOC 1.6/1. This and the other difference regarding the extent of hydroxylation in the presence of DHA may be better appreciated, though not necessarily resolved, after consideration of other regulatory factors influencing steroid hydroxylation.

Sharma, Forchielli & Dorfman (1963) demonstrated that DHA, DHA.S, testosterone and androstenedione will all competitively inhibit 11β-hydroxylation of both DOC and 17α, 21-dihydroxyprogesterone, and of the 4 steroids androstenedione, the immediate metabolite of DHA, was the most active inhibitor. Thus the smaller conversion of both ^{3}H-DOC and ^{14}C-DOC to corticosterone in the presence of DHA, compared with the conversions of ^{3}H-DOC and ^{14}C-DOC to corticosterone without added DHA could be due to a combination of inhibitory processes, that is, inhibition of NADPH production by action of DHA on G-6-P.D.

plus the inhibition of DOC 11β-hydroxylase activity by DHA.

Inspection of fig. 14 shows that the resultant effect of DHA on 17α hydroxylation of progesterone is stimulatory rather than inhibitory. Therefore, since this is a NADPH dependent hydroxylation, there must also be a very strong stimulatory factor in operation to overcome the inhibition known to be mediated through suppression of G-6-P.D. activity by DHA. Further if this anomalous stimulus of 17α-hydroxylation of progesterone by DHA were also true for the 17α-hydroxylation of pregnenolone it would mean that DHA, to a certain extent, stimulates its own biosynthesis. This autocatalytic feature of DHA, if valid, may be shown to be further augmented. Kowal, Forchielli & Dorfman (1964) studying adrenal Δ^5-3β-hydroxysteroid dehydrogenase demonstrated that DHA, as well as its biological oxidation product androstenedione, actively inhibited the conversion of pregnenolone to progesterone. Therefore, more substrate is available for conversion to 17α-hydroxypregnenolone the precursor of DHA. DHA.S was, however, without any inhibitory activity with respects to Δ^5-3β-hydroxysteroid dehydrogenase (Kowal et al, 1964).

The competitive inhibition of 11β-hydroxylase by adrenal steroids is of great interest, especially as one of these steroids is DHA.S. This inhibitory activity of DHA.S contrasts markedly with the complete lack of activity of DHA.S in the inhibition of G-6-P.D or in the inhibition of Δ^5-3β-hydroxysteroid dehydrogenase (Tsutsui et al, 1962 and Sharma et al, 1963). The contrast is further

emphasised when it is realised that as yet nobody appears to have demonstrated any other adrenal regulatory function for DHA.S. This therefore, allows speculation of a possible permissive role for sulphokinase activity in the regulation of steroid biosynthesis. On the other hand, hydrolysis of DHA.S by steroid sulphatases (Burstein & Dorfman, 1963) would probably more effectively check steroid biosynthesis, thus emphasising the importance of the adrenal DHA $\rightleftarrows$ DHA.S equilibrium in the biosynthesis of steroids.

(a) Hypertension. Direct hormonal functions have been described for adrenal steroids such as cortisol and aldosterone, but as yet no direct hormonal function has been described for DHA or DHA.S despite the fact that quantitatively their combined secretion is the most important steroidal secretion by the adrenal gland (Cope & Black, 1958; MacDonald et al, 1965 and Chapdelaine et al, 1965a).

It has been suggested by Baulieu et al (1965) that DHA.S and DHA may function in the adrenal gland as a "safety valve". It is suggested that in situations of stress where suddenly excessive amounts of cortisol are necessary, that its precursor pregnenolone, and possibly pregnenolone sulphate, which are the general precursors of corticoids and adrenal D.17-OS, be shunted towards cortisol production, and vica versa in periods of quiescence and after a period of stress when the demand for cortisol has subsided, the pregnenolone is shunted in favour of

adrenal D.17-OS production. However, though proposed by Baulieu et al (1965) as a function for the DHA $\rightleftarrows$ DHA.S system no example of this mechanism appears as yet to have been demonstrated.

Reconsideration of the discussion dealing with steroids in relation to high blood pressure (Section V.B.3) supplies information relevant to DHA and DHA.S functioning in a "safety valve" mechanism. However, before reconsideration, let the initially proposed "safety valve" mechanism which covers temporary stress changes be extended to cover other stress changes which are of a more permanent nature, such as hypertension. In the idiopathic form of this disease it was stated that pregnenolone was produced in lower amounts by patients as compared with normal individuals and concomitant with this DHA was excreted in lower amounts (Nowaczynski et al, 1968; Sonka et al, 1964 and present report). Therefore, this could represent a situation of not temporary, but of a permanent decrease in the production of corticoids and D.17-OS's principal precursor and necessitating a permanent shunting of pregnenolone through the cortisol pathway. An automatic result of the above shunting would be the stimulation of steroid hydroxylation through the removal of the normal inhibition of G-6-P.D, 11β-hydroxylase and Δ^5-3β-hydroxysteroid dehydrogenase by DHA, DHA.S and androstenedione.

The results of the present section support the latter points regarding the effect of shunting steroid anabolism towards cortisol in idiopathic hypertensive disease. If in the present study:

the control incubation is equated with hypertension with respect to effective DHA levels, that is, the control is without DHA and the hypertensive disease is associated with low urinary DHA levels and by the above "safey valve" theory, hypertension would therefore, represent a condition of reduced adrenal DHA production; and the test incubation is equated with normal health, that is, the test contains DHA as do adrenal of normal individuals. Fig. 14 illustrates that in the control incubation the amounts of 17α-hydroxyprogesterone formed were, relative to the test incubation, extremely small, similarly the progesterone value was extremely small for control relative to test incubation. These findings are in agreement with the studies of Genest et al (1960) and Vermeulen & Van der Straeten (1963), who both found while studying hypertensive patients decreased urinary levels of pregnanetriol, the catabolic product of 17α-hydroxyprogesterone, and are also in agreement with the studies of Nowaczynski et al (1964) who found decreased urinary levels of pregnanediol, the catabolic product of progesterone, in the same disease.

D. Studies on the Effect of Salt Loading, Salt Restriction and Saline Infusion on the Excretion of Individual D.17-OS Sulphates and Glucuronides.

1. Salt loading and salt restriction.

In these studies, subjects were placed on salt diets which generally took the form of 2 days salt ad libitum, 4 days high salt and a final 5 days of low salt diet. Twenty-four hour urine volumes were collected and electrolytes, individual D.17-OS, total 17-OS and total 17-OHCS estimated. Blood pressure was measured and in some subjects plasma electrolytes and plasma cortisol were estimated.

Five normal males and one hypertensive female were studied. Their salt intakes were as below:-

Subjects	Ad Libitum No. days.	300 meq/day No. days.	10 meq/day No. days.
1F	2	9*	6
2M	2	4	5
3M	0	3	7
4M	2	4	5
5M	2	4	4
6M	0	25	4

* In subject 1F high salt intake followed low salt intake. Subjects 2M-6M were normal adult males, 2M & 3M were medical students, 4M & 5M were voluntary patients convalescing after ulcer complaints, and 6M was a member of staff. Subject 1F was a mild idiopathic hypertensive female aged 45 years with an average blood pressure of 160/100 mmHg.

All were free from disorders known to influence urinary 17-OS levels (Section V,A.1). All had normal renal function, as determined by blood urea and serum creatinine levels, and all, excluding subject 1F, had normal blood pressure. None of the subjects was taking medication of any sort for 1 week prior to the studies.

2. Saline Infusion.

A single infusion study was carried out on a healthy medical student. After emptying his bladder the student drank 1 pint of water, 2 hours later he again emptied his bladder, and immediately after this he received by intravenous infusion 2 pints of saline at the rate of 54 ml/minute, that is, 1 hour for the two pints of saline. Urine was collected at 1 hourly intervals for 4 hours, then for a further 2.4 hourly interval.

Urine volumes were measured and urinary electrolytes, creatinine and individual D.17-OS were estimated.

3. Analytical Techniques.

Collection of blood and urine	Section IV,A.5.
Urinary sodium, potassium & chloride	Section IV.A.1.a & b.
Blood urea, urinary & serum creatine	Section IV.A.2.a,b,&c.
17-OS & 17-OHCS	Section IV.A.6.a & b.
D.17-OS & D.17-OS.G	Section IV.C.2.

All analyses were duplicated.

4. Results.

(a) Salt loading and salt restriction. The levels of urinary electrolytes and excretion of urinary D.17-OS for subject 2M while on a varying salt intake are shown in Table 24, and the histogram (fig.15) illustrates the changes in the excretion of the individual D.17-OS sulphates and glucuronides. High salt intake caused definite increase in DHA.S with less definite increases for the other steroid sulphates and glucuronides. Low salt intake caused a marked reduction in steroid sulphate excretion. Comparison of the average urinary steroid sulphate levels during high salt intake with amounts excreted on day 9, the third day of low salt intake, shows a 93% drop in DHA.S excretion and a 83,74 and 50% drop in epiA.S, A.S and E.S respectively. Similarly for the steroid glucuronides, drops of 85, 36 and 32% for DHA.G, E.G and A.G respectively were observed. After 5 days on the low salt diet the urinary D.17-OS sulphates were still very low, however, the D.17-OS glucuronides E.G and A.G had returned to, and even surpassed their highest urinary levels while on high salt diets. It is noteworthy that urine volumes associated with these high glucuronide levels were 53% higher than the average of the previous 4 days of low salt diet.

The ratio of E.G/A.G remained fairly constant throughout the study, varying from 0.50 to 0.57 with no special trend during the different salt intakes. The ratio of E.(S+G)/A.(S+G) was not just as constant varying from 0.38 to 0.5, tending to be higher on the low salt diet. The variation of E.(S+G)/A.(S+G) was determined to great

TABLE 24. THE URINARY EXCRETION OF INDIVIDUAL D.17-OS, TOTAL 17-OS, TOTAL 17-OHCS AND ELECTROLYTES BY SUBJECT 2M WHITE ON VARYING SALT INTAKE.

DAILY Na INTAKE meq.	URINARY ELECTROLYTES meq/24 hr.			D.17-OS SULPHATES mg. free steroid/24 hr.				D.17-OS GLUCURONIDES mg. free steroid/24 hr.			TOTAL 17-OS mg/24 hr.	TOTAL 17-OHCS mg/24 hr.
	Na	K	Cl	epiA	DHA	E	A	DHA	E	A		
1. Ad.lib.	177	53	193	0.26	2.64	0.08	0.94	0.56	1.24	2.34	9.1	7.0
2. Ad.lib.	216	64	234	0.30	2.95	0.10	1.16	0.79	1.51	2.88	10.1	8.0
3. 300	405	86	416	0.39	4.50	0.10	1.43	1.26	1.82	3.32	13.5	10.5
4. 300	310	61	331	0.26	3.45	0.07	1.12	0.97	1.48	2.93	10.8	8.4
5. 300	227	45	241	0.25	3.44	0.08	1.13	0.74	1.52	2.63	8.7	8.6
6. 300	221	54	242	0.27	3.63	0.10	0.98	0.83	1.52	2.67	10.3	9.2
7. 10	75	65	107	0.11	0.99	0.08	0.41	0.37	0.91	1.68	6.8	7.0
8. 10	52	43	40	0.05	0.53	0.04	0.35	0.23	1.06	1.96	5.8	5.3
9. 10	38	46	27	0.05	0.29	0.05	0.30	0.14	1.00	1.98	5.5	5.9
10. 10	9.6	34	16	0.142	0.97	0.08	0.73	0.31	1.71	3.35	7.4	8.3
11. 10	21.3	62	16	0.122	0.75	0.05	0.56	0.47	2.34	4.70	6.5	7.3

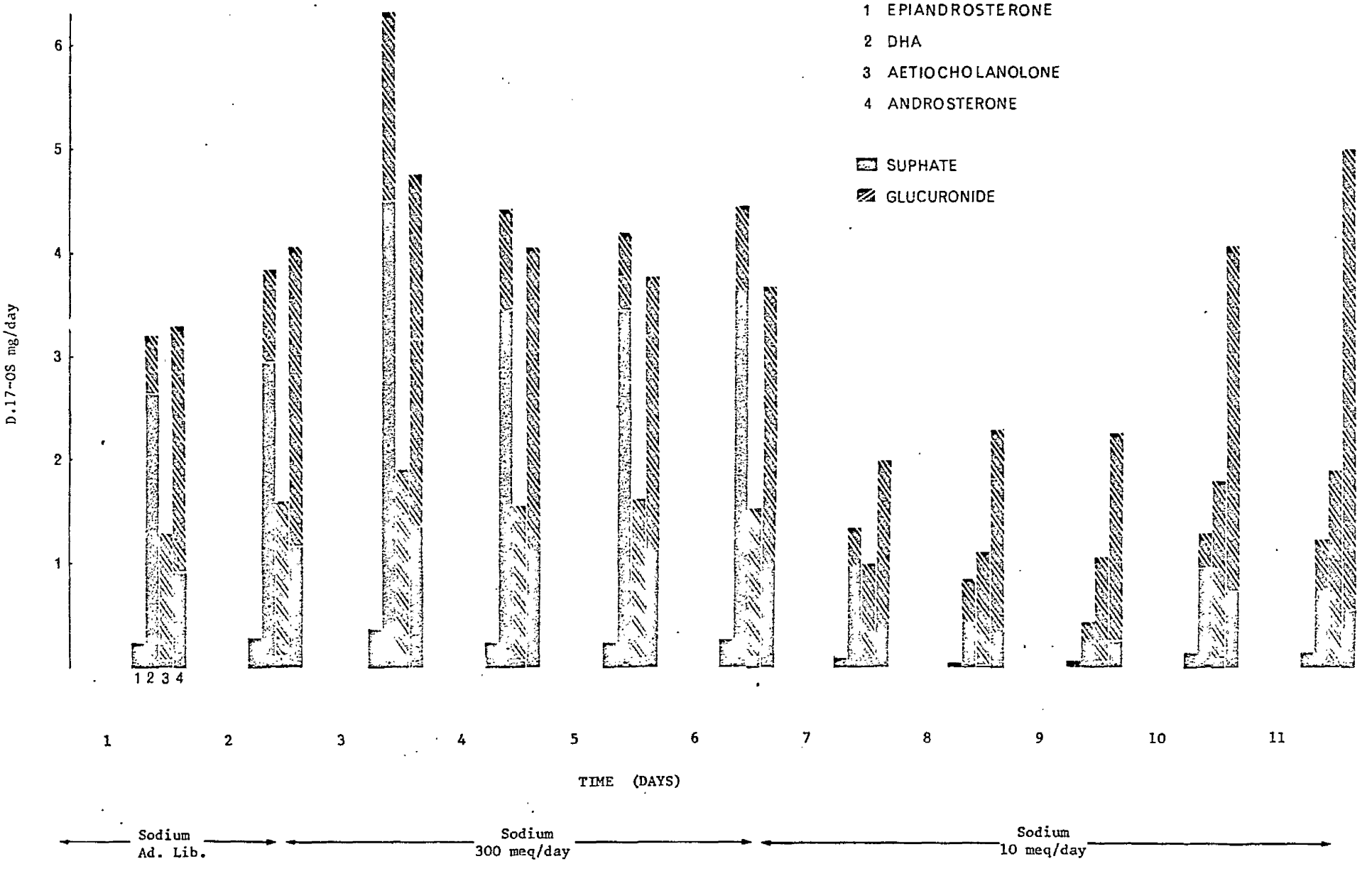


Fig. 15. THE EFFECT OF VARYING SALT INTAKE ON THE EXCRETION OF URINARY D.17-OS SULPHATES AND GLUCURONIDES IN A NORMAL MALE SUBJECT (2M).

extent by the urinary level of A.S which varied to a greater extent than did the other three steroid conjugates involved in this ratio. Comparison of day 4 with day 9 illustrates this point:-

	E.G/A.G	E.(S+G)/A.(S+G)	A.S	A.G	A.G/A.S
Day 4	0.5	0.38	1.12	2.93	2.32
Day 9	0.5	0.46	0.3	1.98	6.60

The most dramatic changes in relative excretion of steroids was that for DHA.(S+G) relative to E.(S+G) and A.(S+G). During all four days of the high salt diet DHA.(S+G) was the major urinary D.17-OS whereas during the low salt diet A.(S+G) was the major urinary D.17-OS (fig.15). Excretion of DHA.(S+G) dropped so low during low salt intake that it fell below that of E.(S+G) thus on high salt diet the urinary excretory pattern of the combined sulphates and glucuronides was DHA>A>E whereas on days 8,9,10 & 11 it was A>E>DHA.

Urine volumes tended to follow the changes in salt intake, that is, urine volumes rose during salt loading and dropped to control levels during salt restriction. Blood pressure followed the same pattern increasing during salt loading and dropping on low salt diet, but never below the blood pressure levels registered while the subject was on salt ad libitum.

The levels of the urinary electrolytes and the excretion of urinary D.17-OS by subject 3M while on high and low sodium intake are shown in Table 25. The results are very similar to those for subject 2M. There was again a marked decrease in the excretion of D.17-OS

TABLE 25. THE URINARY EXCRETION OF INDIVIDUAL D.17-OS, TOTAL 17-OS, TOTAL 17-OHCS and ELECTROLYTES BY SUBJECT 3M WHILE ON VARYING SODIUM INTAKE.

DAILY Na INTAKE *meq.	URINARY ELECTROLYTES meq./24 hr.		D.17-OS. SULPHATES mg. free steroid/24 hr.				D.17-OS. GLUCURONIDES mg. free steroid/24 hr.			TOTAL 17-OS	TOTAL 17-OHCS
	Na	K	epiA	DHA	E	A	DHA	E	A	mg/24 hr.	
5. 300	280	127	0.37	3.90	0.22	1.28	1.67	2.35	4.85	19.1	14.7
6. 300	318	112	0.59	6.43	0.28	1.67	2.62	3.50	6.34	23.2	15.6
7. 300	292	157	0.26	2.30	0.16	0.94	1.19	2.02	3.27	13.1	10.8
8. 10	104	30	0.03	0.12	0.04	0.21	0.11	0.79	1.40	5.6	4.5
9. 10	71	61	0.09	0.42	0.08	0.41	0.28	1.76	3.44	10.9	9.1
10. 10	61	58	0.10	0.64	0.16	0.43	0.31	1.23	2.39	10.0	6.3
11. 10	86	75	0.09	0.82	0.08	0.35	0.56	1.35	2.39	8.4	7.4
12. 10	30	59	0.104	1.11	0.06	0.31	0.49	0.83	1.57	6.8	5.6
13. 10	30	65	0.29	3.15	0.13	0.80	1.28	2.11	3.69	14.9	12.3
14. 10	29	100	0.25	2.53	0.15	1.14	1.27	3.04	4.85	17.3	14.6

* Subject 3M had already been on 300 meq. Na intake for 4 days.

sulphates, on moving from a high to a low salt diet. The lowest levels were registered on day 1 of low salt diet. DHA.S levels dropped by 93% and epiA.S, A.S and E.S by 93, 84 and 80% respectively, and the drop shown by the steroid glucuronides were 93, 70 and 70% for DHA.G, E.G and A.G respectively. After the initial drop in the urinary levels of both the steroid glucuronides and sulphates they increased gradually on the low salt intake to reach levels close to that of respective levels while on high salt intake. Unlike 2M, subject 3M did not show marked changes in the E.(S+G)/A.(S+G) ratio on going from high to low salt intake, though the ratio was not constant, it varied from 0.41 to 0.53. However, the marked change in the relative excretion of DHA(S+G) relative to E.(S+G) and A.(S+G) was demonstrated. On the high salt diet the pattern of excretion of total D.17-OS conjugates was DHA = A>E, which changed to A>E>DHA and remained so for the first 4 days of the low salt diet.

