THE POSSIBLE SIGNIFICANCE OF DEHYDROEPIANDROSTERONE IN CYSTIC FIBROSIS

BY

CHRISTINA E. GRAY, B.Sc..

Thesis submitted to the University of Glasgow
for the degree of
Doctor of Philosophy.
April 1973.

CONTENTS

PREFACE

The introduction to this work is necessarily lengthy. It is partly clinical, being a review of the disease that is cystic fibrosis, and partly biochemical, being an exposition of possible endocrine aspects of the disease.

The biochemical investigations on children with cystic fibrosis were only made possible by the generous laboratory facilities made available by Professor J.H. Hutchison in the University Department of Child Health, Royal Hospital for Sick Children, Glasgow, and also by the co-operation of patients and fully-informed parents attending the hospital. I am indebted to Professor J.H. Hutchison, Dr. Shanks and Dr. W. Hamilton in whose care were the patients who co-operated in this study.

The advice, guidance and encouragement of Dr. W. Hamilton has been unfailing and is greatly appreciated. I am also grateful to Professor R.M.S. Smellie for his interest and encouragement. Valuable experience in the science and art of gas liquid chromatography was gained under the supervision of Dr. T. Simpson, Torry Research Station, Aberdeen.

The /

The expertise of the typist, Mrs. J. Macdonald, aided the smooth progression of this work and the final appearance of the volume has been greatly enhanced by my mother, Mrs. J. Gray, who extended her hobby to the production of the many photographs within this thesis.

The technical skills of Miss M. Thomson and formerly of Mrs. L. Judge were invaluable in the initial purification of urine and plasma extracts. Gifts of steroids were gratefully accepted from Professor W. Klyne, Honorary Curator of the M.R.C. Steroid Reference Collection.

The project was financed by a grant awarded to Dr. W. Hamilton by the Cystic Fibrosis Research Trust. Parts of the reported work were presented at the ninth annual meeting of the European Society for Paediatric Endocrinology, Lyon, 1970 and at the first annual meeting of the European Working Group for Cystic Fibrosis, Stockholm, 1970.

STEROID NOMENCLATURE

To aid fluency, trivial names of steroids have been adopted throughout this thesis. Those trivial names used are listed below together with the approved systematic name according to the IUPAC - IUB 1967 revised tentative rules for steroid nomenclature (Biochem. J. (1969), 113, 5).

TRIVIAL NAME	SYSTEMATIC NAME
Aetiocholanolone	3α-hydroxy-5β-androstan-17-one
Aetiocholanolone glucuronoside	3α-hydroxy-5β-androstan-17-one 3-glucuronoside
Aetiocholanolone heptafluorobutyrate	3α-hydroxy-5β-androstan-17-one 3-heptafluoro-butyrate
Aetiocholanolone sulphate	3α-hydroxy-5β-androstan-17-one 3-sulphate
Δ^5-Androstenediol	5-androstene-3β, 17β-diol
Androstenedione	4-androstene-3, 17-dione
Androsterone	3α-hydroxy-5α-androstan-17-one
Androsterone glucuronoside	3α-hydroxy-5α-androstan-17-one 3-glucuronoside
Androsterone heptafluorobutyrate	3α-hydroxy-5α-androstan-17-one 3-heptafluoro-butyrate
Androsterone sulphate	3α-hydroxy-5α-androstan-17-one 3-sulphate
Cholesterol	5-cholestene-3β-ol
Cortisone /	

Cortisone	17α, 21-dihydroxy-4-pregnene-3, 11, 20-trione
Dehydroepiandrosterone	3β-hydroxy-5-androstene-17-one
Dehydroepiandrosterone glucuronoside	3β-hydroxy-5-androstene-17-one 3-glucuronoside
Dehydroepiandrosterone heptafluorobutyrate	3β-hydroxy-5-androstene-17-one 3-heptafluorobutyrate
Dehydroepiandrosterone sulphate	3β-hydroxy-5-androstene-17-one 3-sulphate
Epiandrosterone	3β-hydroxy-5α-androstan-17-one
Epiandrosterone heptafluorobutyrate	3β-hydroxy-5α-androstan-17-one 3-heptafluorobutyrate
Epitestosterone heptafluorobutyrate	17α-hydroxy-4-androstene-3-one 3,17-heptafluorobutyrate
11β-Hydroxyaetiocholanolone	3α, 11β-dihydroxy-5β-androstan-17-one
11β-Hydroxyandrosterone	3α, 11β-dihydroxy-5α-androstan-17-one
17α-Hydroxypregnenolone	3β, 17α-dihydroxy-5-pregnene-20-one
17α-Hydroxypregnenolone sulphate	3β, 17α-dihydroxy-5-pregnene-20-one 3-sulphate
17α-Hydroxyprogesterone	17α-hydroxy-4-pregnene-3, 20-dione
11-Ketoaetiocholanolone	3α-hydroxy-5β-androstan-11, 17-dione
11-Ketoandrosterone	3α-hydroxy-5α-androstan-11, 17-dione
Oestrone /	

Oestrone	3β-hydroxy-1,3,5 (10)-estratrien-17-one
Pregnanediol	5β-pregnane-3α, 20α-diol
Pregnaretriol	5β-pregnane-3α, 17α, 20α-triol
Pregnenolone	3β-hydroxy-5-pregnene-20-one
Pregnenolone sulphate	3β-hydroxy-5-pregnene-20-one 3-sulphate
Progesterone	4-pregnene-3, 20-dione
Testosterone	17β-hydroxy-4-androstene-3-one
Testosterone mono-heptafluorobutyrate	17β-hydroxy-4-androstene-3-one 17-heptafluorobutyrate
Testosterone di-heptafluorobutyrate	17β-hydroxy-4-androstene-3-one 3,17-heptafluorobutyrate
Δ^4-3-oxosteroids	Steroids having a 3-oxo group and a double bond between C4 and C5
Δ^5-3β-hydroxysteroids	Steroids having a 3β-hydroxyl group and a double bond between C5 and C6
Oxymetholone	17β-hydroxy-17α-methyl-2-hydroxymethylene-5α-androstan-3-one
Ethyl estrenol	17β-hydroxy-17α-ethyl-4-androstene

CHAPTER ONE

INTRODUCTION

Historical Review

Cystic fibrosis of the pancreas is now recognised as probably the most frequent of inherited diseases to affect Caucasian populations. Its lethal nature is well known and it has been described as the most recently recognised of the major chronic diseases of man.

In the early part of this century various clinical facets of the disease seemed to justify the diagnosis of coeliac disease. At this time there was scant information concerning the pathology of the coeliac syndrome and certainly no uniform opinion regarding pathogenesis. Several writers (Garrod and Hurtley (1912); Miller and Perkins (1920); Clarke and Hadfield (1924); Parmelee (1935)) had recognised, in some patients with 'congenital steatorrhea', a clinica course differing from that of coeliac disease. These children presented with large fatty stools, failure to gain weight and a "digestive" disturbance despite dietary management. Nearly all deaths were the result of broncho-pneumonia. Reported as "cystic fibrosis of the pancreas" for the first time by Fanconi of Zurich in 1936, this disease was not differentiated as a distinct disorder from coeliac disease by clinicians until the extensive work of Andersen, Blackfan, May and Harper in 1938.

Andersen /

Andersen recognised a familial pancreatic steatorrhea which was readily distinguishable from idiopathic steatorrhea by the low percentage of split fat in the former as compared to the normal percentage characterising the latter. A study of the clinical histories of forty nine cases on which post-mortem investigation was carried out seemed to show three pathologies. Death in newborn infants was often the result of intestinal obstruction due to inspissated meconium. The pancreas showed extensive fibrosis with cyst formation. Infants dying before the age of six months had either predominantly respiratory or predominantly nutritional difficulties while children of an older age, presenting with symptoms resembling those of coeliac disease, succumbed to chronic respiratory infection. The differences in the expression of the disease were already noted in this first comprehensive report for Andersen observed that some cases were largely troubled by nutritional disturbances and others by infection of the respiratory tract. Microscopic examination of post-mortem tissues from these patients revealed bronchitis in all cases with the bronchial lumina filled with purulent exudate (Fig. 1). The epithelium was often multilayered with squamous metaplasia and keratinization of the superficial layers. Staphylococc (mainly Staphylococcus aureus) were present in the necrotic centres and alveoli showed patchy lobular pneumonia and emphysema (Fig. 2).

The /

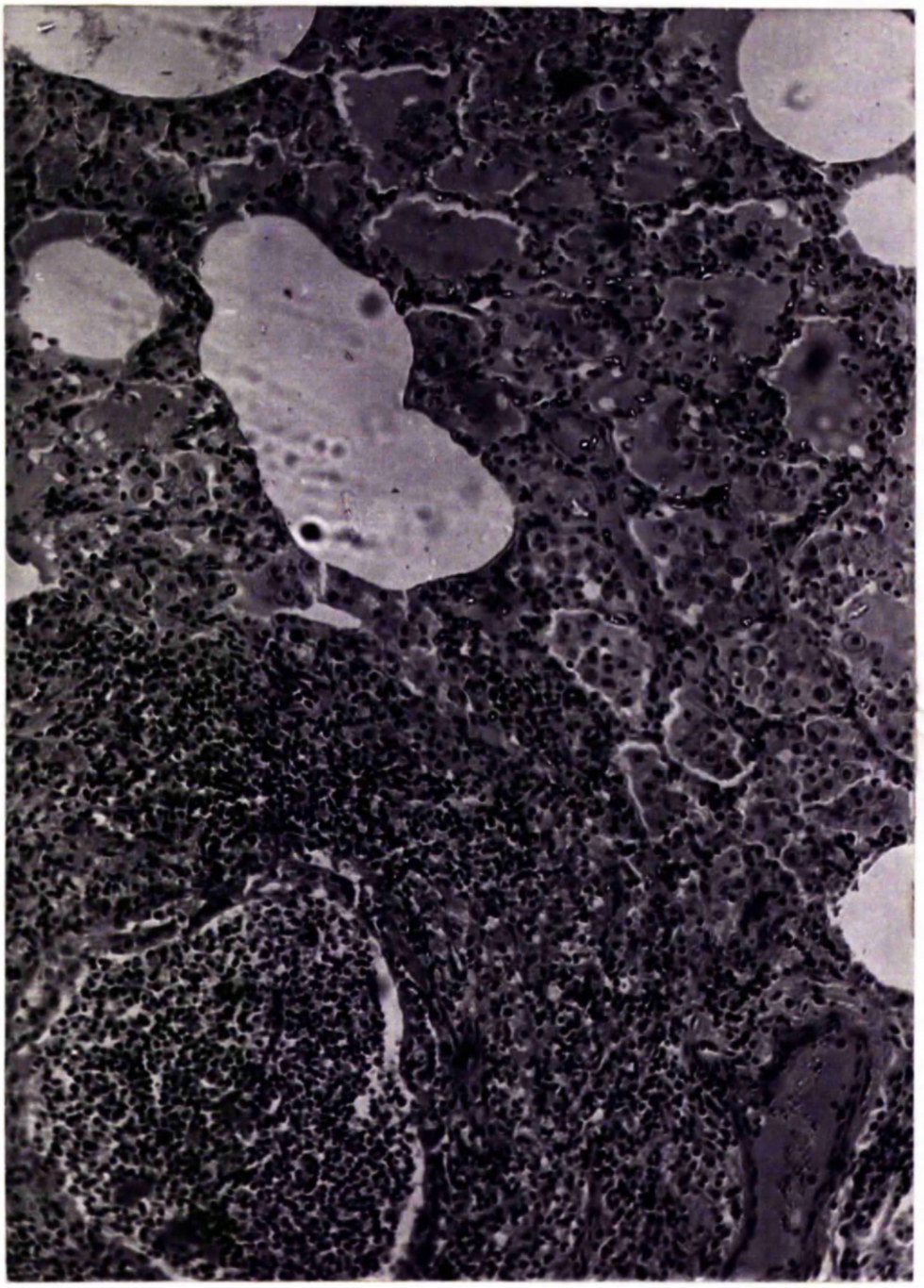


Figure 1. Section of lung from a case of cystic fibrosis showing muco-purulent exudate within bronchioles and alveoli with emphysema of adjacent air spaces.