The level of the urinary electrolytes and the excretion of D.17-OS by subject 4M are as shown in Table 26. A histogram of the urinary D.17-OS sulphate and glucuronide levels while subject 4M was on varying salt intake is shown in fig. 16. The pattern of excretion of urinary steroid conjugates by subject 4M was somewhat different from that of subjects 2M & 3M. There was a very marked increase on the change from salt ad libitum to high salt intake for DHA.S(113%), epiA.S(61%), A.S(48%) and DHA.G(102%), whereas the changes for E.S, E.G and A.G were negligible. There was a drop on going from high to

TABLE 26. THE URINARY EXCRETION OF INDIVIDUAL D.17-OS, TOTAL 17-OS, TOTAL 17-OHCS AND ELECTROLYTES BY SUBJECT 4M WHILE ON VARYING SODIUM INTAKE.

DAILY Na INTAKE meq.	URINARY ELECTROLYTES meq/24 hr.			D.17-OS SULPHATES mg. free steroid/24 hr.				D.17-OS GLUCURONIDES mg. free steroid/24 hr.			TOTAL 17-OS mg/24 hr	TOTAL 17-OHCS mg/24 hr
	Na	K	C1	epiA	DHA	E	A	DHA	E	A		
1. Ad.lib.	127	44	131	0.80	4.72	0.79	2.03	1.19	4.12	6.63	18.7	12.4
2. Ad.lib.	214	68	237	0.96	5.04	0.72	3.31	1.97	5.18	5.84	20.0	16.6
3. 300	240	78	295	1.63	11.34	0.77	4.46	2.75	3.81	5.57	20.9	14.6
4. 300	316	93	338	1.18	9.20	0.56	3.87	3.26	5.08	6.30	20.3	19.1
5. 300	273	65	306	1.40	9.64	0.92	3.66	3.24	5.14	7.94	22.3	15.6
6. 300	330	74	365	1.46	10.15	0.94	3.76	2.95	4.85	7.24	24.6	13.7
7. 10	87	35	85	0.86	5.87	0.63	2.48	1.64	4.22	6.65	18.4	17.0
8. 10	23	46	32	0.74	5.64	0.58	2.14	1.27	3.77	5.95	16.0	15.1
9. 10	12	51	23	0.70	4.52	0.57	2.00	1.38	3.75	5.67	15.4	14.8
10. 10	12	53	11	0.50	3.15	0.54	1.77	0.93	3.59	5.45	16.3	14.0
11. 10	15	91	22	0.69	4.00	0.57	2.52	1.50	3.41	5.93	18.6	16.0

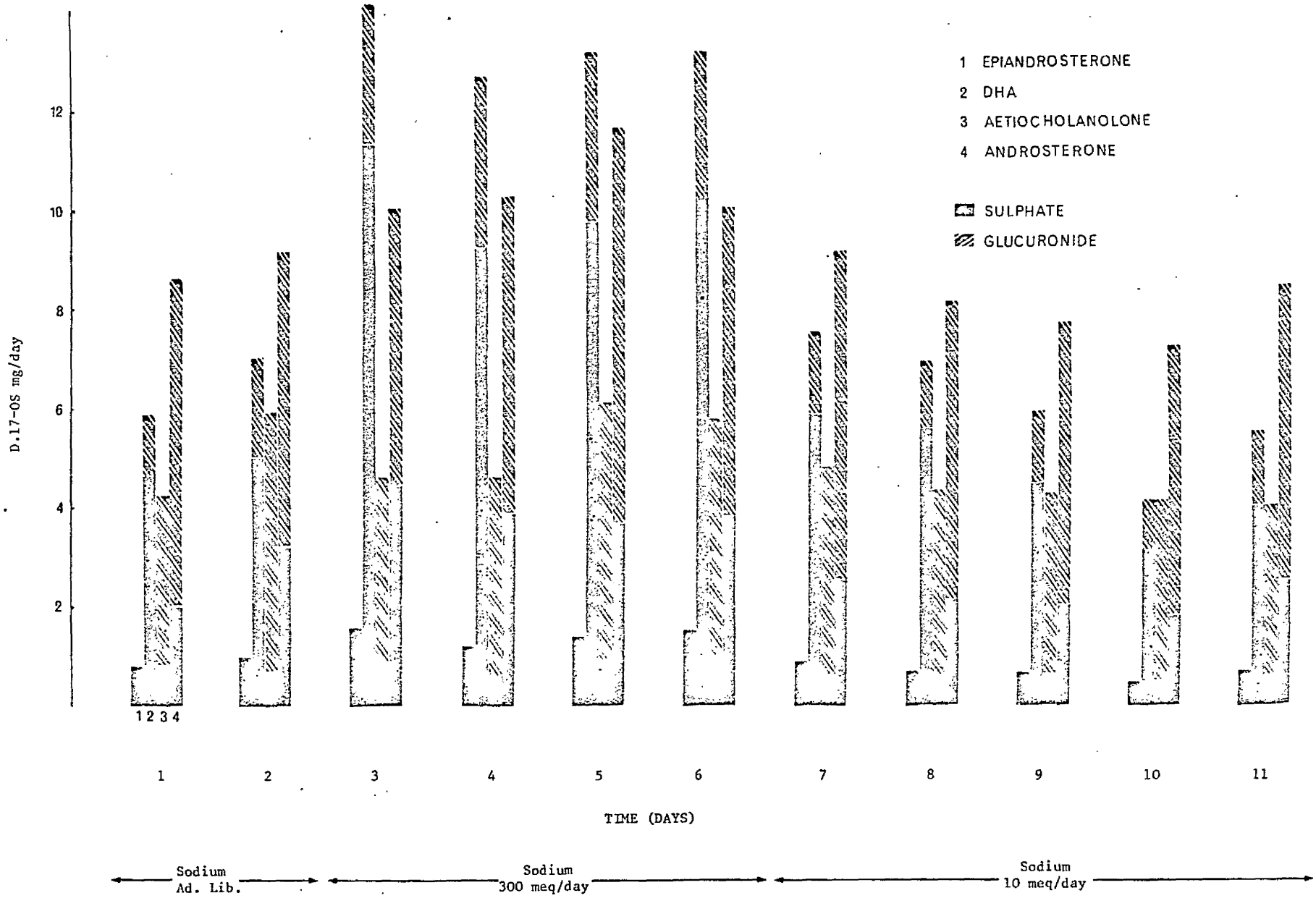


Fig. 16. THE EFFECT OF VARYING SALT INTAKE ON THE EXCRETION OF URINARY D.17-OS SULPHATES AND GLUCURONIDES IN A NORMAL MALE SUBJECT (4M).

low salt diet, the drop however, was not nearly as great as that for subjects 2M & 3M. The D.17-OS urinary levels did decrease gradually reaching their lowest on the fourth day of the low salt intake, levels lower than those of the control days. However, in comparison with the drop in urinary sodium levels, the drops shown by the individual were negligible.

The E.(S+G)/A.(S+G) ratio varied from 0.46 - 0.58 with a tendency to higher values during high salt intake. Similarly the E.G/A.G ratio ranged from 0.57 - 0.8, during high salt intake the mean ratio was 0.7, while over the period of salt ad libitum and low salt intake the mean ratio was 0.63. The pattern of excretion of total D.17-OS conjugates was for salt ad libitum and low salt intake A>DHA>E and for high salt intake DHA>A>E. The blood pressure measurements showed no marked changes with variation in salt diet. Urine volumes were larger during salt loading, and dropped to levels slightly lower than the control levels during salt restriction.

Urinary total 17-OHCS were estimated in subjects 2M, 3M & 4M (see Tables 24, 25 & 26) with purpose of uncovering any possible stress effects which might be induced through extremes of salt intake. Stress through release of ACTH causes secretion of urinary D.17-OS precursors as well as urinary 17-OHCS precursors. Thus, increases in urinary D.17-OS levels could be the result of the stress of varying the salt intake, especially salt loading, and not a direct effect of salt on D.17-OS metabolism. Table 27 demonstrates the effect of stress on

TABLE 27. THE URINARY EXCRETION OF TOTAL 17-OHCS, TOTAL 17-OS AND DHA.S BY 3 MALES BEFORE AND AFTER ADMINISTRATION OF SYNTHETIC ACTH (DEPOT SYNACTHEN, 50 UNITS).

SUBJECT		DAY 1	DAY 2	ΦDAY 3	DAY 4	DAY 5
1	*17-OHCS	14.1	14.9	57.5	23.9	15.3
	*17-OS	7.2	8.3	15.8	9.9	7.8
	$\frac{17\text{-OHCS}}{17\text{-OS}}$	1.96	1.8	3.64	2.41	1.96
	*DHA.S	0.44	0.35	1.77	0.47	0.14
2	17-OHCS	11.8	12.9	33.5	21.2	11.3
	17-OS	9.9	9.9	15.3	13.2	6.1
	$\frac{17\text{-OHCS}}{17\text{-OS}}$	1.19	1.30	2.19	1.61	1.85
	DHA.S	1.34	1.44	3.43	0.84	0.46
3	17-OHCS	8.5	9.4	27.7	8.5	15.0
	17-OS	11.0	11.3	18.1	9.1	15.4
	$\frac{17\text{-OHCS}}{17\text{-OS}}$	0.77	0.83	1.53	0.94	0.97
	DHA.S	4.44	4.5	8.01	0.86	3.35

* mg/24 hours.

Φ Depot synacthen administered (i.m.) on morning of third day.

the excretion of 17-OHCS, 17-OS and DHA.S in man. The injection (i.m.) of depot synacthen into three males resulted in very large increases in the urinary levels of 17-OHCS and moderate increases in 17-OS. Comparison of absolute increases in urinary 17-OHCS and 17-OS together with their 17-OHCS/17-OS ratio with the values obtained from subjects 2M, 3M and 4M (Table 28) would indicate that the changes in urinary D.17-OS levels were not mediated through stress induced ACTH stimulation of the adrenals.

Compared with subjects 2M, 3M & 4M, subjects 5M & 6M did not show marked changes in D.17-OS excretion while on different salt intakes. The levels of the urinary electrolyte and individual D.17-OS for subjects 5M & 6M are shown in Tables 29 & 30 respectively. Subject 5M showed a small increase for DHA.S(21%), E.G(11%) and A.G(14%) in the mean amount of these steroids on going from salt ad libitum to a high salt intake, however, the increases were not statistically significant. The marked decrease expected in the excretion of the D.17-OS on changing from high to low salt diet was not obtained, although in the case of epiA, DHA, E & A sulphates there were statistically significant decreases, $P = 0.02$, $P = 0.02$, $P = 0.10$ and $P = 0.05$ and although there were drops in urinary individual D.17-OS, glucuronide levels they were not statistically significant. Similarly for subject 6M (Table 30) the drop in the excretion of D.17-OS on going from high to low salt intake was very small and only statistically significant in the case of epiA.S($P<0.02$), DHA.S($P = 0.02$), E.S($P = 0.02$) and A.G($P<0.02$).

TABLE 28. THE EFFECT OF VARYING SALT INTAKE COMPARED WITH EFFECT OF ACTH ADMINISTRATION (i.m.) ON THE DAILY URINARY 17-OHCS/17-OS RATIO.

SUBJECTS	DAILY 17-OHCS/17-OS RATIO								
	SALT AD LIBITUM		HIGH SALT INTAKE			LOW SALT INTAKE			
2M	0.77	0.79	0.78	0.78	0.99	1.03	0.91	1.07	1.12
3M	-	-	0.77	0.68	0.82	0.80	0.83	0.63	0.88
4M	0.66	0.83	0.70	0.94	0.70	0.92	0.96	0.86	0.86

	DAY 1	DAY 2	*DAY 3	DAY 4	DAY 5
1	1.96	1.84	3.64	2.41	1.96
2	1.19	1.30	2.19	1.61	1.85
3	0.77	0.83	1.53	0.94	0.97

* Depot synacthen administered (i.m.) on morning of third day.

TABLE 29. THE URINARY EXCRETION OF INDIVIDUAL D.17-OS, TOTAL 17-OS, TOTAL 17-OHCS AND ELECTROLYTES BY SUBJECT 5M WHILE ON VARYING SODIUM INTAKE.

DAILY Na INTAKE meq	URINARY ELECTROLYTES meq/24 hr		D.17-OS SULPHATES mg free steroid/24 hr.				D.17-OS GLUCURONIDES mg free steroid/24 hr.			TOTAL 17-OS	TOTAL 17-OHCS
	Na	K	epiA	DHA	E	A	DHA	E	A	mg/24 hr	
1. Ad.Lib.	142	72	0.20	1.31	0.22	0.57	0.42	2.51	2.35	5.8	6.8
2. Ad.Lib.	111	76	0.11	0.88	0.32	0.35	0.29	2.47	2.34	7.9	8.1
3. 300	131	68	0.12	0.86	0.07	0.24	0.26	2.00	1.93	10.4	8.3
4. 300	196	80	0.14	1.15	0.06	0.31	0.48	2.88	2.86	10.6	10.7
5. 300	289	87	0.16	1.60	0.30	0.50	0.50	2.95	2.90	11.4	12.8
6. 300	265	67	0.18	1.68	0.30	0.51	0.54	3.02	2.98	11.6	12.0
7. 10	168	53	0.11	0.83	0.07	0.26	0.28	2.75	2.98	10.2	11.6
8. 10	66	41	0.12	0.91	0.04	0.24	0.27	2.70	2.91	12.7	11.8
9. 10	73	56	0.11	0.79	0.04	0.14	0.24	2.40	2.52	9.9	10.3
10. 10	46	49	0.12	0.95	0.04	0.20	0.34	2.81	3.00	11.5	9.2

TABLE 30. THE URINARY EXCRETION OF INDIVIDUAL D.17-OS, TOTAL 17-OS, TOTAL 17-OHCS AND ELECTROLYTES BY SUBJECT 6M WHILE ON VARYING SODIUM INTAKE.

DAILY Na INTAKE * meq	URINARY ELECTROLYTES meq/24 hr			D.17-OS SULPHATES mg free steroid/24 hr				D.17-OS GLUCURONIDES mg free steroid/24 hr			TOTAL 17-OS	TOTAL 17-OHCS
	Na	K	Cl	epiA	DHA	E	A	DHA	E	A	mg/24 hr	
1. 300	290	96	290	1.34	10.5	1.08	3.52	2.73	5.95	6.08	22.0	13.7
2. 300	221	99	261	1.23	9.8	1.19	3.71	2.44	6.58	5.82	20.2	11.2
3. 300	215	45	215	1.82	14.5	1.42	4.92	2.45	5.93	5.96	25.5	17.1
4. 300	264	40	206	1.56	12.3	1.26	2.93	1.65	5.22	4.80	21.1	12.8
7. 300	172	75	168	1.17	7.60	1.07	3.29	0.917	5.90	6.02	15.8	11.9
11. 300	264	30	274	1.15	7.77	0.99	2.31	2.57	5.89	6.08	17.6	12.0
14. 300	258	43	264	1.41	9.84	1.05	2.65	3.09	6.35	5.73	20.0	13.3
15. 300	240	45	254	1.32	10.10	0.93	2.53	2.68	4.78	4.25	16.0	12.8
17. 300	243	105	232	0.74	6.52	0.89	2.09	1.13	5.72	5.08	15.3	14.0
22. 300	266	113	254	1.23	10.50	1.02	3.21	3.53	5.47	4.82	19.1	14.0
24. 300	314	119	297	1.18	9.53	1.24	2.93	3.01	5.40	4.73	18.1	12.3
25. 10	277	83	291	1.09	9.52	1.01	2.77	3.78	5.68	5.50	17.9	13.1
26. 10	116	47	139	0.89	7.21	0.97	2.34	2.90	4.91	4.81	16.1	11.9
27. 10	117	106	85	1.17	7.60	0.98	3.29	2.00	4.76	4.60	14.6	14.0
28. 10	66	75	50	1.00	6.50	0.86	2.38	2.68	4.72	4.22	15.8	13.3
29. 10	41	30	49	1.10	9.42	0.86	2.89	2.90	5.68	5.3	18.0	15.1

* The 300 meq sodium intake was maintained on days not recorded.

The results of the electrolyte and D.17-OS urinary levels for subject 1F, an idiopathic hypertensive female, are shown in Table 31. There were definite decreases in the excretion of all the D.17-OS sulphates by subject 1F on changing from salt ad libitum to a low salt intake. The percentage decrease between the mean DHA.S excreted during salt ad libitum and that excreted during low salt intake was 56% and for epiA.S, E.S and A.S the decreases were 66, 80 and 70% respectively, all were statistically significant decreases. On the other hand, the D.17-OS glucuronide levels showed no decreases for the same dietary changes. In fact E.G showed an increase of 30%.

The dietary change from low to high salt intake caused no difference in the excretion of D.17-OS glucuronides. However, the same dietary change caused statistically significant increases in the excretion of all the D.17-OS sulphates at the 0.2% level of significance for DHA.S and epiA.S and at the 0.4 and 0.8% level for E.S and A.S respectively. Only in the case of DHA.S did the urinary levels return to and surpass those obtained on salt ad libitum. The amount of DHA.S excreted on day 14, second day of the high salt diet, showed a remarkable increase over the mean urinary levels on low salt diet. It increased by 2900%, however, it dropped immediately to levels comparable with those of the two control days. The remarkably high levels of DHA.S on day 14 were not reciprocated by the other D.17-OS.

TABLE 31. THE URINARY EXCRETION OF INDIVIDUAL D.17-OS, TOTAL 17-OS, TOTAL 17-OHCS AND ELECTROLYTES BY SUBJECT 1F WHILE ON VARYING SODIUM INTAKE.