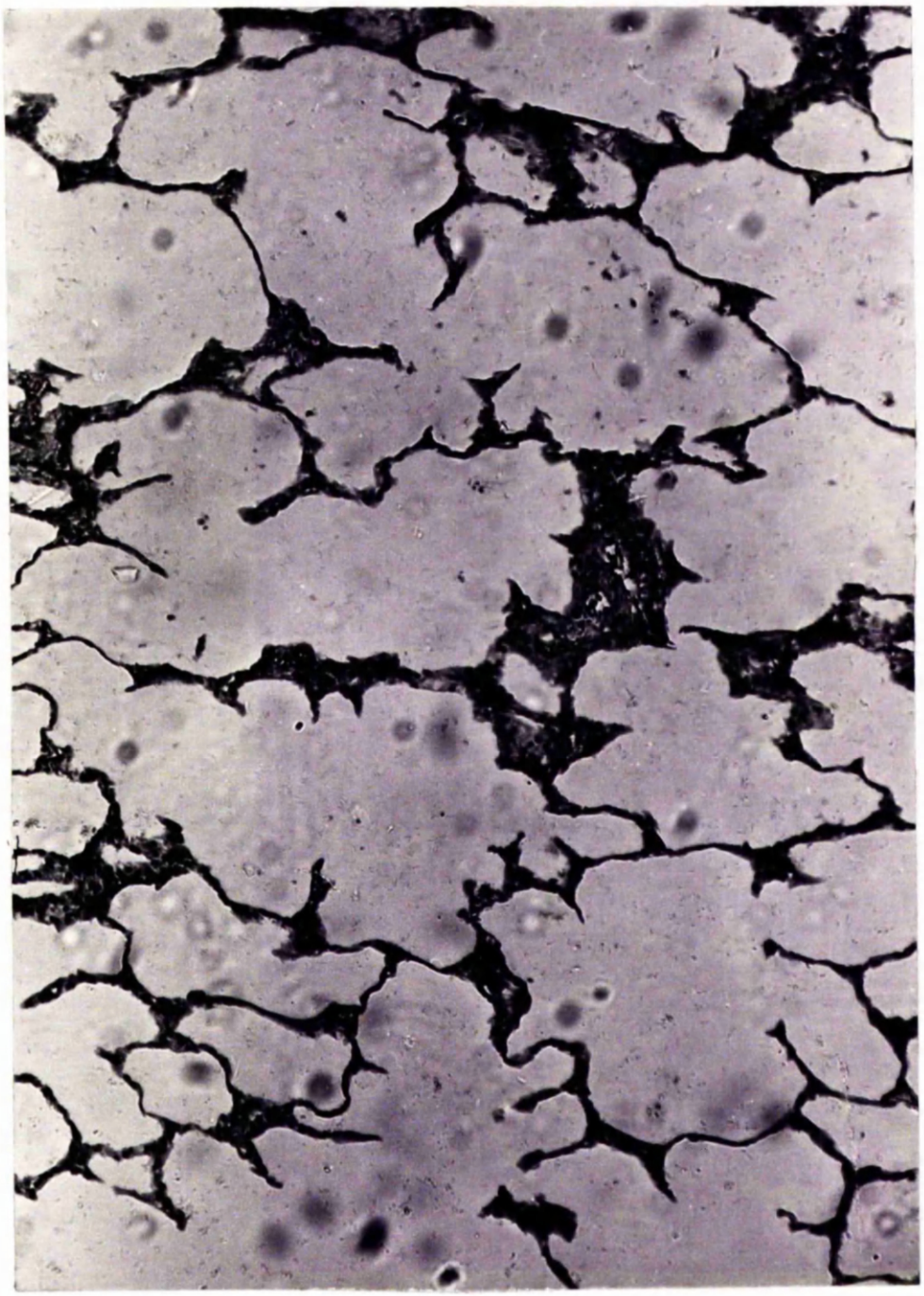


Figure 2. Extensive emphysema from a case of cystic fibrosis.

The microscopic structure of the pancreas was that described by the term "cystic fibrosis" - fibrous tissue encroaching on the exocrine areas of the gland and cyst formation within the acinar areas. In these early reports islets of Langerhans did not appear to be destroyed by the changes taking place within the gland (Fig. 3).

An animal model has often been a useful tool in the investigation of disease and the counterpart of cystic fibrosis has been unsuccessfully sought. However, experiments on dogs by Ivy (1936) and Greenberg (1933) on the exclusion of pancreatic juice from the intestine by ligation of the pancreatic duct or the creation of an external fistula, created a clinical picture closely resembling that of the digestive aspect of cystic fibrosis of the pancreas. Survival and weight gain were maintained by feeding either a normal diet with high vitamin content or raw pancreas. Here was the rationale for the therapeutic administration of pancreatic extracts, in the form of Pancreatin, to patients with this disease. This recommendation was first made by Andersen in 1939.

Vitamin A deficiency was considered as a possible aetiological factor in Andersen's initial report. However, she concluded that a deficiency of such fat-soluble vitamins may be due to the inadequate absorption of lipid from the alimentary tract. In 1942 Andersen proposed the most reliable means of diagnosis of pancreatic deficiency /

Figure 3. Section of pancreas from a case of cystic fibrosis demonstrating fibrosis, cyst formation and atrophy of the exocrine areas of the gland. Surviving islets of Langerhans are clearly seen.

deficiency to be the assay of trypsin in duodenal juice. Farber et al. in 1943 reported a smaller volume and altered viscosity of duodenal juice in patients as compared with normal controls. There was also a marked reduction in tryptic, lipolytic and amylolytic activity. After a number of years it became evident that complete pancreatic achylia was not necessarily a requirement for the diagnosis of this disease.

Ideas of treatment for cystic fibrosis have followed the trend of thought on aetiology. Dietary therapy begun after the onset of respiratory infection appeared to prolong life but when the infection was well established such treatment was only occasionally effective (di Sant' Agnese and Andersen, 1946). Once established, pulmonary infection increased rapidly in severity. Digital clubbing, an index of chronic pulmonary disease, often became apparent. The sputum became thick and mucopurulent and most deaths resulted from asphyxia. Early stages of upper respiratory tract infections were at this time moderately successfully treated with sulphonamides. However, these had little effect once the bronchitic and cyanotic stages had been reached. Intramuscular administration of penicillin had improved some patients with staphylococcic pneumonia. Following the introducti of penicillin aerosol inhalation therapy by Bryson et al. (1944) and Barach /

Barach et al. (1945), di Sant' Agnese applied this means of administration to patients with cystic fibrosis. Those patients responding, often continued to improve after the drug was discontinued. However penicillin was the first drug known to affect the course of fibrocystic disease. Thus by 1946 management of the disease consisted of dietary control, sulphonamide administration during the stage of chronic cough and penicillin for the acute and often terminal suppurative bronchitis. Pathological changes in the respiratory system were well documented by di Sant' Agnese in 1953. Poor alveolar aeration, anoxia and carbon dioxide retention reflected the bronchial obstruction and obstructive emphysema characteristic of the disease.

As early as 1933 Wolbach had suggested that the loss of protective powers of the epithelium often seen in infants, was due to changes in mucus secretion and loss of ciliary motion. The abnormal mucus was implicated in the obstruction of acini and small and large ducts. Then followed cyst formation by pressure and subsequent degeneration of the parenchyma. In 1953 di Sant' Agnese considered this view adequate to account for the pancreatic lesions, the atresia of gall bladder and the fibrotic changes in the liver (Andersen and Hodges, 1946). Thus the concept of cystic fibrosis as a widespread disturbance of mucus secretion affecting many organs of the body was born. Farber (1944), the /

the chief proponent of this theory, coined the term "mucoviscidosis" and suggested that an autonomic imbalance might be responsible for the altered secretion. However histological changes in the pancreas and lung are far from similar and neither the hypothesis of nutritional deficiency nor the concept of viscid mucus explained completely the relationship between pancreatic and pulmonary lesions. The findings of a sweat electrolyte abnormality by di Sant' Agnese and co-workers in 1953 lent further support to the view that this was a generalised disease affecting more than one organ system irrespective of mucus production.

It had already been reported by Kessler and Andersen in 1951 that patients poorly tolerated long periods of high atmospheric temperature. A study of the sweat of these patients showed an elevate Na^+ and Cl^- concentration ($>$ 70 mEq/l) compared with values from normal children. The increase in concentration of these electrolytes in sweat is the most constant and clearly defined abnormality in cystic fibrosis and as such has been exploited for screening purposes. Estimation of sweat electrolytes following localised stimulation by iontophoresis of pilocarpine nitrate (Gibson and Cooke, 1958) is still generally accepted as the most accurate single confirmatory test for the diagnosis of cystic fibrosis. In conjunction with the first sweat studies, /

studies, it was shown that the levels of sodium and chloride in mixed saliva were also significantly higher than normal (di Sant' Agnese, 1953), although the overlap was too great to be of practical value. Thereafter many reports, often with conflicting results, were made concerning the composition of secretions from various exocrine glands.

In 1958 di Sant' Agnese et al. reported a slight increase in the sodium chloride concentration of tears from patients with cystic fibrosis but this finding was not confirmed in a report by Shwachman and Antoniwicz (1962) on observations made by Khaw.

Although the ionic content of mixed saliva was abnormal, further investigation indicated that the defect was expressed differently in the parotid and submaxillary glands. Johnston (1956) and Barbero and Chernick (1958) found an increased rate of parotid secretion in patients and that sodium and chloride concentrations therein were significantly higher than in normal controls. There was however considerable overlap of values. The submaxillary gland on the contrary had a depressed rate of secretion with apparently normal electrolyte concentrations (Chernick et al. 1961).

Thus, since the first description of cystic fibrosis as a disease entity in the late 1930's, an evolution has taken place in its clinical description as a result of an accumulation of clinical and research experience.

Treatment is therefore symptomatic since the basic defect is as /

as yet speculative. Prophylactic administration of drugs to allay pulmonary infection, the decisive factor in determining the mortality of the disease, has led to an increased longevity among patients, albeit posing its own problems. Growth retardation occurs throughout childhood and delayed sexual development is common. Adult females can reproduce while aspermia has been an almost constant finding in males (Kaplan et al. 1968). This latter is a newly described phenomenon. Anatomical defects have been detected in the vas deferens, epididymis and seminal vesicles at post-mortem examinations and during surgical explorations of the area. With increased life span the progressive fibrotic and cystic changes in the pancreas often also affect the endocrine component of the gland resulting in classic diabetes mellitus. The incidence of diabetes in affected children has been reported as being slightly higher than in the general population (Rosan et al. 1962). Milner (1969) believed that the observed increased incidence of impaired glucose tolerance was due to a defect in the release of a glucogon-like substance from the alimentary system, in addition to impaired islet cell function. In a much larger group of patients studied by Handwerger et al. (1969) there were nevertheless individuals presenting with classical symptoms.

Now the generalised nature of cystic fibrosis is well demonstrated by its varied clinical features, most notably progressive bronchiolar obstruction /

obstruction and pulmonary infection leading to atelectasis, hæmoptysis and cor pulmonale, steatorrhea and fat soluble vitamin deficiency, malnutrition and growth failure. Meconium ileus is indicative of the condition. Biliary cirrhosis occurs occasionally giving rise to portal hypertension, hypersplenism and gastrointestinal haemorrhage, (di Sant' Agnese and Blanc, 1956). Sweating can lead to salt depletion. The prognosis is poor particularly due to pulmonary involvement and treatment is but symptomatic. Relatively few patients survive to adulthood. These symptoms of the disease must however be regarded as complications since the basic defect of cystic fibrosis still eludes the investigators.

Aetiological Considerations

The pathogenesis of cystic fibrosis with its many and diverse manifestations is still unknown since the basic genetic defect has not yet been determined. That cystic fibrosis of the pancreas or fibrocystic disease are unsatisfactory names for this disorder is well recognised, but even the physiological terms such as mucosis and mucoviscidosis fail to embrace all aspects of the disease. The changing nomenclature reflected the increased awareness of the varying expressions of the disease together with the desire to link them to a common aetiology. Early workers inclined to the belief that /

that all features of cystic fibrosis could be explained as direct or indirect consequences of pancreatic achylia, that deficient fat absorption from the gut led to vitamin A deficiency which was in turn held responsible for lesions of the respiratory tract. Other authorities upheld a generalised abnormality of mucus secretion as the basic harmful process, hence the terms mucosis and mucoviscidosis. The disease is inherited as a Mendelian recessive characteristic and is thus expressed in the homozygous state. It is truly an inborn error of metabolism. The several, apparently unrelated, abnormalities characterising the disease indicate that they are not themselves the primary defect but are several steps away from the primary molecular abnormality. Any unifying concept would require to explain the abnormality of mucous secretions, the striking electrolyte changes in sweat and to a lesser extent in saliva, the increase in organic secretions as well as calcium in submaxillary saliva, the abnormal serum factor reported by Spock et al. (1967) and the parotid sodium-reabsorption-inhibitory-sweat factor of Mangos and McSherry (1967).

ABNORMALITIES IN MUCUS PRODUCTION.

Although the exocrine glands which produce mainly serous secretions (the eccrine sweat glands and parotid glands) show no gross or /

or histological change, mucus-producing glands throughout the body may show varying degrees of morphological change. The most serious manifestation of the disease is the accumulation of the apparently abnormal "insoluble" secretions responsible for obstruction and resulting in such pathological changes and clinical manifestations as pancreatic fibrosis and achylia, bronchial obstruction, submaxillary enlargement and hepatic cirrhosis. The involvement of the mucus secreting cells in the oral, respiratory and alimentary tracts in cystic fibrosis is well recognised (see Fig. 4 - hypertrophied Brunner's glands) and recent reports by Oppenheimer (1970) and Oppenheimer and Esterly (1970) implicate hypersecretion of uterine cervical mucus in the decreased fertility observed in female patients.