DAILY Na INTAKE * meq	URINARY ELECTROLYTES meq/24 hr			D.17-OS SULPHATES mg free steroid/24 hr				D.17-OS GLUCURONIDES mg free steroid/24 hr			TOTAL 17-OS	TOTAL 17-OHCS
	Na	K	Cl	epiA	DHA	E	A	DHA	E	A	mg/24 hr	
1. Ad.Lib.	136	15	134	0.10	0.20	0.22	0.37	0.15	1.93	1.54	7.5	7.9
4. Ad.Lib.	59	22	55	0.11	0.16	0.50	0.37	0.08	1.55	1.26	6.1	7.1
6. 10	63	46	63	0.04	0.05	0.06	0.10	0.14	2.6	1.86	8.7	7.8
9. 10	8	33	15	0.04	0.06	0.06	0.11	0.07	1.89	1.28	6.1	6.9
12. 10	11	40	15	0.06	0.08	0.09	0.11	0.10	2.10	1.24	8.1	8.4
13. 300	67	37	59	0.08	0.12	0.12	0.18	0.07	1.69	1.18	7.9	5.8
14. 300	211	48	232	0.09	2.11	0.17	0.25	0.07	2.41	1.44	10.3	11.5
15. 300	239	38	219	0.08	0.11	0.09	0.11	0.15	2.53	1.58	11.3	8.6
16. 300	157	34	165	0.08	0.35	0.08	0.13	0.18	1.61	0.90	8.5	7.9
17. 300	190	53	199	0.07	0.15	0.14	0.15	0.16	3.06	1.80	8.4	9.2
18. 300	137	35	153	0.08	0.11	0.10	0.11	0.15	1.84	1.16	7.1	7.3

* Subject 1F remained on the salt diet on days not listed.

(b) Saline infusion study. The effect of saline infusion on individual D.17-OS excretion in normal males is shown in Table 32. The percentages of the sodium load, administered intravenously, and excreted over the first four 1 hourly periods were 18.3, 7.7, 14.3 and 11.0, therefore, the total of the load excreted over the first four hours was 51.3%. This is about 15% greater than the proportion of an administered saline load normally excreted over a four hour period (Rovner, Conn, Knopp, Cohen & Hsueh, 1965). Table 32 and fig. 17ademonstrate that all the D.17-OS sulphates are excreted in a pattern very similar to that followed by sodium. All the steroid sulphates showed two peak excretion periods as did sodium. The first peak shown by all the steroid sulphates occurred at the same time as that for sodium (0 - 1 hours) and the second peak shown by DHA.S, epiA.S and A.S occurred between 3 - 4 hours was after that for sodium and E.S (2 - 3 hours). Thus the excretion of E.S followed that of sodium excretion very closely.

A similar pattern was shown by the D.17-OS glucuronides (Table 32 & fig.17b) though in the case of A.G and E.G their second peaks were not as sharp as their first peaks, they tended to plateau, as did the peak for A.S, over the 2 - 4 hour time intervals (fig. 17a).

The E.G/A.G ratio gradually increased over the 4 one hourly periods of salt excretion as follows - 0.82, 0.91, 0.98 and 1.03 and dropped slightly over the last time interval 4 - 6.4 hours to 0.97. The baseline value and that of the 24 hour urine were 0.87 and 0.9 respectively.

TABLE 32. THE URINARY EXCRETION OF SODIUM AND INDIVIDUAL D.17-OS SULPHATES AND GLUCURONIDES BY A NORMAL MALE BEFORE AND AFTER INTRAVENOUS INFUSION OF NORMAL SALINE.

TIME hr.	SODIUM meq/min	D.17-OS SULPHATES µg free steroid/min				D.17-OS GLUCURONIDES µg free steroid/min		
		epiA	DHA	E	A	DHA	E	A
2	0.16	0.072	0.161	0.240	0.337	0.091	2.16	2.49
1(1)*	0.49	0.152	0.282	0.577	0.805	0.180	4.84	5.90
1(2)	0.21	0.065	0.127	0.175	0.280	0.076	1.69	1.85
1(3)	0.39	0.125	0.262	0.377	0.555	0.136	2.95	3.03
1(4)	0.30	0.192	0.457	0.298	0.595	0.159	3.20	3.12
2.4(6.4)	0.15	0.118	0.266	0.229	0.461	0.085	2.37	2.45
24 Φ	0.13	0.097	0.206	0.252	0.390	0.085	2.29	2.56

* Subject was given 2 pints normal saline intravenously (54 ml/min) at start of this 1 hour period.

Φ Twenty-four hour control period.

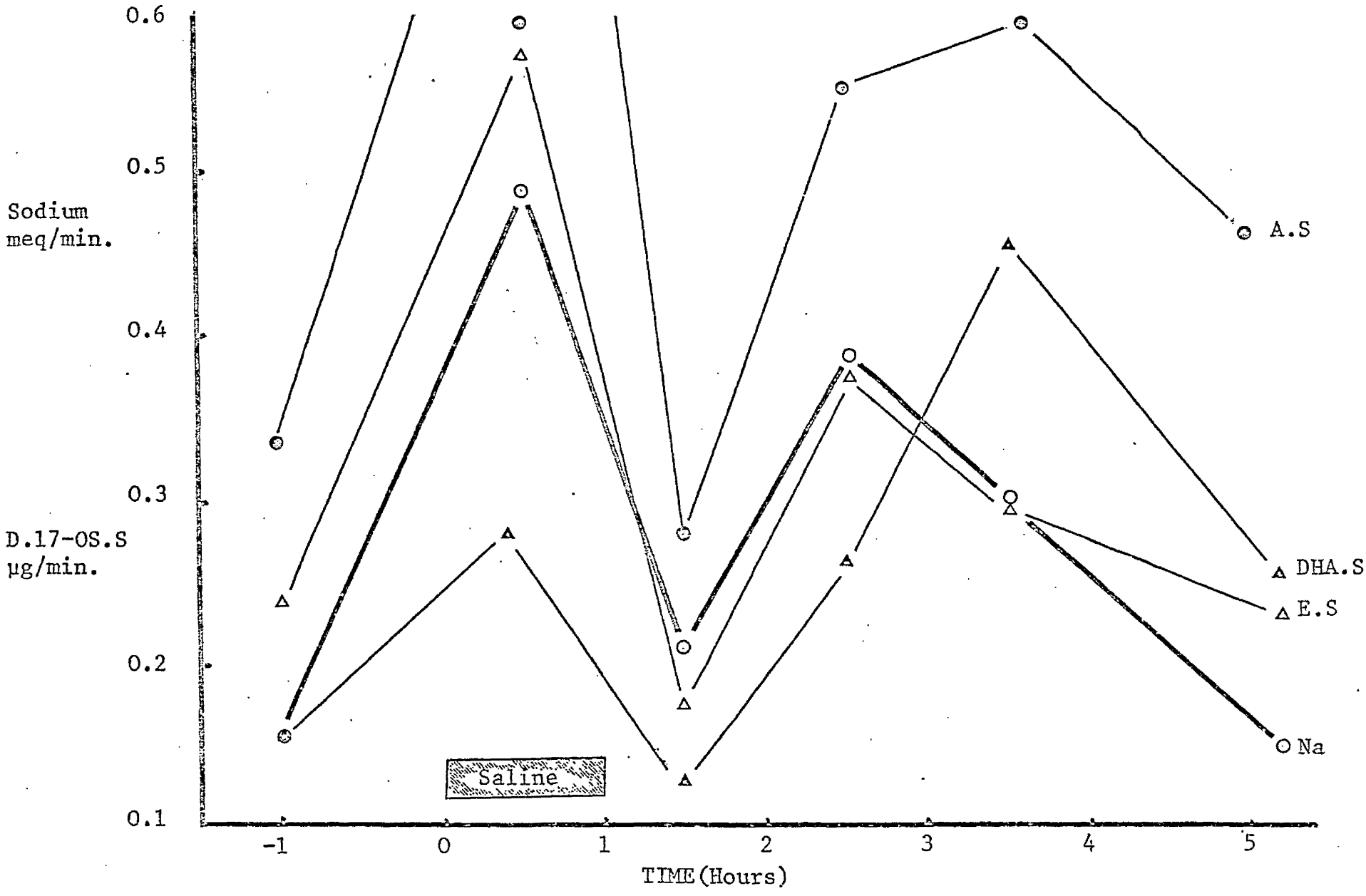


Fig. 17a. The effect of saline infusion (2 pints, 54 ml/min) on the excretion of urinary D.17-OS sulphates in a normal male subject.

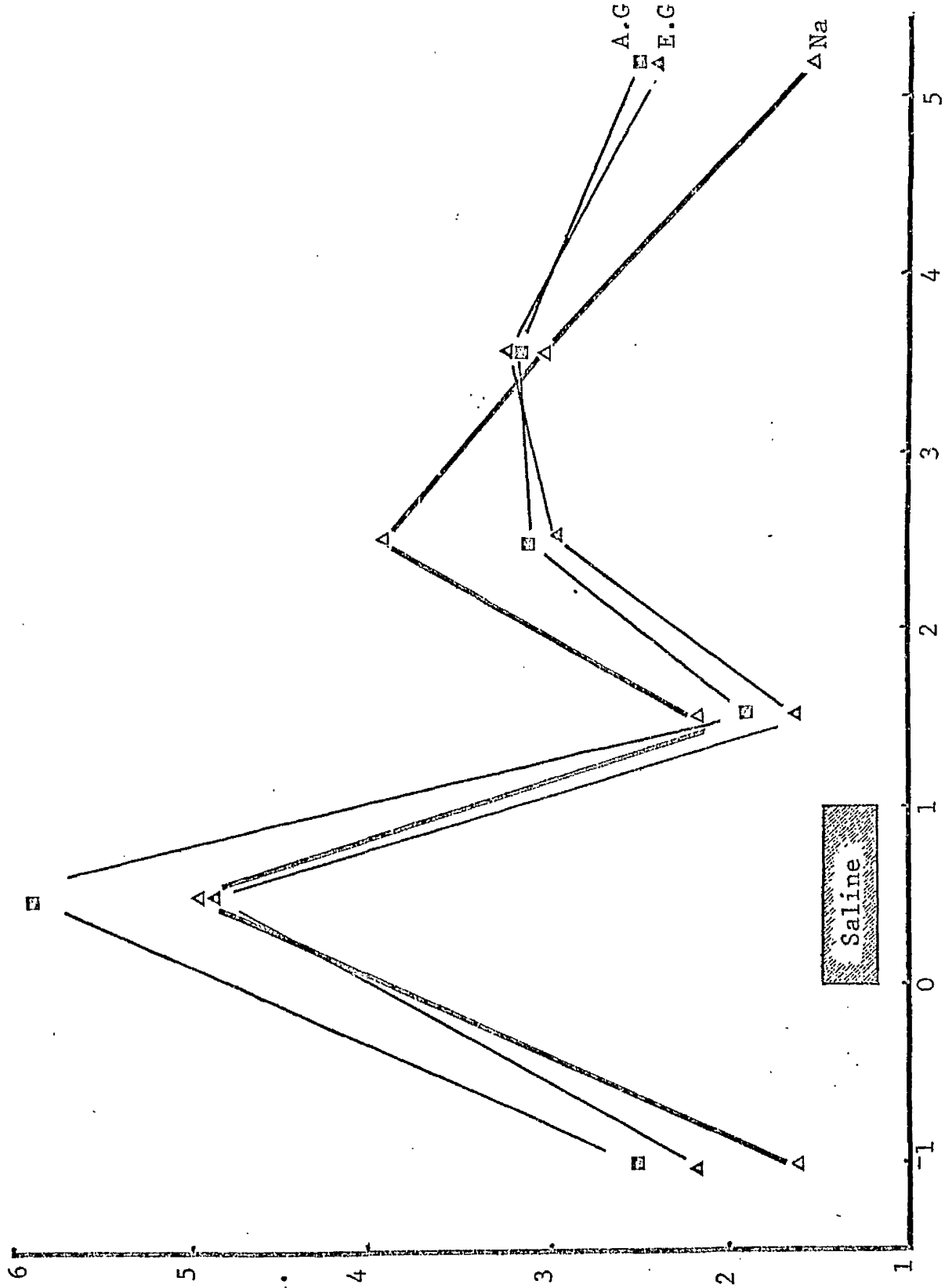


Fig. 17b. The effect of saline infusion (2 pints, 54 ml/min) on the excretion of urinary D.17-OS glucuronides in a normal male subject.

5. Discussion.

The results of the present experiments on the effect of salt loading and salt restriction on urinary D.17-OS levels indicate that in 4 out of 6 subjects, there is a definite relationship between salt and D.17-OS metabolism. The marked increase in the excretion of certain D.17-OS sulphates and glucuronides by subject 4M on changing from salt ad libitum to high salt intake and the very dramatic drop in D.17-OS sulphate excretion by subjects 2M & 3M on going from high to a low sodium intake, are both very suggestive of either salt levels influencing D.17-OS excretion or what is more likely D.17-OS levels influencing salt excretion. Obviously if the latter relationship holds, then the precursors of the urinary D.17-OS are suspect of being the active principle involved in the observed changes in salt metabolism. This assumption is made since the urinary D.17-OS are all conjugated steroids and conjugated steroids are without as yet any known hormonal action.

Since the precursors of urinary D.17-OS are ostensibly testosterone, androstenedione, DHA.S and DHA, it might be expected that one or more of these four steroids may be the active principle. However, there is evidence, some definite and some suggestive that not any of the four steroids is the active principle in question. Testosterone is automatically eliminated since, if there is a salt-loading steroid, it would have to be present equally in both sexes,

and testosterone is not. Besides, it has been known since as early as 1938 that testosterone causes salt retention, not excretion (Thorn & Engel, 1938). The studies of Lipsett, Coffman & Nixon (1965) would appear to rule out DHA and DHA.S as salt losing hormones. They in balance studies on sodium, chloride, calcium and nitrogen found that continuous infusion of DHA at a rate of 50 mg/day for 5 days had no effect on the urinary excretion of sodium, or for that matter on any of the other elements studied, in two normal males. Similar studies involving the continuous infusion of DHA.S were also negative. It is realised that this is not evidence that the reverse holds, that a sodium load does not result in a change in DHA and DHA.S secretion. In fact the results of the present experiments indicate that there is an increase in D.17-OS excretion on salt loading, which probably indicates an increased secretion of urinary D.17-OS precursors(s). The findings of Williamson are suggestive of DHA and androstenedione being involved in salt metabolism. Williamson demonstrated in rats that DHA and androstenedione administered by subcutaneous injection in sesame oil had naturetic properties and that they still possessed the naturetic properties when administered to adrenalectomized rats. From these findings Williamson concluded that DHA and androstenedione were probably physiologic antagonists of aldosterone. Unfortunately the naturetic activity of DHA and androstenedione was low, approximately 80 gm of C_{19} steroid equivalent to 0.1g of aldosterone, too low for the suggested role of a salt-losing steroid. Thus the evidence of

Thorn & Engel (1938), Lipsett et al (1965) and Williamson (1966) would suggest that neither DHA, testosterone, DHA.S nor androstenedione is the active principle.

What then of the present evidence of the effect of salt intake on the excretion of D.17-OS sulphates and glucuronides? Biological compounds are generally formed by a series of intricate biosynthetic steps along a definite pathway, and it is well known that where stimulation results in increased secretion of the final compound of that pathway concomitant increases are observed in the biosynthetic precursors of the compound. ACTH administration results in the increased secretion of 17α-hydroxyprogesterone and 17α, 21-dihydroxy-progesterone, as well as the increased secretion of cortisol. ACTH administration also results in the increased secretion of DHA, DHA.S and androstenedione, which do not lie in the main biosynthetic pathway to cortisol (Chapdelaine et al, 1965a). Angiotensin besides stimulating the increased secretion of aldosterone also stimulates the increased secretion of corticosterone an intermediate in the biosynthetic pathway to aldosterone. Therefore, it is possible that the changes shown by the urinary D.17-OS conjugates are a reflection of the changes in some other "active" compound in the adrenal "androgen" biosynthetic pathways, and the increased or decreased urinary D.17-OS levels are by-products in the chain of events leading to an increased or decreased secretion of an active salt-losing steroid. If the analogies of the effect of angiotensin on aldosterone and ACTH on cortisol are applied

to the hypothetical salt-losing steroid, it would suggest that the hypothetical salt-losing steroid comes at the end of a metabolic pathway as does aldosterone and cortisol. Therefore, it would follow either DHA.S, DHA or androstenedione in the pathway as illustrated in fig. 18. However, if the analogy of the effect of ACTH on cortisol is further considered, it is realised that this is not imperative since ACTH stimulates DHA, androstenedione, and DHA.S and these lie on pathways which are branches leading from the main biosynthetic pathways to cortisol. Therefore, the hypothetical steroid need not necessarily lie at the end of a main pathway as depicted in fig. 18, but could be on a branch leading from a main pathway. Further since aldosterone is produced in small amounts relative to corticosterone, and cortisol in large amounts relative to 17α-hydroxyprogesterone, then either pattern could be shown by the hypothetical salt-losing steroid, that is it could be secreted in either μg or mg amounts.