Structure of Mucus.

The mucins are glycoproteins rich in carbohydrate units and are mostly responsible for the viscosity of these mucous exocrine secretions. The core is proteinous, rich in serine, threonine, glycine, alanine and proline. Carbohydrates are present as oligosaccharide units, in some cases as small as disaccharides. These are attached as numerous side-chains from the protein core as a glycosidic linkage from a hexosamine to the hydroxyl group of the serine and threonine. The di- and oligosaccharides are composed of glucosamine, /

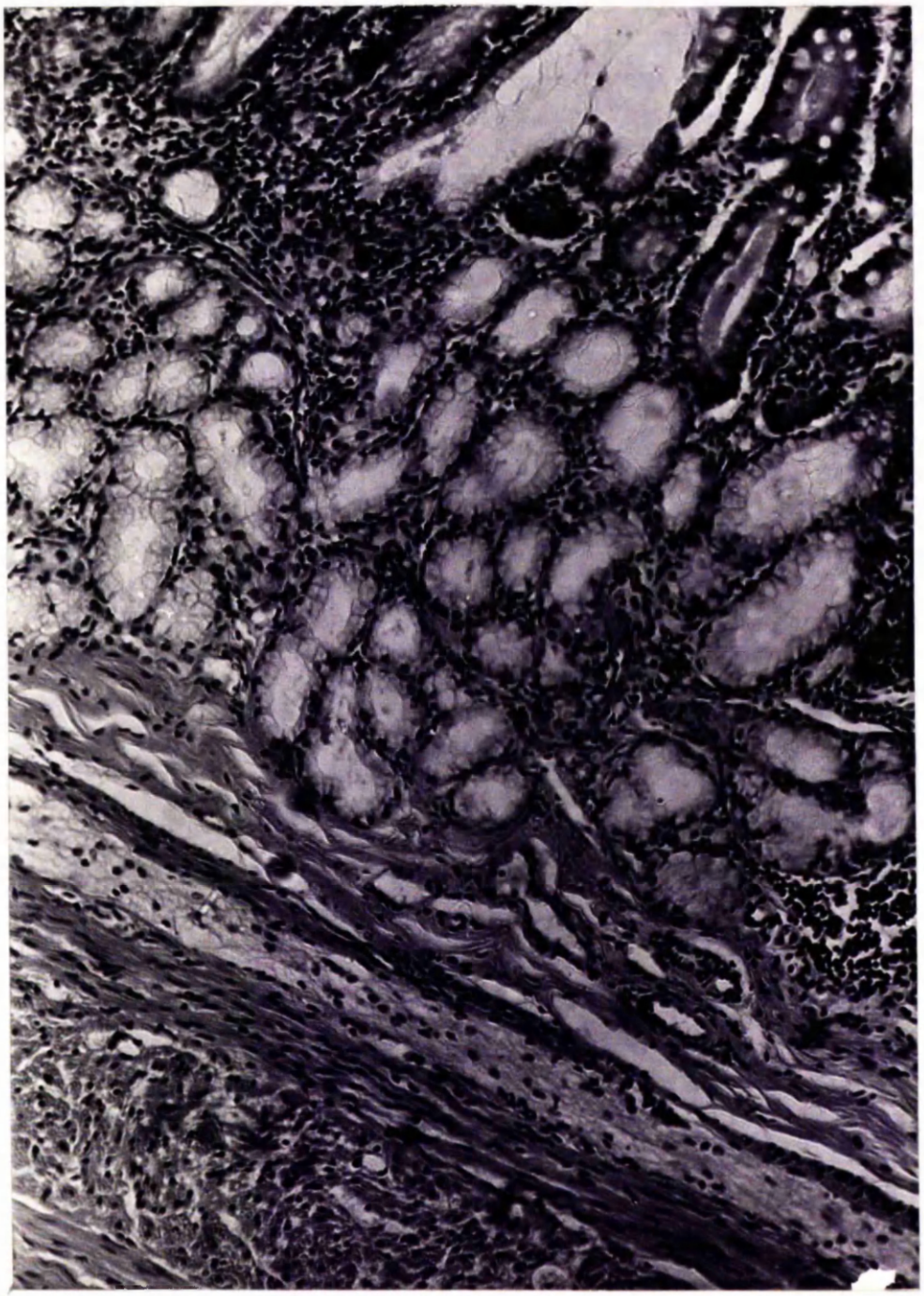


Figure 4. Section of duodenum from a case of cystic fibrosis showing hypertrophy of the Brunner's glands.

glucosamine, galactosamine, D-galactose, sialic acids and L-fucose. The sialic acids or fucose units are the terminal groups. Each tissue apparently has its own characteristic carbohydrate units though the protein core is of similar composition. Thus it is to be expected that each type of cell of the oral, respiratory, gastro-intestinal and reproductive tract secretes mucins of differing compositions The known mucins all have a specificity associated with some blood group.

Chemical and Immunochemical Studies.

No serious attempts at investigation of glycoproteins in cystic fibrosis were made until 1957. At first, attention was focussed on the suggestion of Dische et al. (1959) that the primary alteration in the mucous secretion in this disease might be a quantitative change in the glycoprotein fractions in the form of an increase in fucose in the carbohydrate moiety with a consequent change in the fucose/sialic acid ratio. The first studies by di Sant' Agnese et al. in 1957 had indeed demonstrated that after the treatment of duodenal fluid with ethanol/benzene, a water-insoluble precipitate, unaffected by trypsin and in which the fucose/sialic acid ratio was raised, was present in most patients with cystic fibrosis. Dische postulated that such an abnormal shift in the sugar components of the glycoprotein molecule /

molecule might have led to decreased solubility resulting in widespread obstructive changes. In recent years glycoproteins from glands of patients with cystic fibrosis have been much studied in the hope that the fundamental enzyme abnormality might be identified. Indeed many of the subsequent studies supported these early investigations. A similar increase in fucose/sialic acid ratio was found in urine (Dische 1961), submaxillary saliva (Chomick and Barbero 1963; Dische 1970), sweat (Pallavicini et al. 1963) and rectal mucus (Roelfs et al. 1967). Conflict has arisen in analysis of tracheobronchial secretions. Johansen (1963) reported an elevated fucose content in relation to sialic acid whilst Potter et al. (1963) found consistently elevated sialic acid and decreased fucose levels. Although it now seems that such chemical analyses will not reveal the underlying abnormality, nevertheless the production of mucus is still of prime importance to the clinical status of the patient. Considering the insolubility of mucus in this disease, it is well recognised that elevated concentrations of calcium in the submaxillary gland are associated with the turbidity of its secretions (Chernick and Barbero 1961). In his own studies Pallavicini (1970) has noted that with the addition of small amounts of calcium, submaxillary saliva of normal subjects became turbid and gave an electrophoretic pattern resembling that obtained from /

from cystic fibrosis patients. Addition of EDTA to both calcium-treated and patients' saliva removed the turbidity and produced similar electrophoretic patterns.