There is no direct evidence relating salt metabolism with that of D.17-OS metabolism, further no studies have been carried out with the specific purpose of attemtping to demonstrate such a relationship. However, there is indirect evidence available which would suggest that such a relationship does exist. Urinary 17-OS levels in congenital adrenal hyperplasia when considered in relation to concomitant changes in salt excretion provides such indirect evidence. In this disease urinary 17-OS levels are markedly elevated in all patient and

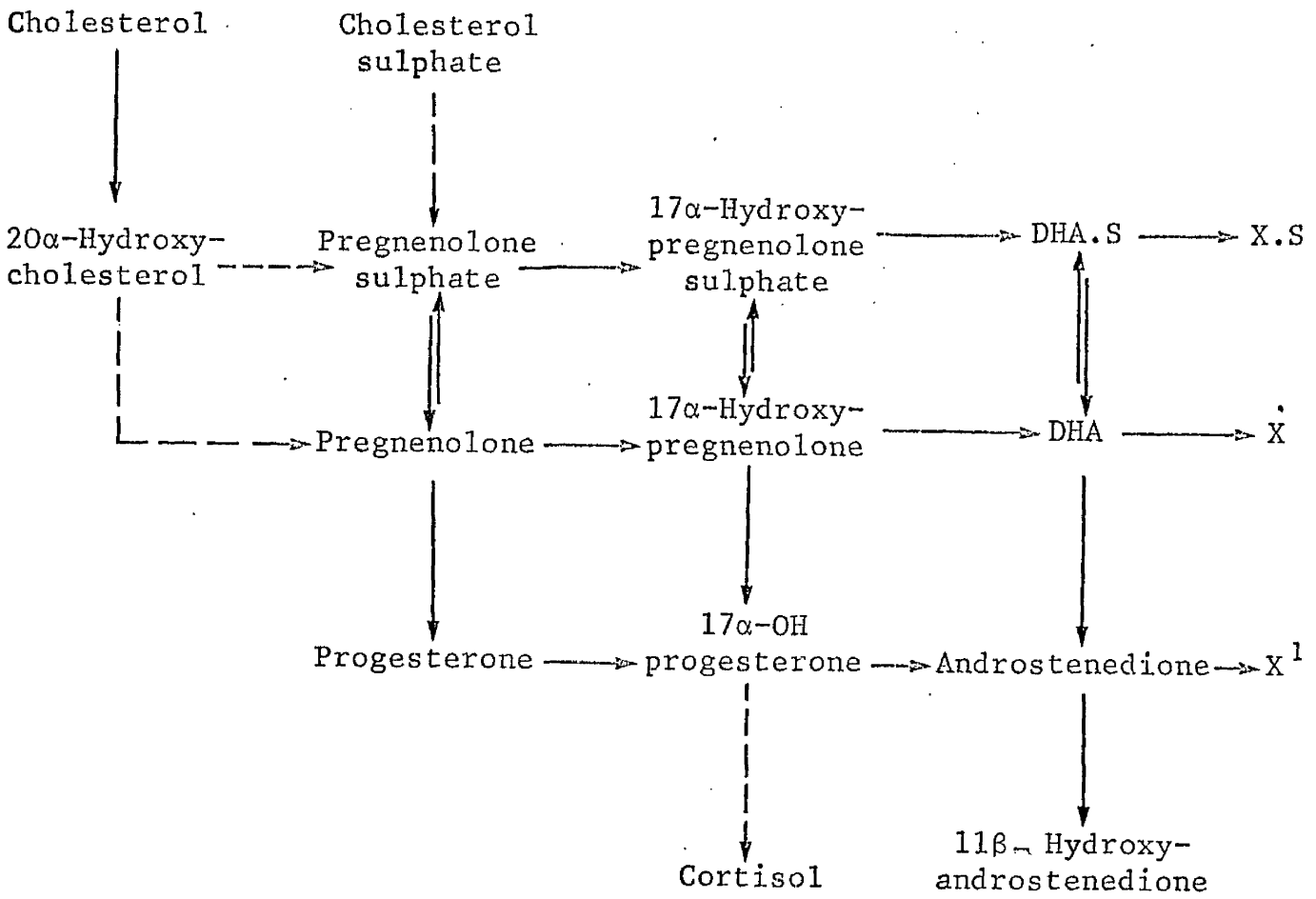


Fig. 18. Suggested biosynthetic pathway for the formation of the hypothetical salt-losing steroid (X, X^1 or X.S).

about 33% of these patients display severe salt loss and often the salt-loss is accompanied by normal or elevated aldosterone secretion rates (Jailer et al, 1959 and Visser & Degenhart, 1967). The other 67% of the patients have normal salt balances, but often these normal salt balances are associated with elevated aldosterone secretion rates (Jailer et al, 1959 and Kowarski et al, 1965). Further, in this disease the urinary 17-OS levels are decreased to normal levels by cortisone therapy, treatment which is known to bring some salt-losers into sodium balance and lower the elevated aldosterone excretion and secretion rates in non-salt-losers (Wilkins et al, 1950; Wilkins, 1965 and Visser & Degenhart, 1967). Also 17-OS are excreted in greater amounts after ACTH stimulation, treatment which has been shown to aggravate the salt-loss in the salt-losing types and to induce salt-loss in the non-salt-losing types of congenital adrenal hyperplasia.

Indirect evidence other than that from congenital adrenal hyperplasia which also substantiates the present findings is available. The elevated urinary levels of free aldosterone (New et al,1966), together with the high urinary 17-OS levels found in the new born infant could be explained by the existence of a salt-losing steroid. The elevated urinary aldosterone level could be a compensatory mechanism initiated in the adrenal adult zone, in order to maintain salt balance, as a result of the high production of 17-OS precursors by foetal adrenal zone.

Thus evidence from three different sources, congenital adrenal hyperplasia, steroid levels in newborn infants and the present findings

all implicate 17-OS with salt metabolism and the present report restricts the 17-OS involved to the 11-deoxy, 17-OS. However, as already pointed out the hypothetical steroid does not appear to be any of the known urinary D.17-OS precursors.

Several studies have been made in attempt to find a salt-losing steroid. Neher et al (1958) isolated 3β, 16α-dihydroxy-allopregnan-20-one, however Cope & Parry (1959) found it ineffective as a naturetic hormone on patients with congestive cardiac failure. Klein et al (1958) isolated from the urine of infants with salt-losing congenital adrenal hyperplasia, a steroid with salt excreting properties, and interestingly enough the urinary steroid extracts seemed more potent after ACTH stimulation. The steroid was shown to have a polarity somewhere between tetrahydro A and 11-deoxycortisol. Rosemberg et al (1960) isolated from the urine of salt-losing patients with congenital adrenal hyperplasia a mixture of steroids composed mainly of 17α-hydroxypregnenolone which actively antagonised the salt retaining properties of DOC, unfortunately the antagonist was thought to be an impurity. Regrettably none of the above studies were brought to a fruitful conclusion.

If there is such an entity as a salt-losing steroid, it might be expected to contribute to the aetiology of idiopathic hypertension. Hypertension is recognised as having high incidence in communities consuming large quantities of salt (Dahl, 1960), also salt restriction has long been known to ameliorate hypertension (Ambard & Beauyard, 1905; Allen & Sherrill, 1922 and Volhard, 1931). Therefore, inadequate.

excretion of salt could be a causative factor in the aetiology of hypertension. A decreased production of the hypothetical salt-losing steroid might present such a symptom as inadequate salt excretion. In Section V.B. an explanation for the various steroid abnormalities presented in hypertension was given - an enzymatic block in the conversion of pregnenolone to 17α-hydroxypregnenolone. If as suggested the hypothetical salt-losing steroid may be in the biosynthetic pathway leading from 17α-hydroxypregnenolone then its secretion would probably be reduced in idiopathic hypertension. Therefore, the proposed explanation for the steroidal abnormalities presented in hypertension would also have an aetiological basis.

Most of the previous steroids proposed as the hypothetical salt-losing steroid were active only in the presence of mineralocorticoids, therefore, they were all probably competitive inhibitors of aldosterone. Obviously, if the steroid in question only acts through competitive inhibition its potential is limited and its action will always be secondary to that of mineralocorticoids. Williamson (1966) showed DHA and androstenedione to be physiologic antagonists of aldosterone, that is, having naturetic properties independent of aldosterone. They are antagonists of aldosterone only in the sense that they have the reverse action of aldosterone on salt metabolism. Since it is possible that DHA and androstenedione may have biosynthetic pathway common to the hypothetical salt-losing steroid, then it may be structurally similar to these two D.17-OS and therefore also display physiologic antagonism.

The existence of a salt-losing steroid with physiologically antagonism towards aldosterone would be in harmony with the results recorded for the saline infusion experiment. In the saline infusion experiments it was shown that during the first hour of the excretion of the saline load all the D.17-OS showed increased excretion, concomitant with that of sodium, suggesting that D.17-OS steroids may be involved in the increased excretion of sodium. Acute studies performed by other investigators have shown that the changes observed in sodium excretion took place before any changes in plasma and urinary aldosterone levels were registered (Ross, 1964 and Fraser, 1967). Also other studies have shown that there is a two hour delay before the effect of aldosterone on sodium excretion by the kidney is observed in man. On considering this evidence it might, therefore, be concluded that the observed changes in sodium metabolism are initially dependent on a steroid other than aldosterone and the evidence is further suggestive that the steroid might be a D.17-OS displaying physiologic antagonism and, therefore, closely structurally related to DHA and androstenedione.

Of the six patients involved in studies concerning the D.17-OS salt relationship, two, 5M and 6M (see Tables 28 & 29), did not show results which truly indicate such a relationship. This apparent disagreement could be explained in terms of a dual control of salt metabolism. Aldosterone inducing salt retention and the hypothetical steroid inducing salt loss, and a balance between them set up for each individual. In the subjects 5M and 6M it is proposed that the

hypothetical steroid plays a lesser role than it does in the other four subjects studied.

Experimental results can often be interpreted in more than one way. The existence of a salt-losing steroid is one explanation of the results presented here, however, any other possible explanations must also be considered.

ACTH has been shown to increase the secretion of DHA, DHA.S and androstenedione (Chapdelaine et al, 1965a) and the excretion of individual D.17-OS. Stress stimulates the release of ACTH. Gross variation in salt intake, especially salt loading, might possibly be regarded as stressful. Therefore, increases in urinary D.17-OS levels could be the result of stress from salt loading and decreases due to the removal of the stress by changing to low salt intake. ACTH administration which may in certain respects be equated to stress, results in a far greater increase in urinary 17-OHCS levels than it does in 17-OS levels, such that urinary 17-OHCS/17-OS ratio is approximately doubled as compared with normal (Table 27). The results of the present report indicate that even though individual urinary D17-OS levels were elevated by high salt intake and lowered by low salt intake, the urinary 17-OHCS levels did not show the same variation, and in no case did the urinary 17-OHCS/17-OS ratio double or nearly double (Table 28). Therefore, the changes observed in the individual urinary D.17-OS levels while on varying salt intake cannot be attributed to stress release of ACTH.

Diet has been shown to greatly influence the excretion of individual D.17-OS. Cawley et al (1965) studying a single normal male and Hendrikx et al (1965) studying 7 obese females have both shown that starvation and low calorie diets cause a marked reduction in the excretion of DHA(S+G), A.(S+G) and E.(S+G) and Cawley et al (1965) further demonstrated that starvation had much less effect on the excretion of 11-oxy, 17-OS. Unfortunately details of salt intake were not available for either of the two studies. However, contrary to these two reports Lopez-S & Krehl (1967) demonstrated an increased excretion of DHA.(S+G) by 5 young obese females when they were placed on a diet restricted in calories and very low in carbohydrate.

Thus it may be possible that in the present report the changes observed in D.17-OS excretion by the test subjects while they were on varying salt intake were through unnoticed changes in calorific intake due to changes in the diet necessary to obtain high and low salt intake. This reasoning might explain the marked drop in excretion of individual D.17-OS shown by subjects 2M & 3M on going from high to low salt intake. This reasoning might also explain subject 1F's marked increase in DHA.S excretion on changing from a low to high salt intake, if low salt intake is partially equated with low calorie intake and high salt intake equated with normal diet. In high salt intake the supplementary salt was given in the form of tablets. However, the idea of calorific intake changing in accordance with salt intake

does not explain the very marked increase in DHA excretion shown by subject 4M on going from salt ad libitum to high salt intake. Further, what of the two patients showing very little change in D.17-OS excretion while on varying salt intake.

If salt dieting or dieting in general does influence the excretion of individual D.17-OS, then reconsideration of the results discussed in Section V.B. on hypertension are necessary. In the studies carried out by Kolbel et al (1965) the patients were placed on low purine, low cholesterol diets prior to their studies, this being necessary since besides the estimation of urinary DHA, S.U.A. and plasma cholesterol levels were also estimated. In the studies of Nowaczynski et al (1968) the patients were placed on salt diets prior to the estimation of plasma aldosterone and urinary DHA.S. In the present studies on hypertension the patients and control subjects remained on their normal hospital diets. Is a hospital diet a normal diet for all individuals?

Therefore, the low urinary DHA levels in hypertension demonstrated by the three reports could be attributed to changes in diet. The markedly low urinary DHA levels found by Kolbel et al, and Nowaczynski et al, compared with the moderately low DHA levels found by the present report could be attributed to the definite dietary measures imposed upon the patients by the two former groups and the difference between the individually normal and hospital diets in the studies of the latter investigation.

However, the suggestion that the changes in D.17-OS excretion induced by varying salt intake were actually due to changes in calorific intake, could be considered from a different aspect, namely weight reduction. In starvation and low calorie diets there is a weight loss which is always associated with marked elevation of sodium excretion and further this sodium excretion is associated with increased aldosterone excretion, presumably in attempt to maintain sodium balance (Haag, Reidenberg, Shannon & Channick, 1967). Therefore, if there is a salt-losing steroid and its excretion is mirrored by the excretion of D.17-OS, then a decrease in this might be expected in starvation and low calorific diet in an attempt to maintain salt balance. Thus the decreased excretion of D.17-OS shown in starvation and low calorie diets may be due directly to salt loss and indirectly to low calorie intake. The results of Haag et al (1967) interrelate weight loss, salt excretion and 17-OS excretion along with calorie intake in form of glucose, and demonstrated that during starvation there is weight loss concomitant with increased sodium excretion, increased aldosterone excretion and decreased 17-OS excretion and that glucose, but not fat consumption could stop the weight loss and salt loss, and brought about a plateau of the falling 17-OS levels. Thus these findings together with the fact that D.17-OS, especially DHA and androstenedione are known to have some control over the pentose phosphate pathway through inhibition of the rate limiting enzymatic step (G-6-P.D.) suggests a tie-up among the metabolisms of salt, D.17-OS and carbohydrates.

E. Individual Urinary D.17-OS Sulphates and Glucuronides: Values for Male Patients Suffering from Gout.

Urinary D.17-OS sulphate and glucuronide levels and serum uric acid levels were estimated in male patients suffering from gout and compared with the corresponding levels obtained in Section V.A.3 for normal males.

1. Patients studied.

Seven patients with gout were studied. Gouty arthritis was diagnosed on clinical grounds with supporting evidence of hyperuricaemia defined as a serum uric acid level greater than 7.0 mg/100 ml. The mean age of the patients was 43 ± 9 years (range 31 - 57). The duration of the gout in the 7 patients varied from 3 months to 10 years. Two of the patients with the longer standing gout were also hypothyroid and one of them was also a mild diabetic. The % overweight of each patient was calculated from the tables of Kemsley et al (1962), on this basis all the patients were overweight with a mean % overweight value of 132.6 ± 20.8 (range 110 - 170). One patient was hypertensive with blood pressure of 220/130 mmHg. All patients had satisfactory renal function as estimated by blood urea (mean 32 ± 8 mg/100 ml) and serum creatinine (mean 0.87 ± 0.11 mg/100 ml). All patients were on normal hospital diets and no medication was given for 1 week prior to the study.

2. Analytical techniques.

2. Analytical techniques.

Collection of blood and urine	-	Section IV.A.5.
Blood urea and serum creatinine	-	Section IV.A.2 a & b.
Serum uric acid	-	Section IV.A.4.
Plasma cholesterol & glycerides	-	Section IV.A.3 a & b.
17-OS & 17-OHCS	-	Section IV.A.6 a & b.
D.17-OS.S & D.17-OS.G	-	Section IV.C.2.

All estimations were duplicated.

Urinary D.17-OS.S and D.17-OS.G levels were estimated in 50 ml volumes of urine, not 25 ml volumes.

3. Results.

The urinary levels of the individual D.17-OS for gouty males are shown in Table 33.

(a) Sulphate conjugates. The values for the individual urinary D.17-OS sulphates were extremely low and on statistical comparison with the values for the normal controls reported in Section V.A. the values proved to be significantly very much lower (Table 34). All the steroid sulphate values except E.S. had P values of 0.0002 or less.

The urinary D.17-OS.S levels did not show the same degree of variation as the normal controls. Inspection of Table 34 indicates that the normal controls had standard deviations greater than or equal to the respective mean values, whereas in the case of gouty males the

TABLE 33. INDIVIDUAL URINARY D.17-OS LEVELS AND SERUM URIC ACID LEVELS FOR MALE PATIENTS SUFFERING FROM GOUT.

	AGE	DHA			EpiA.S	E			A			S.U.A.
		S	G	S+G		S	G	S+G	S	G	S+G	mg%.
1	35	0.095	0.272	0.367	0.038	0.114	1.450	1.564	0.053	2.610	2.663	11.0
2	57	0.047	0.000	0.047	0.041	0.175	1.130	1.305	0.047	0.490	0.537	8.0
3	42	0.077	0.010	0.087	0.042	0.085	0.351	0.436	0.054	0.559	0.613	8.4
4	31	0.094	0.025	0.119	0.016	0.035	0.328	0.363	0.041	0.313	0.354	8.6
5	46	0.163	0.048	0.211	0.014	0.025	1.960	1.985	0.023	1.150	1.173	9.0
6	50	0.098	0.000	0.098	0.029	0.216	1.440	1.656	0.133	1.051	1.183	8.0
7	43	0.105	0.000	0.105	0.000	0.010	3.540	3.550	0.010	5.700	5.710	10.8
MEAN	43.4	0.097	0.051	0.148	0.026	0.094	1.457	1.551	0.052	1.696	1.748	9.1
S.D.	8.7	0.035	0.099	0.109	0.016	0.079	1.095	1.073	0.039	1.925	1.910	1.3

TABLE 34. COMPARISON OF D.17-OS URINARY LEVELS AND % OVERWEIGHT VALUES FOR MALES SUFFERING FROM GOUT WITH NORMAL MALE CONTROLS.

	NORMAL MALES MEAN ± S.D.	GOUTY MALES MEAN ± S.D.	P VALUE
EpiA.S	0.30 ± 0.326	0.026 ± 0.016	0.0002
DHA.S	2.75 ± 3.75	0.097 ± 0.035	0.001
E.S	0.23 ± 0.212	0.094 ± 0.079	0.066
A.S	0.82 ± 0.838	0.052 ± 0.039	0.0002
DHA.G	0.37 ± 0.365	0.051 ± 0.099	0.001
E.G	2.61 ± 1.36	1.457 ± 1.095	0.028
A.G	3.05 ± 1.89	1.696 ± 1.925	0.024
DHA.(S+G)	3.12 ± 4.07	0.148 ± 0.109	0.0004
E.(S+G)	2.89 ± 1.36	1.551 ± 1.073	0.032
A.(S+G)	3.87 ± 2.46	1.748 ± 1.910	0.010
17-OS	12.3 ± 4.3	7.1 ± 1.8	0.006
17-OHCS	12.7 ± 3.7	11.6 ± 3.2	0.62
% O.WT.	116 ±15	132.6 ± 20.8	0.058

standard deviations were all less than the respective mean values.

E.S was quantitatively the most important D.17-OS.S in 4 (57%) of the 7 gouty males, whereas this held for only 1 (4.5%) of the 22 normal controls. Thus for 4 gouty males the urinary excretion pattern was E>DHA>A>epi (fig. 19), whereas in normal males it was DHA>A>epi>E (fig.5). The fact that E.S was the major D.17-OS.S in 4 patients and never the minor D.17-OS.S in any of the 7 gouty males is possibly related to the fact that, of all the D.17-OS.S excreted by the gouty males, E.S levels showed the smallest deviation from those of normal controls (Table 34).

The E.S/A.S ratios for gouty males were markedly different from that of normal males, this is clearly illustrated by comparison of the gas-chromatographic tracings for gouty and normal males (figs. 19 & 5). Gouty males show E>A whereas normal males show very much the reverse with A>E. This is further exemplified by the regression equations of E.S on A.S for:-

Normal males E.S = 0.20 A.S + 0.068 = 0.79 P = 0.001

Gouty males E.S = 1.68 A.S + 0.00 = 0.84 P = 0.02

The value of the slopes of the regression lines indicate that more E.S was excreted relative to A.S by the gouty males whereas the reverse holds for normal males.

(b) Glucuronide conjugates. The values for the individual urinary D.17-OS glucuronides were very low when compared with normal controls, although excluding E.G, they were not, relative to normal controls, as

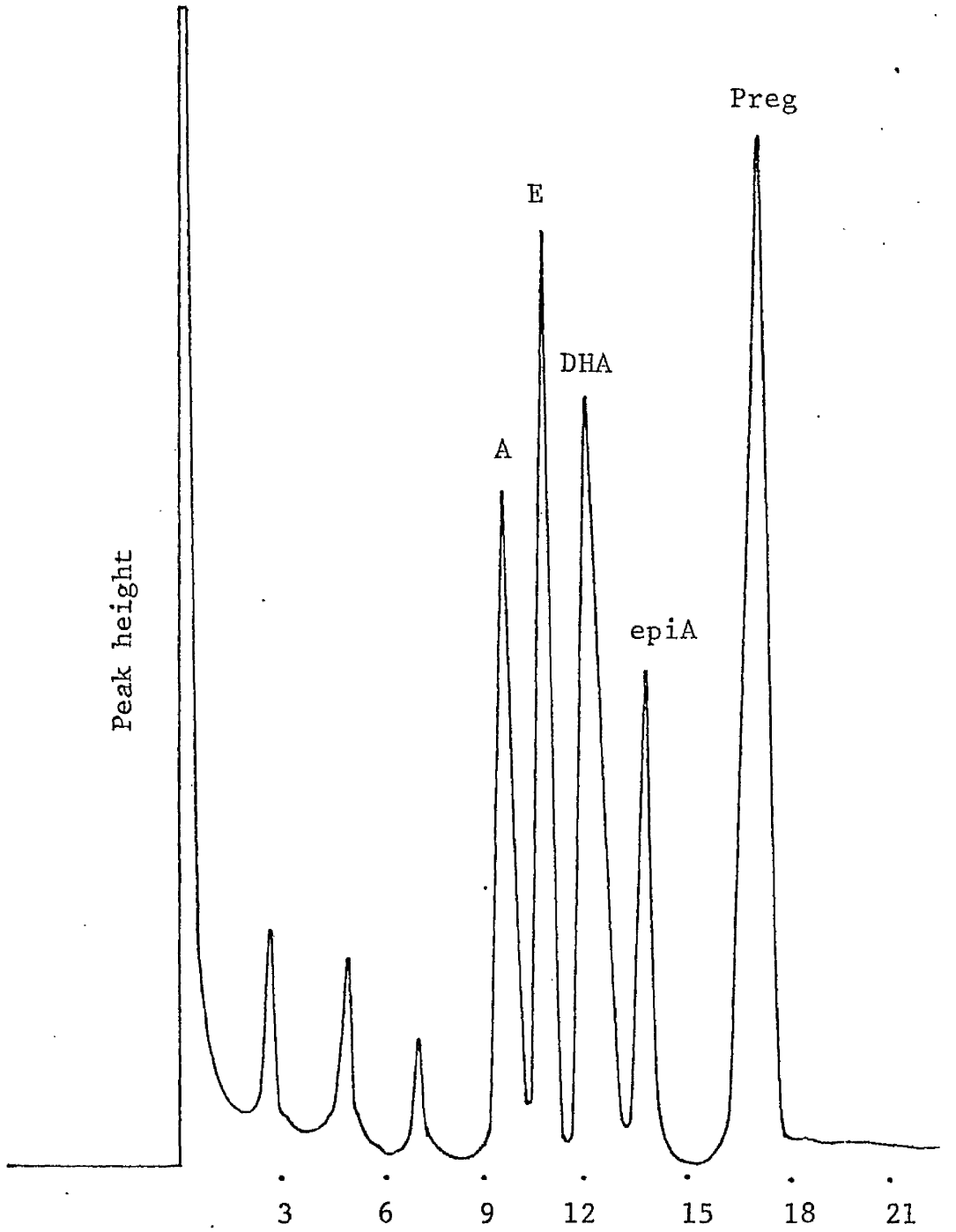


Fig. 19. Gas chromatographic tracing of the D.17-OS sulphate fraction obtained from a gouty male urine. 3% QF-1 column at 200°. A) Androsterone, E) Aetiocholanolone, DHA) Dehydroepiandrosterone, epiA) Epiandrosterone and Preg) Pregnenolone all as TMSE derivatives.

low as the urinary D.17-OS sulphates. Also the urinary glucuronide levels unlike the D.17-OS.S levels showed more variation about their respective mean values than did the corresponding values for the normal controls.

The urinary E.G/A.G ratios showed no definite pattern, 4(57%) of the 7 patients showed E.G as their major steroid glucuronide while the other 3 showed A.G to be their major steroid glucuronide. Of the seven patients, the patient who also suffered from hypertension (No.6, Table 33) showed the expected elevated E.G/A.G ratio for a hypertensive patient. However, of the 2 gouty males with hypothyroidism (No.2 & 3, Table 33) only patient No. 2 showed an elevated E.G/A.G ratio with a markedly high value (2.3). Patient No.3 had normal E.G/A.G ratio (0.59), but very low urinary levels of both A.G and E.G.

(c) Sulphate plus glucuronide conjugates. They showed exactly the same pattern as that shown by the urinary D.17-OS glucuronides.

(d) Discriminant function analysis. This was carried out as described in Section IV.D.4. For each of the two groups, gouty males and normal males, values for 14 variable [17-OS, 17-OHCS, epiA.S, DHA.S, E.S, A.S, DHA.G, E.G, A.G, DHA.(S+G), E.(S+G), A.(S+G), (epiA.+DHA+E+A).S, (DHA+E.+A).G] were tabulated and statistically compared using a simple test of significance (t-test or U-test). The three variables showing the most significant differences - epiA.S, A.S and (epiA.+DHA+E+A).S were employed in the discriminant function analysis. Complete discrimination between the two groups was obtained.

Discussion.

Of the 3 previous investigations on the levels of urinary 17-OS in gout, 2 found decreased levels while the other one found normal levels. Wolfson et al (1949) the first to investigate urinary 17-OS in this disease found very low levels, well below the lower limits of their normal controls. In addition to this, two gouty females included in the study had urinary 17-OS levels of less than 1 mg/24 hr. From these results Wolfson et al concluded that the 17-OS deficiency was of an adrenal origin and in their opinion the investigation excluded hyperuricaemia and arthritides as causitive factors of this deficiency since normal 17-OS urinary levels were found in both non-gouty hyperuricaemia and mild rheumatoid arthrites. Robinson et al (1949) confirmed the findings of Wolfson et al (1949), however, although they obtained urinary 17-OS levels that were low, they were still within the lower limits of normal.

The more recent studies of Sonka et al (1964) and the present report, both confirm the earlier findings of Wolfson et al (1949). Sonka et al (1964) besides confirming the earlier results demonstrated that the low 17-OS urinary levels found in gout were the result of total absence from the urine of DHA. They reported zero urinary levels of DHA in 26 male gouty patients and a mean DHA level of 2.5 ± 0.46 mg/24 hr in normal individuals. Unlike Wolfson they associated the high S.U.A. levels found in gout with the absence of urinary DHA. The validity of this association they based on the following: 26 hyperuricaemic gouty

patients all showed an absence of urinary DHA; 4 non-gouty arthritic patients with uric acid stones had hyperuricaemia concomitant with an absence of urinary DHA; 3 non-gouty non-arthritic patients with uric acid stones had normal S.U.A and normal urinary DHA levels; one gouty patient with normal S.U.A levels had normal urinary DHA levels. Other studies by the same group of investigators on hypertension also substantiate the association of hyperuricaemia with an absence of urinary DHA (Kolbel et al, 1964 and Kolbel et al, 1965a).

Later studies on urinary DHA levels in gout by Beck et al (1967) also demonstrated a urinary DHA deficiency. However, their results were not as "clear cut" as those of Sonka et al (1964). Beck et al (1967) found no DHA in 7 (54%), a trace in 4 (31%) and normal amounts in 2(15%) in a study of 13 gouty male patients. Unfortunately they did not report S.U.A levels, but it is presumed that all the patients were hyperuricaemic since they further reported that 4 arthritic, but normouricaemic patients had low urinary DHA levels and that only one such type of patient had normal urinary DHA levels. Results which, therefore, do not lend support to an association between hyperuricaemia and an absence of urinary DHA.

The present report which provides the only available data on the urinary levels of individual D.17-OS sulphates and glucuronides in this disease supports within limits both the findings of Sonka et al (1964) and Beck et al (1967). In the present report it was found that the 7 gouty males studied had very low urinary DHA.(S+G) levels, but in none of their urines was it not detectable. As a group the urinary DHA (S+G)

levels were significantly much lower than normal controls (P = 0.0004), but a more dramatic difference was demonstrated for urinary DHA.S levels, where normal controls had a mean level of 2.75 mg/24 hr, whereas that for the gouty group was only 0.097 mg/24 hr.

Despite these highly significant differences the unique feature of the gouty males in relation to D.17-OS levels was not just the very low levels of DHA, but the fact that all the D.17-OS sulphates were present in extremely small quantities. In fact, utilising the variables epiA.S, A.S and (epiA+DHA+E+A).S complete discrimination between normal individuals and gouty patients was possible.

With minor reservations, that is, the difference between zero and very small amounts, the results of the present report agree with those of Sonka et al (1964) with respects to urinary DHA. However, this is not so when A and E are considered. Sonka et al (1964) found normal levels of urinary E.(S+G) in gouty patients whereas by the present report both steroids were present in diminished amounts both as the sulphate and as the glucuronide conjugates. Thus, obviously the values for E.(S+G) and A.(S+G) were also low, their differences from normal were significant at 3% and 1% levels respectively.

What is the meaning of the markedly low urinary D.17-OS levels, especially the D.17-OS.S levels, found in gout?

In all 7 cases of gout studied there was very low urinary DHA.S levels and in all 7 cases the S.U.A levels were elevated. This

would appear to support Sonka et al (1964) theory of an association between hyperuricaemia and an absence or very low urinary DHA levels. However, this is invalidated when the hyperuricaemic normotensive subjects of Section V.A. and the hyperuricaemic hypertensive patients of Section V.B. are considered. In both these sections it was demonstrated that there was no obvious association between hyperuricaemia and low urinary DHA levels, further there was no correlation between S.U.A. levels and urinary DHA levels. Also the results in Section V.A. & B. shows that low urinary DHA levels are often present in individuals with normal S.U.A. levels, for example, Table 7 lists the following values for normal males.

	DHA.(S+G) mg/24 hr	S.U.A. mg%
Patient 17	0.13	5.3
Patient 18	0.10	5.8

Similarly, Table 14 lists the following values for idiopathic hypertensive males.

	DHA.(S+G) mg/24 hr	S.U.A. mg%
Patient 7	0.11	5.0
Patient 17	0.05	5.4

Thus contrary to the results of Sonka et al (1964) and confirmatory with those of Beck et al (1967), hyperuricaemia does not appear to be in close association with low urinary DHA levels.

Duration of gout does not appear to influence the urinary

D.17-OS.S levels. For example, two of the 7 gouty patients studied had suffered from gout for 3 months and 12 years respectively, yet both had very low urinary D.17-OS.S levels and of the two the former patient had the lower urinary D.17-OS.S levels.

Sonka et al (1964) reported that gouty patients produced significantly more urine than normal controls. In Section V.A. significant correlations between urine volume and individual D.17-OS urinary levels in normal females was demonstrated. Therefore, it was decided that closer inspection of this observation was merited. However, by the present report there was no correlation between urine volume and the individual D.17-OS urinary levels, nor was there any difference between urine volumes of gouty males (1570 ml) and normal males (1467 ml).

Numerous investigators have reported the close association between gout and obesity (Getler, Garn & Levine, 1951; Sonka et al, 1964; Benedek, 1967 and Barlow, 1968). The present report supports these findings, a significant difference being observed between normal and gouty males for % overweight values ($P = 0.058$).

This association is of further interest in that Lopez-S & Krehl (1967) while studying urinary DHA levels in a series of obese individuals graded by increasing % overweight from 0 - 160%, demonstrated a decrease in urinary DHA levels which were inversly related to an increase in % overweight. Also Hendrikx et al (1965) reported significantly lower urinary E and A levels, expressed as mg/100 Kg/24 hr, when comparing

values for obese females with those for normal females. Both studies would suggest that urinary D.17-OS studies in obesity may be of importance in attempting to explain the abnormally low urinary D.17-OS levels found in gout, that is, overweight may be the link between gout and low urinary D.17-OS levels.

On the basis of this premise, the 22 normal male controls were divided into two groups according to % overweight (Table 35), and the values for each of the 10 individual D.17-OS urinary levels of the two groups statistically compared. No significant difference between the two groups for any of the 10 variables could be demonstrated nor could a significant correlation be demonstrated between any of the individual D.17-OS urinary levels and % overweight when treated as two groups or as one combined group. Further although the 7 gouty males studied were all overweight (mean % overweight 32.6 ± 20.8) and they all excreted very small quantities of D.17-OS, their % overweight values showed no significant correlation with the D.17-OS urinary levels.

However, it must be emphasised that in the present study the 7 gouty males and the overweight normal males involved were not nearly as obese as the subjects studied by Lopez-S & Krehl and Hendrikx et al, and therefore the influence of overweight on the individual D.17-OS urinary levels might not be significant except in really obese individuals. Consideration of the results of Lopez-S & Krehl shown below will illustrate this point.

TABLE 35. THE URINARY D.17-OS LEVELS (mg. free steroid/24 hr). FOR NORMAL MALE SUBJECTS DIVIDED INTO TWO GROUPS ACCORDING TO % OVERWEIGHT (% O.WT).

	% O.WT.	DHA			epiA	E			A		
		S	G	S+G	S	S	G	S+G	S	G	S+G
Under 120% overweight:											
1	-14	0.66	0.40	1.06	0.08	0.25	2.07	2.32	0.39	1.26	1.65
2	- 6	0.14	0.03	0.17	0.06	0.24	1.03	1.27	0.13	0.86	1.09
3	- 5	0.58	0.17	0.75	0.07	0.05	1.02	1.07	0.28	1.85	2.13
4	0	0.82	0.12	0.94	0.22	0.21	1.60	1.81	0.56	1.25	1.81
5	8	11.93	1.00	12.93	0.84	0.24	5.86	6.10	1.84	7.42	9.26
6	8	0.71	0.11	0.82	0.23	0.61	2.52	3.15	0.70	1.75	2.45
7	8	2.69	0.31	3.00	0.43	0.21	4.02	4.23	1.16	6.46	7.62
8	8	0.07	0.06	0.13	0.01	0.03	0.36	0.39	0.05	1.48	1.53
9	11	1.20	0.17	1.37	0.43	0.25	2.25	2.50	1.02	3.15	4.17
10	12	10.03	1.04	11.07	0.94	0.68	3.68	4.36	2.18	5.51	7.69
11	13	0.46	0.13	0.59	0.13	0.07	2.97	3.04	0.33	2.63	2.96
12	17	11.63	1.38	13.01	1.29	0.80	4.47	5.27	3.74	4.02	7.76
MEAN	5	3.41	0.41	3.89	0.39	0.31	2.65	2.96	1.03	3.14	4.18
S.D.	9	4.76	0.46	5.21	0.41	0.25	1.62	1.75	1.08	2.22	3.01
Over 120% overweight:											
1	20	2.32	0.29	2.61	0.30	0.21	2.01	2.22	0.67	2.42	3.09
2	21	0.19	0.14	0.33	0.11	0.08	4.15	4.23	0.60	5.70	6.30
3	23	1.44	0.53	1.97	0.22	0.09	3.74	3.84	0.62	5.36	5.98
4	23	6.54	0.54	7.08	0.35	0.06	2.89	2.95	0.61	3.51	4.12
5	24	3.74	0.77	4.51	0.29	0.39	2.63	3.02	1.08	3.27	4.35
6	29	0.06	0.04	0.10	0.06	0.21	1.92	2.13	0.30	1.69	1.99
7	31	1.75	0.36	2.11	0.12	0.08	1.66	1.74	0.65	2.12	2.77
8	31	1.07	0.20	1.27	0.08	0.06	1.30	1.36	0.12	1.61	1.73
9	40	0.39	0.09	0.48	0.19	0.20	3.85	4.05	0.67	2.19	2.86
10	45	2.08	0.29	2.37	0.10	0.08	1.52	1.60	0.28	1.61	1.89
MEAN	28	1.96	0.32	2.28	0.18	0.15	2.57	2.71	0.56	2.95	3.51
S.D.	8	1.96	0.23	2.14	0.10	0.11	1.05	1.06	0.27	1.51	1.64

% Overweight	No. Patients	DHA mg/24 hr. MEAN ± S.E.M.
0 - 20	18	1.13 ± 0.05
20 - 50	9	1.08 ± 0.03
50 - 80	7	0.90 ± 0.01
80 - 120	7	0.63 ± 0.02
120 - 160	6	0.75 ± 0.01

Therefore, although obesity does appear to influence the urinary DHA levels it does not affect them as markedly as would be necessary to explain the very low urinary DHA levels found in gout.

Recently there has been much interest in the inter-relationships of gout and hyperlipidaemia (Berkowitz, 1966; Hansen, 1966; Benedek, 1967 and Barlow, 1968). The latter investigator, through comparing the serum concentration of various lipids in healthy normals and in patients with atherosclerosis obtained criteria for defining hyperlipidaemia. By applying these criteria to the serum lipid results obtained for the gouty patients he showed that as many as 77% of gouty patients were hyperlipidaemic, yet only 15% at that time showed evidence of atherosclerosis.

Thus, the high incidence of hyperlipidaemia in gout could possibly be of importance in the interpretation of the very low D.17-OS.S levels found in gout. Of interest in this respect are the studies of Hellman et al (1958a,b) in myxoedema, a condition characterised by hypercholesterolaemia. They reported very low urinary levels of E and A together with elevated urinary E/A ratios. Further, Hellman et al associated the hypercholesterolaemia found in this disease with the very low urinary A levels and demonstrated that parentally administered A was an effective hypocholesterolaemic agent/..

when administered to normocholesterolaemic patients as well as to hypercholesterolaemic patients. This work was subsequently confirmed by Cohen et al (1961) and Furman & Howard (1962). In support of this is the evidence that another D.17-OS (DHA) has been shown to have a hypocholesterolaemic effect when administered orally to rats rendered hypercholesterolaemic by propylthiouracil treatment (Ben-David et al, 1967). Finally Kotasek et al (1967) demonstrated low urinary DHA levels in women with toxaemia of pregnancy, a condition characterised by elevated phospolipids and β-lipoproteins (Boyd, 1935, and Kotasek et al, 1965).