Glycoproteins may be regarded as highly charged anionic particles exerting their forces by forming intermolecular calcium bridges. In this process of aggregation calcium seems to play an important linking role (Boat 1970). Experimental evidence would also seem to implicate disulphide linkages in mucins as among the factors contributing to their insolubility. It is accepted that mucus is a complex chemical substance. Human mucins have not been well studied chemically. Nonetheless, except for insolubility, no difference have been observed in secretions from cystic fibrosis patients and controls. Pallavicini (1963) had investigated carbohydrates in the sweat of cystic fibrosis patients and in normal subjects but could demonstrate no immunological differences. Johansen (1970) using histochemical techniques and autoradiography, studied mucus production in rectal biopsies from forty patients with cystic fibrosis and from normal controls. No consistent differences in the mucus glands were observed. Likewise exhaustive histochemical examination of rectal mucus failed to reveal any abnormality in the cells of the patients.

Extensive investigations have been made on urinary glycoproteins - particularly on the Tamm-Horsfall urinary glycoprotein. These studies were intended as a logical model system of structural abnormalities

of /

of glycoproteins in cystic fibrosis since this glycoprotein is easily separable by methods which do not destroy its chemical or immunological properties. Investigations by Maxfield and Wolins (1962) indicated that the Tamm-Horsfall glycoprotein was present in an abnormally aggregated state in the urine of cystic fibrosis patients. However, subsequent intensive studies have failed to substantiate this finding. Friedmann and Johnson (1967) by ultracentrifugation, starch-gel electrophoresis, assessment of amino acid composition, immunodiffusion and haemagglutination inhibition techniques could demonstrate no difference between patients with the disease and control subjects. Neuberger (1970) also reported no gross difference in the chemical structure or sub-unit structure in this glycoprotein in the urine of patients with cystic fibrosis.

The viscosity of mucus secretions is thought to be caused by mucins with a fibrillar structure. Destruction is effected by mild cystein reduction, and ultimate analysis of bronchial mucus by electrophoresis has demonstrated the presence of proteins, blood group substances acid glycoproteins (sialic acid or sulphate) and nucleic acids. In chronic pulmonary disease such as asthma and bronchitis an increase of the acid glycoprotein fraction has been recorded. Human

tracheal /

tracheal mucosa from pathological conditions in incubation studies using ^{35}S synthesise sulphated acid glycoprotein in excess of the sialic acid fraction. Reid and de Haller (1967) have described the changing patterns of secretion of the sulpho- and sialomucins in children. In the foetus and neonate there is an increase in sulphomucin production while after a few months of life there is a shift to the secretion of sialomucin.

Before pulmonary infection, cystic fibrosis patients show normal secretion patterns for their age but once infection is established sulphomucin production increases to the point of eliminating sialomucin. This swing in production cannot be attributed to hypertrophy of the gland alone since in patients with chronic bronchitis the glands are enlarged but the shift to sulphomucin is not so complete. Reid (1967) has also reported the biochemical analysis of sputum by Keal and Sturgess which showed that in cystic fibrosis cases there was a higher sulphate and lower sialic acid level than in chronic bronchitics.

Localization of Mucins.

Histological quantitation of sulphomucins by staining procedures presents problems. A careful correlation between stains and uptake of radioactive sulphate is necessary prior to histochemical analyses (Reid 1967; Spicer 1970). Often stains for sulphate only /

only identify heavily sulphated compounds. Spicer (1970) investigating sections of pancreas showing the classical changes associated with cystic fibrosis, concluded that in this disease the zymogenic epithelium is lost and is replaced by mucigenic epithelium which is normally only present as a lining of ducts but in cystic fibrosis also lines the cystic spaces. This epithelium appeared to be hypersecretory, the secretions having an increased content of sialo- and sulphomucins. Patients dying early as a result of pulmonary disease but with a mild pancreatic lesion, had epithelium normal in type and distribution but had also large amounts of insoluble concretions in the lumina of the ducts. This precipitation of zymogen secretion is believed to result in the obstruction and dilatation of ducts typical of the disease.

Mucopolysaccharide Metabolism.

Abnormal mucopolysaccharide metabolism, as an aetiological factor, has been popularised by Danes and Bearn (1968, 1969). Fibroblasts and later white cells, cultured from cystic fibrosis patients and from some parents, showed metachromatic staining with toluidine blue for acid mucopolysaccharide. It was hoped that this observation could act as a cell marker for the identification of the homozygote cases and perhaps also for the heterozygote carriers of the disease. This recognition of cytoplasmic abnormality might then facilitate genetic /

genetic and biochemical studies. It was reported that fibroblasts from patients cultured in a medium containing radioactive sulphate and acetate accumulated excess radioactive mucopolysaccharides. These observations had also been made on fibroblasts from patients with mucopolysaccharidosis (Hurler and Hunter syndromes). Thus it was felt that the primary defect in cystic fibrosis might also be a disturbance of mucopolysaccharide metabolism. Unfortunately the results of Danes and Bearn have not been wholly confirmed. Wiesmann and Neufeld (1970) studying the turnover of sulphated mucopolysaccharide in fibroblasts from thirteen patients with cystic fibrosis failed to show an accumulation of radioactive sulphate.

However, Brown et al. (1970) demonstrated that the mean uptake of ^{35}S by cells from a group of fibrocystic patients was significantly greater than the corresponding mean of a group of control patients even although there was considerable overlap. Sulphate uptake rates of parents were indistinguishable from controls. Reed et al. (1970) also studied metachromasia as an in-vitro model for the disease and were indeed able to demonstrate an increase within the fibroblasts cultured from patients. However, the amount of metachromasia was found to be dependent on culture conditions and varied from one subculture to another even from the same patient. Such diverse observations obviously detract from the possible potential of tissue culture for diagnosing /

diagnosing the disease.

Rheology of Mucus.