Thus there would appear to be a causal relationship between plasma lipids and D.17-OS levels particularly for A and DHA, which is often manifested as patients with elevated plasma lipids concomitant with low urinary D.17-OS levels.

Unfortunately, plasma lipid values for only 4 of the gouty patients studied are available. These are presented below, together with the corresponding D.17-OS urinary levels (mg/24 hr).

ΦPLASMA LIPIDS			DHA		epiA	E		A	
	CHOL.	GLY.	S	G	S	S	G	S	G
*2	458	384	0.047	0.000	0.041	0.175	1.130	0.047	0.490
4	213	288	0.163	0.048	0.014	0.025	1.960	0.023	1.150
5	374	249	0.098	0.000	0.029	0.216	1.440	0.133	1.051
6	236	260	0.105	0.000	0.000	0.010	3.540	0.010	5.700

* Refers to numbers given to patients as in Table 33.

Φ Normal male plasma lipids - cholesterol (Chol). - 15-250 mg/100 ml
- glycerides (Gly) . - 80-150 mg/100 ml

All four of the gouty males were hyperglyceridaemic and two of them also hypercholesterolaemic, and as demonstrated in the result section all 7 have exceptionally low D.17-OS levels and especially low sulphate levels. Thus the evidence, though only in 4 cases is 100% in support of a relationship between elevated plasma lipid levels and low urinary D.17-OS.S levels.

On the basis of the above evidence it was decided to compare the urinary D.17-OS levels of normolipidaemic individuals with those of hyperlipidaemic individuals. In this study which is presented in the next section, it was attempted to standardise the other two factors which might also be implicated with low urinary D.17-OS levels, namely over-weight and elevated S.U.A. levels.

F. Individual Urinary D.17-OS Sulphates and Glucuronides:

Values for Normolipidaemic and Hyperlipidaemic Males.

Urinary D.17-OS sulphate and glucuronide levels were estimated in normolipidaemic males, and the results compared with those for hyperlipidaemic males. Normolipidaemia was defined as plasma glyceride levels of less than 150 mg/100ml and hyperlipidaemia as plasma glyceride levels of greater than 210 mg/100ml.

1. Subjects studied.

Twenty male subjects were studied, 13 normolipidaemic and 7 hyperlipidaemic. The mean plasma lipid levels for the normolipidaemic males were glyceride 126 ± 25 mg/100ml, cholesterol 229 ± 38 mg/100ml, and phospholipids 234 ± 28 mg/100ml and for the hyperlipidaemic males the values were glyceride 327 ± 75 mg/100ml, cholesterol 316 ± 78 mg/100ml, phospholipids 317 ± 52 mg/100 ml.

The normolipidaemic group consisted of 9 normal subjects, 3 idiopathic hypertensive patients and 1 patient recovering from a myocardial infarction. The hyperlipidaemic group consisted of 5 patients recovering from myocardial infarction and 2 idiopathic hypertensive patient. The mean age of the normolipidaemic group was 49 ± 7 years (range 40 - 59) and for the hyperlipidaemic group was 48 ± 7 years (range 40 - 59). The two variables, % overweight and serum uric acid, referred to in the last section were:-

	Normolipidaemic. mean ± S.D.	Hyperlipidaemic. mean ± S.D.
S.U.A.mg%.	5.1 ± 1.0	5.0 ± 0.9
% overweight	13 ± 1.3	23 ± 1.1

The attempt to control the weight variable was not successful. However, when the results of the study of Lopez-S & Krehl (1967) referred to in the last Section are considered, it is realised that a difference of 23 - 13 = 10% overweight should have little influence on the urinary DHA levels.

All the subjects had normal renal function as estimated by blood urea and serum creatinine except for one normolipidaemic patient with a serum creatinine of 1.5 mg/100 ml. All subjects were on normal or hospital diets and none were taking or being given medication of any sort for one week prior to the study.

2. Analytical techniques.

Collection of blood and urine	-	Section IV.A.5.
Blood urea & serum creatinine	-	Section IV.A.2 a & b.
Serum uric acid	-	Section IV.A.4.
Plasma cholesterol, phospholipids and glycerides	-	Section IV.A.3 a,b & c.
17-OS & 17-OHCS	-	Section IV.A.6 a & b.
D.17-OS.S & D.17-OS.G	-	Section IV.C.2.

All analysis were duplicated.

3. Results.

The urinary levels of the individual D.17-OS together with the plasma lipid levels for normolipidaemic and hyperlipidaemic males studied are shown in Tables 36 & 37 respectively. The values are listed in order of increasing age of the subjects studied. In both cases there is a wide range of values for all the urinary D.17-OS, though not nearly as wide as was found for normal males (Section V.A., Table 7). This disparity is probably due partly to the fact that in both Section V.A and the present Section the excretion of D.17-OS correlates negatively with age. Therefore, a difference in the ranges of urinary D.17-OS urinary levels would be expected since the normolipidaemic and hyperlipidaemic males ages range from 40 - 60 years whereas in normal males the ages range from 29 - 69 years.

A statistical comparison between the urinary D.17-OS and the plasma lipid levels for normolipidaemic and hyperlipidaemic males is shown in Table 38. Although the subjects studied were divided into two groups on the basis of their plasma glyceride levels, it is obvious that the plasma phospholipid, total plasma lipids (cholesterol + glyceride + phospholipid) and to lesser extent cholesterol show equally large statistical differences. However, inspection of the actual values in Tables 36 & 37 shows that complete discrimination into two groups by a single lipid parameter can only be achieved by employing plasma glyceride levels.

TABLE 36. INDIVIDUAL URINARY D.17-OS LEVELS (mg. free steroid/24 hr), PLASMA LIPID LEVELS (mg./100 ml), SERUM URIC ACID LEVELS AND % OVERWEIGHT VALUES FOR NORMOLIPIDAEMIC MALES.

	AGE	DHA			epiA.	E			A			PLASMA LIPIDS			S.U.A.	%	
		S	G	S+G	S	S	G	S+G	S	G	S+G	P.LIPID	CHOL.	GLY.	mg%	O.WT.	
1	40	1.44	0.53	1.97	0.22	0.09	3.74	3.83	0.62	5.36	5.98	278	258	123	4.8	123	N
2	42	2.21	0.23	2.44	0.11	0.08	1.30	1.38	0.26	1.90	2.16	240	213	120	4.9	105	M.I.
3	43	2.32	0.29	2.61	0.30	0.21	2.01	2.22	0.67	2.42	3.09	263	269	177	4.5	121	N
4	43	3.93	0.90	4.83	0.12	0.08	2.03	2.11	0.27	1.09	1.36	220	219	120	4.5	106	H.BP.
5	44	1.75	0.36	2.11	0.12	0.08	1.66	1.74	0.65	2.12	2.77	200	175	147	5.2	131	N
6	45	0.71	0.11	0.82	0.23	0.61	2.54	3.15	0.70	1.75	2.45	218	244	104	6.2	111	N
7	46	3.69	0.54	4.23	0.61	0.38	3.40	3.78	1.81	4.38	6.19	230	254	78	7.4	105	N
8	52	0.58	0.17	0.75	0.07	0.05	1.02	1.07	0.28	1.85	2.13	249	259	120	4.7	96	N
9	52	0.46	0.13	0.59	0.13	0.07	2.97	3.04	0.33	2.63	2.96	240	283	148	5.6	113	N
10	54	0.46	0.09	0.55	0.07	0.18	1.75	1.93	0.11	1.22	1.33	185	150	120	4.6	134	H.BP.
11	55	0.06	0.04	0.10	0.06	0.21	1.92	2.13	0.30	1.69	1.99	245	238	150	5.8	129	N
12	59	0.15	0.04	0.19	0.01	0.14	1.65	1.79	0.36	0.58	0.94	200	208	115	4.7	107	H.BP.
13	61	1.43	0.03	1.46	0.06	0.24	1.03	1.27	1.33	0.86	2.19	270	205	120	3.8	94	N
MEAN	49	1.48	0.28	1.74	0.16	0.19	2.08	2.26	0.59	2.14	2.73	234	229	126	5.1	113	
S.D.	7	1.27	0.26	1.50	0.16	0.16	0.86	0.91	0.48	1.36	1.61	28	38	25	1.0	13	

N = Normal males. M.I. = Male recovering from a myocardial infarction. H.BP. = Male with high blood pressure.

TABLE 37. INDIVIDUAL URINARY D.17-OS LEVELS (mg. free steroid/24 hr), PLASMA LIPID LEVELS (mg/100 ml), SERUM URIC ACID LEVELS AND % OVERWEIGHT VALUES FOR HYPERLIPIDAEMIC MALES.

	AGE	DHA			epiA.	E			A			PLASMA LIPIDS			S.U.A.	% O.WT.	
		S	G	S+G	S	S	G	S+G	S	G	S+G	P.LIPID	CHOL.	GLY.	mg%		
1	40	1.07	0.20	1.27	0.08	0.06	1.30	1.36	0.12	1.61	1.73	330	295	358	4.8	130	I.HL.
2	42	0.39	0.09	0.48	0.19	0.20	3.85	4.05	0.67	2.19	2.86	265	270	208	4.0	140	I.HL.
3	43	0.58	0.57	1.15	0.03	0.03	0.67	0.70	0.11	0.72	0.83	386	315	436	5.5	127	M.I.
4	45	0.19	0.12	0.31	0.04	0.16	2.13	2.29	0.17	0.94	1.11	383	472	330	3.8	112	M.I.
5	51	0.66	0.09	0.75	0.05	0.13	1.22	1.35	0.08	0.87	0.95	281	338	281	6.0	106	M.I.
6	56	0.99	0.11	1.10	0.07	0.05	2.04	2.09	0.22	1.82	2.04	263	220	275	5.0	127	M.I.
7	59	0.03	0.00	0.03	0.00	0.03	0.32	0.35	0.03	0.54	0.57	310	305	371	5.9	120	M.I.
MEAN	48	0.56	0.17	0.73	0.06	0.09	1.65	1.74	0.20	1.24	1.44	317	316	327	5.0	123	
S.D.	7	0.39	0.19	0.47	0.06	0.07	1.17	1.23	0.22	0.63	0.81	52	78	75	0.9	11.5	

I.HL. = Idiopathic hyperlipidaemic males. M.I. = Male recovering after myocardial infarction.

TABLE 38. COMPARISON OF INDIVIDUAL D.17-OS URINARY AND PLASMA LIPID LEVELS OF NORMOLIPIDAEMIC AND HYPERLIPIDAEMIC MALES.

	Normolipidaemic Males MEAN ± S.D.	Hyperlipidaemic Males MEAN ± S.D	P VALUE
epiA.S	0.16 ± 0.16	0.06 ± 0.06	0.052
DHA.S	1.48 ± 1.27	0.56 ± 0.39	0.10
E.S	0.19 ± 0.16	0.09 ± 0.07	0.074
A.S	0.59 ± 0.48	0.20 ± 0.22	0.01
DHA.G	0.28 ± 0.26	0.17 ± 0.19	0.48
E.G	2.08 ± 0.86	1.65 ± 1.17	0.42
A.G	2.14 ± 1.36	1.24 ± 0.63	0.08
DHA.(S+G)	1.74 ± 1.50	0.73 ± 0.47	0.12
E.(S+G)	2.26 ± 0.91	1.74 ± 1.23	0.28
A.(S+G)	2.73 ± 1.61	1.44 ± 0.81	0.029
PHOSPHOLIPIDS	234 ± 28	317 ± 52	<0.0005
CHOLESTEROL	229 ± 38	316 ± 78	<0.002
GLYCERIDES	126 ± 25	323 ± 75	<0.0005
TOTAL LIPIDS	589 ± 70	952 ± 162	<0.0005

(a) Sulphate conjugates. The urinary D.17-OS.S levels for normolipidaemic males were all significantly higher than the respective values for hyperlipidaemic males, and of these A.S was significantly higher at the 1% level. On quantitative comparison of the mean urinary D.17-OS.S levels, DHA.S showed the greatest difference (1.48 - 0.56 = 0.92 mg/24 hr). However, despite this, the difference between the DHA.S levels of the two groups was only significant at the 10% level.

In both groups the major steroid sulphate was DHA. In the normolipidaemic group this applied to 11 (85%) subjects, in the other 2 (15%), a normal male and an idiopathic hypertensive male, A was the major steroid sulphate. Of the above 11 subjects, 10 showed the typical D.17-OS.S urinary excretion pattern for normal males, namely DHA>A>epiA>E. In the other group which was predominantly patients recovering from myocardial infarctions there was no typical D.17-OS.S excretion pattern, the only semi-consistent feature was DHA.S as the major steroid sulphate in 6 (86%) of the group.

(b) Glucuronide conjugates. All the D.17-OS.G were excreted in greater amounts by the normolipidaemic males but A.G was the only D.17-OS.G excreted in amounts significantly greater ($P = 0.08$) than that by the hyperlipidaemic group.

In the normolipidaemic group E.G was the major steroid glucuronide in 7 (54%) and A.G in the other 6 (46%). Similarly, in the hyperlipidaemic group E.G was the major steroid glucuronide in 4(57%) and A.G in the other 3 (43%).

(c) Sulphate and glucuronide conjugates. Like the D.17-OS glucuronide levels the mean urinary levels of the D.17-OS.(S+G) were higher for the normolipidaemic group, with A.(S+G) showing a significant difference between the two groups ($P = 0.029$). The urinary excretion of the D.17-OS.(S+G) like the D.17-OS.G showed no definite pattern in either group.

(d) Correlations. Correlation coefficients (Section IV.D.2) were calculated by computer for any combination of 2 of the 31 variables collected for all the subjects. The variables involved were age; serum uric acid; serum creatinine; blood urea; urine volume; height; surface area; weight; % overweight; systolic blood pressure; diastolic blood pressure; mean blood pressure; total 17-OS; total 17-OHCS; epiA.S; DHA.S; E.S; A.S; DHA.G; E.G; A.G; DHA.(S+G); E.(S+G); A.(S+G); (epiA. + DHA + E + A).S; (DHA + E + A).G; (epiA. + DHA + E + A).S+G; plasma phospholipids; plasma cholesterol; plasma glycerides and total plasma lipids (phospholipids + cholesterol + glycerides). Correlation coefficients for the most highly correlated of the 31 variables are shown for normolipidaemic and hyperlipidaemic males in Tables 39 & 40 respectively. Correlation coefficients significant at the 5% level or less are underlined.

Like the normal males (Section VA, Table 10) the age of the normolipidaemic males showed extensive negative correlations with the excretion of individual D.17-OS and total 17-OS (Table 39). The hyperlipidaemic males showed the same trend, however in no case was the correlation statistically significant.

TABLE 39. MATRIX OF CORRELATION COEFFICIENTS FOR NORMOLIPIDAEMIC MALES.

	AGE	%O.WT	S.U.A.	17-OS	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	P.LIPID	CHOL.	GLY.
AGE	1.00													
%O.WT.	-0.22	1.00												
S.U.A.	-0.19	0.37	1.00											
17-OS	-0.75	-0.08	-0.24	1.00										
epiA.S	-0.48	-0.05	0.01	0.57	1.00									
DHA.S	-0.59	-0.24	-0.40	0.69	0.61	1.00								
E.S	0.00	-0.05	0.38	-0.13	0.47	0.00	1.00							
A.S	0.00	-0.33	-0.27	0.17	0.73	0.46	0.50	1.00						
DHA.G	-0.69	-0.04	-0.23	0.81	0.44	0.84	-0.19	0.16	1.00					
E.G	-0.46	0.25	0.32	0.55	0.65	0.22	0.26	0.31	0.41	1.00				
A.G	-0.58	0.18	0.10	0.62	0.69	0.29	0.03	0.40	0.41	0.82	1.00			
P.LIPID	-0.13	-0.32	-0.22	0.34	0.16	0.10	-0.11	0.28	0.05	0.17	0.46	1.00		
CHOL.	-0.24	-0.30	0.27	0.47	0.42	0.07	0.10	0.18	0.12	0.49	0.51	0.65	1.00	
GLY.	-0.03	0.48	0.11	-0.03	-0.04	-0.25	-0.40	-0.44	-0.19	-0.20	-0.14	0.24	0.13	1.00

Correlation coefficients significant at 5% level or less are underlined.

TABLE 40. MATRIX OF CORRELATION COEFFICIENTS FOR HYPERLIPIDAEMIC MALES.

	AGE	%O.WT.	S.U.A.	17-OS	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G	P.LIPID	CHOL.	GLY.
AGE	1.00													
%O.WT.	-0.39	1.00												
S.U.A.	0.56	-0.38	1.00											
17-OS	-0.48	0.75	-0.38	1.00										
epiA.S	-0.51	0.70	-0.60	0.87	1.00									
DHA.S	-0.24	0.24	0.06	0.37	0.23	1.00								
E.S	-0.39	-0.03	-0.64	0.32	0.63	-0.25	1.00							
A.S	-0.39	0.67	-0.65	0.70	0.94	-0.43	0.68	1.00						
DHA.G	-0.51	0.22	-0.10	-0.16	-0.13	0.26	-0.36	-0.15	1.00					
E.G	-0.38	0.48	-0.79	0.61	0.89	0.33	0.80	0.93	-0.27	1.00				
A.G	-0.33	0.71	-0.59	0.83	0.90	0.49	0.40	0.80	-0.19	0.82	1.00			
P.LIPID	-0.41	-0.23	-0.14	-0.50	-0.50	-0.26	-0.20	-0.43	0.61	-0.41	-0.58	1.00		
CHOL.	-0.24	-0.63	-0.31	-0.51	-0.36	-0.52	-0.38	-0.27	0.00	-0.09	-0.52	0.69	1.00	
GLY.	-0.05	-0.19	0.40	-0.53	-0.74	-0.08	-0.73	-0.74	0.63	-0.83	-0.72	0.79	0.25	1.00

Correlation coefficients significant at 5% level or less are underlined.

With respect to the relationships of S.U.A levels - urinary D.17-OS levels as seen in gout and hypertensives (Sonka et al, 1964 and Kolbel et al, 1964, 1965a), and % overweight - urinary DHA levels as seen in obesity (Lopez-S & Krehl, 1967) the results shown in Tables 39 & 40 lend little or no support. Of the normolipidaemic and hyperlipidaemic males neither of their DHA levels exhibited a correlation with S.U.A levels. However, the hyperlipidaemic group displayed a negative correlation for E.G versus S.U.A. ($r = -0.79$, $P<0.05$). Similarly for % overweight versus DHA levels, there were no significant correlations, although there was a significant positive correlation ($r = 0.75$, $P<0.05$) between % overweight and urinary 17-OS levels for hyperlipidaemic males.

Like the normal males (Table 10) both the normolipidaemic and the hyperlipidaemic males showed a marked degree of intercorrelation between the individual D.17-OS.

However of greatest interest was the marked and extensive negative correlations between plasma glyceride levels and certain individual urinary D.17-OS for hyperlipidaemic individuals. These negative correlations were as significant as the positive correlation demonstrated for plasma glyceride levels versus plasma phospholipid levels, two understandably interdependent variables, since both incorporate fatty acid molecules in their respective biosynthesis. The negative correlation between plasma glyceride levels and urinary D.17-OS levels held for all the D.17-OS sulphates and glucuronides

except DHA.S and DHA.G. The highest correlation was for E.G versus plasma glyceride ($r = -0.83$, $P = 0.02$), this negative correlation is appreciated by the mere appraisal of the values for the two variables.

Urinary E.G levels. mg/24 hr.	Plasma glyceride levels. mg/100 ml.
320	371
670	436
1300	358
2130	330
1220	281
2040	275
3850	208

(e) Discriminant function analysis. This was carried out as described in Section IV.D.3. For each of the two groups, normolipidaemic and hyperlipidaemic males, values for 14 variable - 17-OS; 17-OHCS; epiA.S; DHA.S; E.S; A.S; DHA.G; E.G; A.G; DHA.(S+G); E.(S+G); A.(S+G); (epiA +DHA + E + A).S and (DHA + E + G).G. were tabulated and statistically compared using a simple test of significanct (t - test or U - test). The three variables showing the most significant differences epiA.S, A.S and A.(S+G) were employed in the discriminant function analysis. Employing these three variables it was possible to discriminate between hyperlipidaemic and normolipidaemic males, for all but one individual, an idiopathic hypertensive in the normolipidaemic group (Patient 10, Table 36) who fell within the elipsoid surrounding the hyperlipidaemic group.

4. Discussion.

The object of this study was to determine, if the expected differences between the urinary D.17-OS levels for hyperlipidaemic and normolipidaemic males were real or unreal, that is, to test the hypothesis based on the results for gouty males.

A real difference was found. The D.17-OS.S urinary levels of the hyperlipidaemic group were much lower than the respective levels of the normolipidaemic group, especially in the case of A.S where the difference was significant at the 1% level. Similarly A.G and A.(S+G) urinary levels showed significant differences at the 8% and 3% levels respectively.

Comparison of the above differences, with respect to steroids involved and level of significance, with those found for gouty males relative to normal controls would further test the hypothesis. Inspection of Table 34 (gouty versus normal males) and Table 38 (hyperlipidaemic versus normolipidaemic males) shows certain similarities. Both test groups show lower D.17-OS.S levels than the respective control groups and in both cases epiA.S and A.S show the most significant differences. Also in both cases A.G and A.(S+G) in both studies showed significant differences. The only disparity is that the gouty males showed lower levels of DHA.G and DHA.(S+G) relative to the control group whereas this difference was not manifested by the other study.

Though the steroids involved are the same, the levels of significance of the differences shown in the two studies is not. The

P values in the gout study were much lower, due simply to the fact that the urinary D.17-OS levels in the gouty males were much lower than those for the hyperlipidaemic males and that the values for the normolipidaemic male controls were lower than the respective values for the normal controls of the gouty males.

The above consideration of the results of the two studies supports the hypothesis of a relationship between low urinary D.17-OS levels and elevated plasma lipid levels, but does not explain why gouty males have such low urinary D.17-OS levels, markedly lower than the respective values for the hyperlipidaemic males. This disparity might be in some way related to the markedly elevated S.U.A levels or the obesity normally found in gout, though as previously stated this explanation is doubtful since by the present report there is no significant correlation between either urinary D.17-OS levels and hyperuricaemia or D.17-OS levels and obesity. However, a strong negative correlation between plasma glyceride levels and urinary D.17-OS levels has been demonstrated for hyperlipidaemic males. Thus, by the present report, the plasma lipid levels, unlike the levels of the other two variables S.U.A and % overweight may be influenced by the excretion rate of certain D.17-OS. Plasma glyceride the lipid in question is elevated in gout (Berkowitz, 1964, 1966 and Feldman & Wallace, 1964), therefore this may be a common variable in gout and hyperlipidaemia, the elevation of which may be a direct result of low D.17-OS urinary levels in both diseases.

The normolipidaemic males did not show any significant correlations between plasma lipid levels and D.17-OS urinary levels, which is contrary to the findings of Sonka, Fassati, Gregorova & Picek (1968) who found that serum lipid levels (cholesterol, phospholipids, β-lipoproteins and free fatty acids) were negatively correlated with urinary DHA levels in healthy blood donors. However, they suggested that the correlations were more a function of the dependence of DHA excretion and serum lipid levels on age than a direct inverse relationship between urinary DHA and serum lipid variables. But, despite this the authors still advocated a causal relationship between DHA and lipid synthesis, and by manipulation of their results they presented evidence. They divided the group of healthy blood donors (47 men, 47 females) into two groups according to urinary DHA level, "DHA excretors" (mean 1.44 mg/24 hr, mean age 34 years) and "DHA nonexcretors" (mean 0.08 mg/24 hr, mean age 40 years). The serum lipid levels were significantly lower, cholesterol ($P<0.005$), phospholipids ($P<0.025$) and free fatty acids ($P<0.05$) in the "DHA nonexcretors" compared with the "DHA excretors".

The above procedure utilised by Sonka et al (1968) whereby the normal group was divided into two, according to their urinary DHA levels, and then differences in plasma lipids sought, is exactly the same as that for the present exercise except in reverse, that is, division according to plasma lipid levels and then differences in urinary D.17-OS levels sought. By both procedures significant results were found, both therefore, strongly supporting a relationship

between plasma lipids and urinary D.17-OS.

The discriminant function analysis presented further support of a plasma lipid - urinary D.17-OS relationship in that by employing only three variables epiA.S, A.S and A.(S+G) it was possible to discriminate between hyperlipidaemic and normolipidaemic males for all but one individual, an idiopathic hypertensive patient (patient 10, Table 36) in the normolipidaemic group, who fell within the elipsoid of the hyperlipidaemic group. This patient although normolipidaemic had steroid levels more in keeping with the hyperlipidaemic males,

	epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G
Patient 10	0.07	0.46	0.18	0.11	0.09	1.75	1.22
Hyperlipidaemic males (mean)	0.06	0.56	0.09	0.20	0.17	1.65	1.24
Normolipidaemic males (mean)	0.16	1.48	0.19	0.59	0.28	2.08	2.14

and in addition to this patient 10 had a % overweight value of 34%, the highest value in that group and, therefore, more suited to the hyperlipidaemic group.

Thus the hypothesis of a plasma lipid - urinary D.17-OS relationship seems well founded, but obviously requiring further confirmatory evidence. It was, therefore, decided to study urinary levels in myocardial infarction, since the majority of the males in the hyperlipidaemic group were males recovering after a myocardial infarction and since numerous other investigators have demonstrated

a high incidence of hyperlipidaemia in myocardial infarction (Morrison et al, 1948; Getler & Garn, 1950; Steiner et al, 1952; Albrink, Meigs & Man, 1961; Albrink, 1962; Getler, White, Cady & Whiter, 1964; Benedek, 1967 and Kuo, 1968).

G. Individual Urinary D.17-OS Sulphates and Glucuronides: Values for Male Myocardial Infarct Patients.

Urinary D.17-OS sulphates and glucuronides levels were estimated in male patients recovering after a myocardial infarction and compared with the corresponding levels in normal males as reported in Section V.A.3.

1. Patients studied.

Fifteen male patients all recovering after a myocardial infarction were studied. The myocardial infarction was diagnosed by clinical examination, electrocardiographic findings and serum glutamic - oxaloacetic transaminase levels. The mean age of the patients was 48 ± 6 years (range 42 - 56). The % overweight of each patient was calculated from the tables of Kemsley et al (1962), the mean % overweight value was 114 ± 9 (range 100 - 127). None of the patients had elevated blood pressure and all had satisfactory renal function as estimated by blood urea (32 ± 6 mg/100 ml) and serum creatinine (0.89 ± 0.10 mg/100 ml). All patients were on normal hospital diets and no medication was given for 3 days prior to the study.

2. Analytical techniques.

Collection of blood and urine	-	Section IV.A.5.
Blood urea and serum creatinine	-	Section IV.A.2 a & b.
Serum uric acid	-	Section IV.A.4.

(Analytical techniques continued):

17-OS & 17-OHCS - Section IV.A.6 a & b.

D.17-OS & D.17-OS.G - Section IV.C.2.

All estimations were duplicated.

3. Results.

The urinary levels of the individual D.17-OS sulphates and glucuronides for the 15 male myocardial infarct patients are shown in Table 41.

(a) Sulphate conjugates. The urinary levels of the individual D.17-OS sulphates for myocardial infarct males, were lower than the respective levels found in the normal males controls (Table 42).

With the exception of E.S the steroid sulphate conjugates were excreted in significantly lower amounts by the myocardial infarct patients and in the case of A.S the difference was significant at the 0.03% level.

DHA.S, as in normal males, was quantitatively the most important D.17-OS.S in 73% of the myocardial infarct patients. But unlike normal males, for whom the typical urinary D.17-OS.S excretory was DHA>A>epiA>E, the myocardial infarct patients showed no typical pattern other than DHA>A, epiA or E and that in 73% of the patients epiA was the minor steroid sulphate excreted. However, despite this lack of obvious pattern, the E.S/A.S ratios for the myocardial infarct patients were consistently higher ($P = 0.0028$) than those

TABLE 41. INDIVIDUAL URINARY D.17-OS LEVELS (mg. free steroid/24 hr.) FOR MALE MYOCARDIAL INFARCT PATIENTS.

NO.	AGE	DEHYDROEPIANDROSTERONE			epiA	AETIOCHOLANOLONE			ANDROSTERONE		
		S	G	S+G	S	S	G	S+G	S	G	S+G
1.	43	0.58	0.57	1.15	0.03	0.03	0.67	0.70	0.11	0.72	0.83
2.	51	0.66	0.09	0.74	0.05	0.13	1.22	1.35	0.08	0.87	0.95
3.	45	0.19	0.12	0.31	0.04	0.16	2.13	2.29	0.17	0.94	1.11
4.	49	0.71	0.15	0.87	0.07	0.04	2.57	2.61	0.25	2.47	2.72
5.	54	0.35	0.16	0.51	0.10	0.41	2.78	3.19	0.34	1.70	2.04
6.	59	0.03	0.00	0.03	0.00	0.03	0.32	0.35	0.03	0.54	0.57
7.	42	2.21	0.23	2.44	0.11	0.08	1.31	1.39	0.26	1.89	2.15
8.	56	0.99	0.11	1.11	0.07	0.05	2.04	2.06	0.22	1.82	2.04
9.	56	0.19	0.21	0.40	0.06	0.12	1.84	1.96	0.24	0.87	1.11
10.	48	0.28	0.10	0.38	0.07	0.26	1.94	2.12	0.17	1.43	1.60
11.	43	0.68	0.32	1.00	0.00	0.11	3.04	3.15	0.17	2.03	2.20
12.	44	0.48	0.19	0.67	0.23	0.34	3.77	4.11	0.37	2.10	2.47
13.	46	0.43	0.15	0.59	0.07	0.49	1.63	2.12	0.25	1.07	1.32
14.	55	0.37	0.22	0.59	0.02	0.09	0.60	0.69	0.12	0.54	0.66
15.	52	0.28	0.14	0.42	0.13	0.27	0.89	1.12	0.51	0.74	1.23
MEAN	48	0.56	0.18	0.75	0.07	0.17	1.78	1.95	0.22	1.31	1.53
S.D.	6	0.51	0.13	0.56	0.06	0.14	0.98	1.05	0.12	0.64	0.69
RANGE	43-59	0.03-2.21	0.00-0.57	0.03-2.44	0.00-0.23	0.03-0.49	0.32-3.77	0.35-3.19	0.03-0.51	0.54-2.47	0.66-2.72

TABLE 42. COMPARISON OF INDIVIDUAL D.17-OS URINARY LEVELS FOR MALE MYOCARDIAL INFARCT PATIENTS WITH NORMAL MALE CONTROLS.

	NORMAL MALES MEAN ± S.D	MEAN ± S.D	P VALUE
epiA.S	0.30 ± 0.326	0.07 ± 0.06	0.001
DHA.S	2.75 ± 3.75	0.56 ± 0.51	0.01
E.S	0.23 ± 0.212	0.17 ± 0.14	0.52
A.S	0.82 ± 0.838	0.22 ± 0.12	0.0003
DHA.G	0.37 ± 0.365	0.18 ± 0.13	0.19
E.G	2.61 ± 1.36	1.78 ± 0.98	0.07
A.G	3.05 ± 1.89	1.31 ± 0.64	0.001
DHA.(S+G)	3.12 ± 4.07	0.75 ± 0.56	0.03
E.(S+G)	2.89 ± 1.36	1.95 ± 1.05	0.07
A.(S+G)	3.87 ± 2.46	1.53 ± 0.69	0.0004
17-OS	12.3 ± 5.3	8.5 ± 3.0	0.03
17-OHCS	12.7 ± 3.7	10.7 ± 2.8	0.084

for normal males (fig.20a). Similarly for E.S/epiA.S ratios which were also significantly higher at the 0.28% level.

(b) Glucuronide conjugates. The urinary D.17-OS.G levels for the myocardial infarct patients were low compared with the normal controls (Table 42), and were significantly lower in the case of A.G and E.G ($P = 0.001$ and $P = 0.07$ respectively).

In the myocardial infarct patients E.G was the major steroid glucuronide in 73% and A.G in the other 27%. Thus the typical urinary excretory pattern of the steroid glucuronide conjugates for the myocardial infarct patients was E>A>DHA, whereas in normal controls it was A>E>DHA. The E.G/A.G ratios of the infarct group was significantly higher ($P = 0.014$) than the same ratios for normal controls (fig. 20b).

(c) Sulphate plus glucuronide conjugates. Like the D.17-OS.G levels the urinary D.17-OS.(S+G) levels for the myocardial infarct patients were low compared with normal controls, in fact, relatively they were much lower with A.(S+G) significantly lower at the 0.04% level.

E was the major D.17-OS excreted by 66% of the infarct group, whereas in the control group it was A (46%) followed by E (27%) and DHA (27%). The difference between the urinary E.(S+G)/A.(S+G) ratios for the two groups was highly significant ($P = 0.002$, fig.20c).

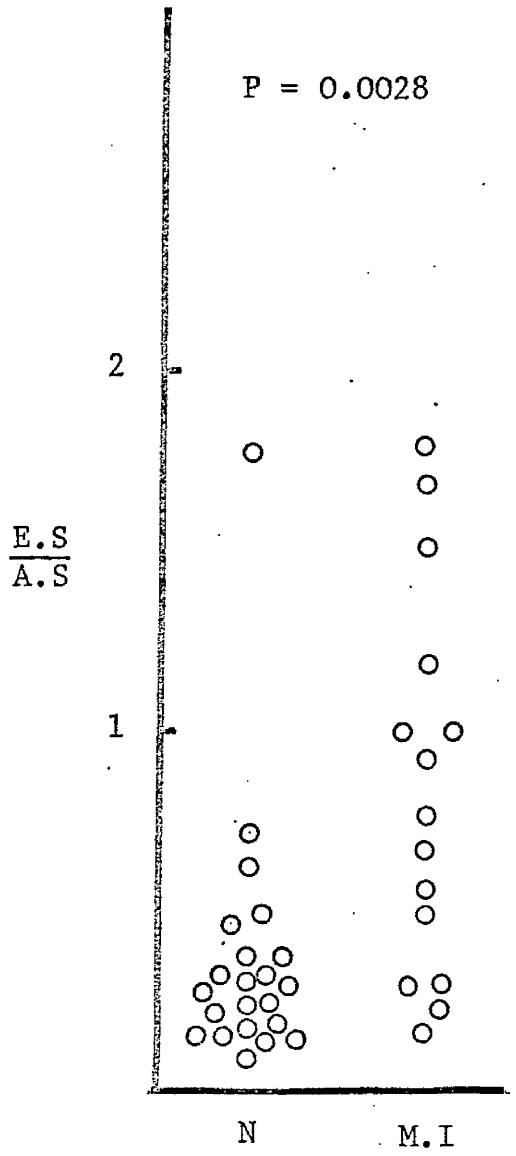


Fig. 20a.

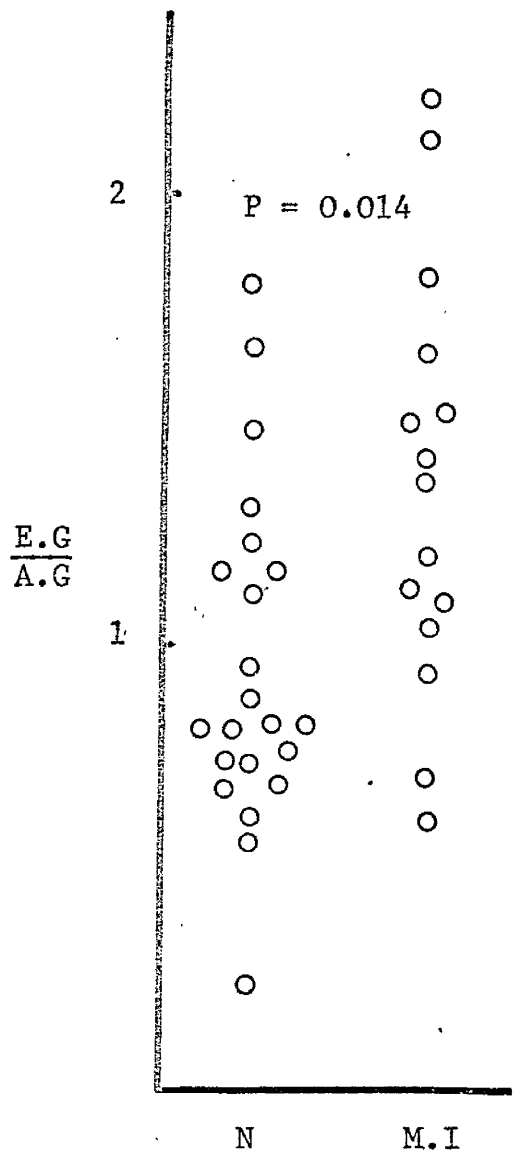


Fig. 20b.