The clearance of mucus from the bronchial tree is of prime importance in cystic fibrosis and the physical nature of the secretion is a critical factor in the process. Viscosity and elasticity are the two major physical characteristics determining the rheological or flow properties. The production of excessive quantities of bronchial secretion, sufficient to cause sputum production, is characteristic of other chronic pulmonary diseases such as chronic bronchitis and bronchiectasis. Airways obstruction and progressive pulmonary involvement in cystic fibrosis have been attributed to the abnormal characteristics of the bronchial secretion (Farber 1944). Sturgess and Reid (1969) and Feather and Russell (1970) have compared the viscosity of sputum by shearing methods in these disease states. Mucoid sputum from cystic fibrosis patients was not found to be more viscous than in the other conditions. Viscosity increases with purulence and so changes appear to be associated with infection rather than with an abnormal mucus production. Also quantitative changes in bronchial secretion can be attributed to the pus formation. The clinical pulmonary problems of the disease are related to the high incidence of Staphylococcus aureus infection so characteristic of the disease. /

disease. An increased level of p-hydroxyphenylacetic acid (May and Roberts, 1969) has been thought to favour colonisation by these organisms.

Attention has recently been given to Pseudomonas aeruginosa because there has been an increase in incidence of infection by this bacterium in cystic fibrosis patients. The organism is not normally encapsulated but in children with cystic fibrosis up to 70 per cent of Pseudomonas infections are caused by a mucoid encapsulated strain (Saggers, 1970). In post-mortem studies Doggett and Harrison (1969) have observed that the purulent material in the bronchi and pulmonary abscesses yields pure cultures of "mucoid" Pseudomonas aeruginosa. Such strains produce profuse viscous capsular material containing polysaccharide similar to alginic acid (the marine algal polysaccharide). The possible obstructive role of this capsular polysaccharide in the tracheobronchial tree cannot be ignored. Alginic acid is known to form viscous solutions at relatively low concentrations, to form gels and fibres, to behave as polyelectrolytes and to form films on surfaces thus controlling viscosity and water penetration (Percival and McDowell, 1967). The mucoid strains of this organism are difficult to control (no enzymes from higher animals and only a few from lower animals will degrade alginates). An increase in serum /

serum precipitins specific to mucoid Pseudomonas aeruginosa have been found in patients infected with this organism. Pseudomonas-specific precipitins could not be demonstrated in the sera of patients carrying rough strains of the species. Indeed, it has been noted that cystic fibrosis patients without specific precipitins or mucoid Pseudomonas in their sputa generally reflect a better clinical condition than those having high precipitins and mucoid Pseudomonas.

ELECTROLYTIC DEFECTS.

The most constant and characteristic abnormality expressed in patients with cystic fibrosis is still the increase of sodium and chloride in eccrine sweat first noted in 1953 by di Sant' Agnese et al. albeit relatively late in the history of the disease. These elevated electrolyte levels are also found, though to a much lesser extent, in submaxillary saliva but in other biological fluids the electrolytes are normal. Urinary electrolytes behave normally (di Sant' Agnese et al. 1962) and indeed it is the disassociation between the inability of the sweat gland to retain electrolytes and the preserved ability of the kidney to conserve sodium chloride that makes the sweat defect the unique feature of cystic fibrosis. Serum sodium, chloride and potassium levels are normal except in patients with severe pulmonary distress /

distress where impaired gaseous exchange gives rise to accumulation of carbon dioxide with a decrease in chloride. Massive salt depletion by excessive sweating may also lead to hypoelectrolytaemia.

Until the work of Balfe et al. (1968), it was considered that the sodium pump in erythrocytes of patients with cystic fibrosis was normal. However these workers detected defective transport across the membranes of red cells in homozygotes and also the heterozygote parents. They reported that the ouabain-sensitive adenosine triphosphatase is diminished in erythrocyte ghosts of cystic fibrosis patients and that the ouabain-sensitive and ethacrynic acid-sensitive efflux from erythrocytes of these patients are both decreased. The parents of affected children also demonstrated a reduced ethacrynic acid-sensitive sodium efflux. Gardner (1969) in Bethesda has confirmed that sodium outflux from the erythrocytes of patients is decreased in post-pubertal males and females. He was, however, unable to distinguish heterozygotes from normal individuals. The difficulties inherent to the interpretation of such studies are the differing rates of sodium transport observed during the life cycle of the red cell.

Faecal sodium and chloride levels have been reported as being normal (di Sant' Agnese et al. 1953) as have sodium, chloride and potassium concentrations in the duodenal contents (di Sant' Agnese et al. 1958). However investigation of secretion rates of fluid volume /

volume, protein and electrolytes by the pancreas after hormonal stimulation, using pancreozymin and secretin (2 I.U./Kg body wt. of each), has revealed a reduced fluid volume and electrolyte output (particularly bicarbonate) relative to the organic enzymic fraction produced by the pancreas (Hadorn et al. 1968; Zoppi et al. 1970).

Conflicting reports on comparable numbers of patients have been made on the sodium content of tears. di Sant' Agnese et al. in 1958 found elevated levels in their patients whereas normal concentrations were observed by Shwachman and Antonowicz in 1962.

Hope for a further diagnostic test was raised when Kopito et al. (1965) discovered sodium and potassium to be increased in nails of children with the disease. Neutron activation analyses of nail clippings have shown the test to be reliable for diagnosis, after the first month of life. In the first few days of life considerable overlap occurs between normal and cystic fibrosis patients, this probably being related to contamination of the nails with sodium chloride from amniotic fluid (Kollberg, 1971).

Sweat Gland Abnormality.

The sweat gland abnormality is present from birth, throughout life and is unrelated to the heterogeneity of the clinical manifestations of the disease. The sweat test therefore remains the cornerstone for the diagnosis of cystic fibrosis. The range in sweat electrolyte values for both affected children and normal controls is /

is quite broad but the overlap is minimal. Normally, chloride concentration is less than sodium but Lobeck (1966) has observed that in cystic fibrosis patients the chloride concentration approximates to the sodium levels because of the higher potassium concentration in these subjects. Therefore the chloride levels are less likely to overlap tho: of controls and may be more useful than sodium levels in diagnosis. Other diseases may be associated with elevation of sweat electrolytes but these are clinically distinct. High sodium or chloride levels have been reported for some patients with chronic pulmonary disease such as chronic bronchitis, bronchiectasis and emphysema. Addison's disease and congenital adrenal hyperplasia during salt depletion are well known to result in an increase in sweat electrolytes. There are also indications of elevated levels in patients with glucose-6-phosphatase deficiency (hepatorenal glycogen storage disease), vasopressin-resistant diabetes insipidus and familial ectodermal dysplasia though numbers reported are small. A recent report has also indicated elevated sweat chloride levels in mucopolysaccharidosis (Spock and Renuart, 1970). Lobeck and Huebner (1962) failed to demonstrate a difference in the rate of sweating between children with cystic fibrosis and control children.