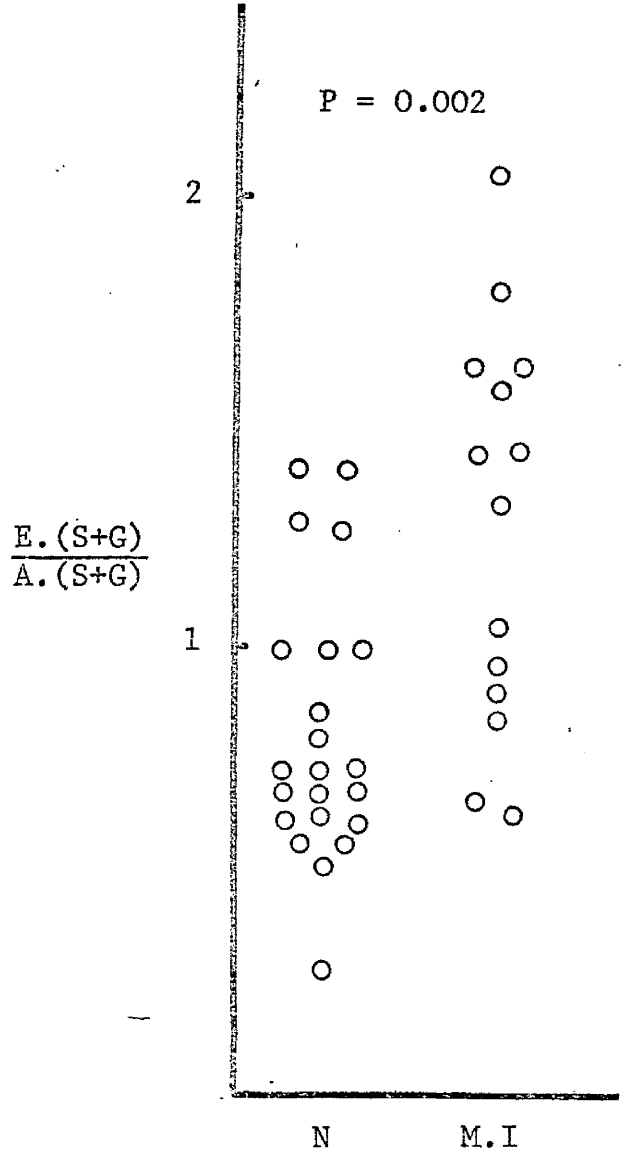


Fig. 20c.

Figs 20a,b,c. Comparison of the ratios of E.S/A.S, E.G/A.S and E.(S+G)/A.(S+G) for myocardial infarct males (M.I) and normal males (N).

4. Discussion.

There is very little previous work on urinary steroid levels in myocardial infarction. Marmorston, Lewis, Bernstein, Sobel, Kuzma, Alexander, Magidson & Moore (1957) estimating urinary 17-OS in this disease found levels markedly decreased in male patients. Their findings were later confirmed and extended by Garrett, Creech, Horning & DeBakey (1962), who estimated DHA, E and A in both the sulphate and glucuronide fractions in urine from a group of atherosclerotic patients which included individuals with myocardial infarcts. Their results though of great interest, were unfortunately completely lacking in normal control values.

They studied individual urinary D.17-OS levels in 20 atherosclerotic patients (8 with myocardial infarcts) prior to surgical treatment and in another 20 atherosclerotic patients (5 with myocardial infarcts) 6 months to 3 years following surgical treatment. No normal subjects were studied. Their results in both groups, that is pre and post-operative, were very similar to the results of the present study:-

		epiA.S	DHA.S	E.S	A.S	DHA.G	E.G	A.G
Present study	N	0.30	2.75	0.23	0.82	0.37	2.61	3.05
	M.I	0.06	0.70	0.20	0.24	0.18	1.60	1.30
Garrett et al	A	-	0.3	0.2	0.3	-	2.7	1.2

N = Normal. M.I. = Myocardial Infarction. A = Atherosclerosis.

Not only, are the results of Garrett et al (1962) very similar to those

of the present study in that the urinary D.17-OS levels are very low in both studies but also with respect to the excretion of E.G relative to A.G and the excretion of E.S relative to A.S. Garrett et al (1962) results indicate that 95% of the atherosclerotic patients studied had values for E.G > A.G, this compares favourably with 73% for present study and contrasts markedly with 34% for normal males. Similarly for urinary E.S/A.S ratios Garrett et al (1962) and present study found 65 and 66% respectively of patients to have E.S/A.S ratios greater than 0.5 whereas this held for 18% of the normal males. A unique feature emerging from both studies is the fact that in vascular disease urinary E.S levels are not depressed.

Some of the above findings in both studies bear a strong resemblance to those found in Section V.B.3 for idiopathic hypertensive males, where elevated urinary E.S/A.S, E.G/A.G and E.(S+G)/A.(S+G) ratios were demonstrated, and in all cases the ratios were significantly higher for the hypertensive group than for the respective ratios of the normal males.

Since the results for hypertensive and myocardial infarct patients bear such a close similarity, yet display a marked divergence from normal, it might be expected that the proposed explanation for abnormalities of steroid metabolism in hypertension holds also for myocardial infarction, namely an enzymatic block between pregnenolone and 17α-hydroxypregnenolone (fig.13). This proposal of a pregnenolone 17α-hydroxylase block, if accepted, may very satisfactorily explain the

abnormal urinary D.17-OS patterns found in hypertension and myocardial infarction, but it does not explain why in one case the block manifests itself as elevated blood pressure and in another case as a myocardial infarct.

Why the urinary steroidal patterns seen in idiopathic hypertension may be explained on the basis of pregnenolone to 17α-hydroxy-pregnenolone block, and why this block might result in elevated blood pressure have been discussed in Section V.B.4.

Assuming the block between pregnenolone and 17α-hydroxy-pregnenolone explains the urinary steroid patterns shown in myocardial infarction, what then is the relationship of D.17-OS to myocardial infarction. A lipid - D.17-OS relationship was the outcome of earlier discussions.

From the available evidence there definitely appears to be a relationship between urinary D.17-OS levels and plasma lipid levels. However, is it a causal relationship, are the plasma lipid levels dependent on the in vivo D.17-OS levels and, therefore, indirectly on the D.17-OS urinary levels, or is it a coincidental relationship, the variables being independent of one another?

There are numerous studies reporting low urinary D.17-OS levels in diseases characterised by elevated plasma lipids. Hellman et al (1959a,b) reported low urinary levels of E and A concomitant with an elevated urinary E/A ratio in myxoedema. Garrett et al (1962) reported low urinary D.17-OS.S (DHA and A) and D.17-OS.G (A and E) in

atherosclerosis. Sonka et al (1964) reported zero levels of urinary DHA in gout, whereas Beck et al (1967) reported low levels of urinary DHA in the same disease and the present report found very low levels of all the urinary D.17-OS, especially the D.17-OS.S. Kotasek et al (1967) reported low urinary DHA levels in toxaemia of pregnancy. Sonka & Gregorova (1963) reported zero, and Lopez-S & Krehl (1967) low urinary DHA levels in obesity, whereas Hendrikx et al (1965) reported low urinary A and E levels. The present studies report low urinary D.17-OS.S (epiA, E and A) and low A.G in hyperlipidaemia, and very low levels of D.17-OS.S (epiA, DHA and A) and low levels of A.G and E.G in myocardial infarction. Therefore, this supplies the evidence necessary to justify the conclusion that there is a definite relationship between urinary D.17-OS levels and plasma lipid levels, though not necessarily proof of a causal relationship.

There is evidence suggesting a causal relationship between D.17-OS levels and plasma lipid levels, but it is not nearly as conclusive as the evidence simply demonstrating a relationship between the two variables. While studying the control of plasma lipid levels, Hellman et al (1959a,b), Cohen et al (1961) and Furman & Howard (1962) all demonstrated the hypocholesterolaemic effect of parentally administered A, and Cohen et al (1961) further demonstrated a depression of plasma triglyceride concomitant with the depression of plasma cholesterol. Kotasek et al (1967) reported a negative

correlation between urinary DHA and serum β-lipoprotein levels as studied in normal females, pregnant females and toxaemia of pregnancy. Sonka et al (1968) reported negative correlations between urinary DHA levels and plasma, lipid levels, though the authors suggested the correlations might have been more a dependence of DHA and plasma lipids on age rather than on each other. The present report describes a negative correlation between each of several urinary D.17-OS and plasma glyceride levels and the correlations could not be attributed to a mutual dependence of the variables on age. Experiments on rats by Ben-David et al (1967) and Kahn (1966) describe the hypocholesterolaemic effects induced by administration of DHA and A respectively.

Therefore, from the aforegoing evidence, the proposal of a relationship and possibly a causal one between D.17-OS and plasma lipids seems fairly reasonable, but what of the mechanism by which the two variables are interrelated, and why are the plasma lipids elevated in the afore-mentioned diseases?

Expressions such as "the thyromimetic effect" of A have been bandied about, but the expression merely equates the hypocholesterol-aemic effect of thyroxine and A and gives no indication of mode of action of A, merely of the end result. Orally administered A was shown to be inactive in the depression of plasma cholesterol without the vehicle clofibrate (Oliver, 1963 and Hellman et al, 1963). However, studies on the mode of action of clofibrate indicated that it acted by potentiating the effect of endogenous hormones particularly thyroxine and A. This evidence though favouring the hypocholesterdaemic

properties of A does not give any insight into its mode of action.

The explanation proposed by Kotasek et al (1967) to interpret the elevated plasma lipid levels in toxaemia of pregnancy is of relevance here. They suggested that the removal of the inhibitory effect of DHA on G-6-P.D activity would result in increased NADPH production and, therefore, ultimately in increased lipid biosynthesis in circumstances of low in vivo DHA levels.

The above explanation with modifications suggested by the results of other studies, could possibly form the basis of a working hypothesis for the mechanism of action of D.17-OS in control of lipid biosynthesis.

Marks & Banks (1960) demonstrated the inhibition of G-6-P.D by not only DHA, but also by E,A and epiA. In fact, epiA showed greater inhibition than DHA, but unfortunately later experiments untilising the inhibitory effect of D.17-OS appear to be restricted to DHA.

G-6-P.D activity has been shown to decrease in liver during starvation and increase to a peak activity several times the level of non-starved controls 72 hours after refeeding with a high glucose diet, (Potter & Ono, 1961; McDonald & Johnson, 1965, and Johnson, Moser & Sassoon, 1966). Following the demonstration of the inhibitory effect of DHA on the G-6-P.D activity in vitro by Marks & Banks (1960), Willmer & Foster (1965) showed that the injection of DHA into rats sharply limited the increase in G.6.P.D activity that occurs on starvation and refeeding. There is good circumstantial evidence from inhibitory studies to suggest that the increased enzyme activity

represents de novo synthesis of new enzyme (Potter & Ono, 1961 and Tepperman & Tepperman, 1963). It is not yet certain whether the decreased shunt enzyme (G-6-P.D) activity seen in the steroid-treated rats is due to repression or due to inhibition or both. However, preliminary observations by Tepperman, De La Garza & Tepperman (1968) suggest that the decreased enzyme activity probably represents decreased enzyme synthesis.

Therefore, it is possible that there could be present in the liver a dual inhibitory control over G-6-P.D activity which would result in decreased NADPH production in circumstances of high in vivo DHA levels and, therefore, probably in circumstances of high D.17-OS in vivo levels. The dual control limiting NADPH production would be a short term control, by D.17-OS enzyme inhibition and a longterm control, by D.17-OS enzyme repression.

The liver has been shown to be intimately involved in lipid synthesis: fatty acids (Tietz & Popjak, 1955); cholesterol (Bloch, Borek & Rittenberg, 1964; Little & Bloch, 1950); glycerides (Weiss & Kennedy, 1956) and phospholipids (Kennedy, 1961). All of these lipids require NADPH for their biosynthesis, therefore their production would be regulated by any mechanism regulating NADPH production, namely D.17-OS in vivo levels. Under normal conditions there is no storage of lipids by the liver, thus the plasma will be the next immediate site of liver synthesised lipids. Therefore, plasma lipids would

to some extent come under the control of NADPH levels in the liver.

The liver is the major site of DHA, DHA.S androstenedione and testosterone metabolism. There they are metabolised to the D.17-OS, DHA, epiA, A and E (fig. 1). Steroid hormones are active only as free steroids and not as their steroid conjugates. Since the liver is the site of the metabolism of D.17-OS precursors, it is most likely that the highest concentration of free D.17-OS in the body will be found in the liver cells. Therefore, any regulation by D.17-OS over G-6-P.D activity by either or both inhibition and repression will be manifested to the greatest degree in the liver. The end result of this would be regulation of lipid synthesis, therefore of plasma lipid levels.

Thus in conditions characterised by elevated plasma lipids and low D.17-OS levels it is suggested that the removal of inhibitory effects of D.17-OS on G-6-P.D activity would result in increased liver NADPH production and, therefore, ultimately in increased lipid biosynthesis.

VI. SUMMARY

1. A method is described for determining the 11-deoxy-17-oxosteroids (D.17-OS), dehydroepiandrosterone (DHA), aetiocholanolone (E), androsterone (A) and epiandrosterone (epiA) in the sulphate (S) and glucuronide (G) fractions of urine. Internal standards pregnenolone and pregnenolone sulphate were added to separate aliquots of urine and the steroid conjugates isolated and cleaved separately. The steroid extracts were purified by alumina column chromatography, then the steroids were separated and quantitated by gas-liquid chromatography as their trimethylsilyl either derivatives.

Absolute and relative recoveries for the method were satisfactory. The method was specific and reproducible and sufficiently sensitive to determine 0.1 µgm of steroid/25 ml of urine.

2. Thirty-seven normal males (22) and females (15) were studied. Their urinary steroid levels (mg/24 hr) were:-

	Males Mean ± S.D.	Females Mean ± S.D.	P value
epiA.S	0.30 ± 0.33	0.05 ± 0.05	0.0008
DHA.S	2.75 ± 3.75	0.45 ± 0.99	0.0008
E.S	0.23 ± 0.21	0.16 ± 0.15	0.2
A.S	0.82 ± 0.84	0.16 ± 0.15	0.00006
DHA.G	0.37 ± 0.36	0.27 ± 0.52	0.044
E.G	2.61 ± 1.36	1.12 ± 0.82	0.0008
A.G	3.05 ± 1.89	0.87 ± 0.69	0.00002

The above values for males compare favourably with those

for other investigators whereas those for the females are low. The study revealed hitherto unrecognised correlations between individual urinary D.17-OS. Therefore, understandably, the urinary excretion of steroid conjugates showed definite patterns:-

males - DHA.S>A.S>epiA.S>E.S

- A.G>E.G>DHA.G

females - E.G>A.G>DHA.G

The E.S/A.S, E.G/A.G and E.(S+G)/A.(S+G) ratios were significantly higher for females. Possible reasons for the above differences were discussed.

3. Thirty-two hypertensive males (15) and females (17) were studied. The hypertensive males excreted significantly less epiA.S ($P = 0.056$), DHA.S ($P = 0.067$) and A.G ($P = 0.028$), than normal controls. They also showed significantly higher ratios for epiA.S/A.S ($P = 0.0008$), DHA.S/E.S ($P = 0.0001$), A.S/E.S ($P = 0.0004$), E.G/A.G ($P = 0.00004$).

As with normal males the individual D.17-OS were highly intercorrelated, but no set pattern for steroid sulphate excretion arose, that for glucuronide conjugates was E.G>A.G>DHA.G.

The urinary steroid levels for the hypertensive females showed no significant differences compared with normal controls.

On the basis of the present results together with those for previous investigations a theory to explain the abnormal urinary D.17-OS patterns in hypertension is presented. The theory also

attempts to accommodate the other steroid abnormalities presented in hypertension.

4. Incubation studies on the effect of DHA on steroid hydroxylation in human adrenal tissue demonstrated an inhibitory action by DHA. ^{14}C-progesterone and ^{3}H-DOC were metabolised to a much lesser extent by incubations containing DHA than those without. The results supported the increased steroid hydroxylation found in hypertension.

5. Studies on the effect of varying salt intake on the excretion of D.17-OS conjugates were carried out. The results of 4 of the 6 subjects demonstrated a relationship between salt and D.17-OS metabolism. One subject showed a marked increase in D.17-OS excretion after increasing his salt intake and in 2 other subjects there was a very dramatic drop in D.17-OS sulphate excretion on decreasing their salt intake. The hypertensive female showed significant decreases in the excretion of D.17-OS sulphates after decreasing her salt intake and significant increases after increasing her salt intake.

Intravenous saline infusion resulted in increased salt excretion concomitant with increased excretion of D.17-OS.

These results were interpreted in terms of a "salt-losing" steroid and its possible role in hypertensive disease.

6. Seven gouty males were studies. Their urinary D.17-OS sulphate levels were remarkably low relative to normal controls (epiA.S P = 0.0002, DHA.S P = 0.001, E.S P = 0.066, A.S P = 0.0002). The glucuronide levels were also low (DHA.G P = 0.001, E.G P = 0.028, A.G P = 0.024). Complete discrimination from normals was obtained using the variables epiA.S, A.S and total sulphates.

A common factor between the low D.17-OS Levels found in gout and in other diseases was sought. Hyperuricaemia, obesity and hyperlipidaemia, all characteristics of gout, were considered and hyperlipidaemia favoured.

7. Thirteen normolipidaemic and 7 hyperlipidaemic males were studied. The urinary D.17-OS sulphate levels were significantly lower for the hyperlipidaemic group (epiA.S P = 0.05, DHA.S P = 0.1, E.S = 0.07, A.S P = 0.08). Of the glucuronide conjugates only A.G was significantly lower (P = 0.08). But for 1 normolipidaemic individual, complete discrimination between the two groups was achieved, using the variables epiA.S, A.S and A.(S+G). For the hyperlipidaemic group there was significant negative correlation between plasma glyceride levels and certain urinary D.17-OS levels.

The results were considered as evidence for a D.17-OS-hyperlipidaemic relationship. As confirmatory evidence of this relationship a study of D.17-OS levels in myocardial infarction was carried out, since this is a disease characterised by a high incidence of hyperlipidaemia.

8. Fifteen male myocardial infarct patients were studied. Their urinary D.17-OS sulphate levels were remarkably low relative to normal controls (epiA.S P = 0.001, DHA.S P = 0.01, E.S P = 0.52, A.S P = 0.003). Their glucuronide levels were also low (DHA.G P = 0.19, E.G P = 0.07, A.G P = 0.001) and the urinary excretion pattern of their glucuronides was E>A>DHA. They also showed significantly higher ratios for E.S/A.S (P = 0.002), E.G/A.G (P = 0.014) and E.(S+G)/A.(S+G) (P = 0.002).

The results supported a D.17-OS-hyperlipidaemic relationship. A theory based on the inhibitory action of D.17-OS on glucose-6-phosphate dehydrogenase is proposed to explain the relationship.

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