As well as high electrolyte values in eccrine sweat there is also an inability to restrict loss under stressful conditions such as hot weather, /

weather, salt restriction and administration of steroids. Massive salt-loss and vascular collapse, as a result of failure to acclimatise to hot weather, was first noted by Kessler and Andersen (1951) in New York in 1948. Normally following administration of large doses of deoxycorticosterone acetate or 9α-fluorohydrocortisone high levels of sweat sodium are reduced to low levels. Children with cystic fibrosis fail to regulate on this treatment which is suggestive of sweat gland insensitivity to mineralocorticoids (Lobeck and McSherry 1963; Grand et al. 1967). After adequate doses of salt-retaining steroids sweat sodium levels remain high while on the other hand kidney retention of sodium is preserved. All aspects of adrenal function studied in cystic fibrosis patients have been normal (Chodos et al. 1965). Increased excretion of urinary 17-hydroxycorticosteroids has been demonstrated in response to ACTH administration. Secretion and excretion of aldosterone are normal as is the response to the metyrapone test used as an index of pituitary corticotrophin production. The degree of insensitivity to salt-retaining steroids is variable. Grand and colleagues (1967) were able to achieve significant decreases in sweat sodium levels following the administration of large doses of aldosterone though the response was not of the same order as shown by normal subjects.

Morphologically no difference has been found between the eccrine sweat /

sweat glands of patients with cystic fibrosis and those of normal children. Ultrastructural studies made by Munger et al. (1961), Landing et al. (1970) and Mangos and McSherry (1967) have revealed no anatomical variance between the two groups.

The composition of sweat is considered to be the elaboration at the base of the gland of a secretion (the precursor secretion) which is modified as it passes through the sweat duct on to the skin surface. It is believed that the precursor fluid secreted in the coil of the sweat gland is isotonic with plasma and is modified in the duct by reabsorption of sodium chloride in excess of water resulting normally in hypotonic sweat. Using micropuncture techniques (Schulz 1967) it has been demonstrated that in cystic fibrosis patients the newly formed sweat in the coil is isotonic with plasma. However, the final secretion is always significantly less hypotonic than in control subjects and this phenomenon has been attributed to defective reabsorption of sodium chloride in the ducts.

The human sweat gland duct has coiled proximal and straight distal components, differentiated not by cell type, but functionally regarding sodium transport. It would appear that in the distal portion, sodium is mainly exchanged for potassium, a process regulated by aldosterone. However, it is the coiled region that is normally /

normally responsible for most of the sodium reabsorption. Sodium and chloride ions and some water diffuse into the duct cell by a rate limited process. Grand et al. (1967) demonstrated some response after aldosterone administration and therefore the sweat gland defect in cystic fibrosis would seem to be located in the coiled region of the duct. Active transport of sodium does occur in the kidney, erythrocytes and sweat glands following aldosterone administration and it would be in accord with the theory of Gordon and Cage (1966) if the passive diffusion phase rather than active sodium transport were affected in patients with cystic fibrosis.

Salivary glands.

In conjunction with the first sweat studies, electrolytes in mixed saliva were analysed (di Sant' Agnese et al. 1953) and it was shown that sodium and chloride levels were significantly higher than normal. Controversy arose concerning the results obtained from the analysis on each of the component parts - the submaxillary and the parotid saliva. Some investigators reported an elevated secretory rate and increased sodium and chloride levels in the parotid gland while others found these parameters to be normal (Mandel et al. 1967). The secretion rate of the submaxillary gland has undisputedly been regarded as normal though discrepancies have been described in electrolyte studies. However, Mandel studying a large number of patients, /

patients, found values to be significantly elevated.

Most of the information concerning the elaboration of normal saliva is based on animal studies. Comparison of the structure and composition of the salivary secretions suggests that human glands are probably very similar (Mangos and Braun-Schubert, 1967) to those of animals. Micropuncture studies of the submaxillary glands of rats have established the existence of a gradient from isotonic or slightly hypertonic secretory fluid in the acini to a hypotonic secretion in the mouth. Although there are distinct differences in the structure of sweat and salivary gland excretory ducts, they are both regions of reabsorption of electrolytes by the active transport of sodium ions.

Pancreatic Function.

In 1968 in a study of pancreatic function on cystic fibrosis patients lacking the malabsorptive symptoms of the disease, Hadorn et al. (1968) observed an unusual secretion produced in response to pancreozymin and secretin stimulation (2 I.U./Kg body wt. of each). The scanty viscous secretion had a low bicarbonate ion content in contrast to the watery fluid with high bicarbonate levels found normally. Under normal conditions the final secretory product of the pancreas is a mixture of a small amount of concentrated enzyme solution (organic fraction) and a larger volume of secretion containing mainly /

mainly electrolytes and bicarbonate (electrolyte fraction). Secretion of the organic fraction can be stimulated by pancreozymin and is usually attributed to the acinar cells of the pancreas. Secretion of the electrolyte fraction is stimulated by secretin acting on the centro-acinar and intercalary duct cells. The abnormal pattern in cystic fibrosis is the decrease in the electrolyte fraction resulting in a final secretion largely organic in content. High enzyme concentrations reported are due to a reduction in the dilution of the organic phase. Total output of pancreatic enzymes are usually within the normal range. Hormonal stimulation of the exocrine pancreas has also been studied by Zoppi et al. (1970) with regard to water and electrolyte movement. Control children had higher sodium and bicarbonate secretion rates following secretin stimulation whereas those of protein, calcium and magnesium were higher after pancreozymin stimulation. Secretion rates of fluid volume and potassium did not show any significant difference. In cystic fibrosis patients secretion rates of sodium, potassium and magnesium were more reduced after secretin whereas those of calcium and protein were more reduced after pancreozymin. The secretion rates of fluid volume and bicarbonate were reduced after both hormonal stimulations.

The mechanisms of secretion of water and the various electrolytes were /

were apparently altered to different degrees, However sodium, bicarbonate and fluid volume are all reduced following intravenous injection of hormone. The lower bicarbonate concentrations contributing to the duodenal juice must be an important factor in the digestive disturbance of the disease since the pH of the fluid is lower than that required for optimal enzyme activity in the duodenum.

HUMORAL FACTORS.

Spock et al. (1967) observed that serum from cystic fibrosis patients and their parents had a ciliostatic effect on rabbit respiratory epithelial cells. In the same year Mangos et al. (1967) and Mangos and McSherry (1967) noted an inhibition of sodium reabsorption in the rat parotid gland after perfusion with saliva or sweat from patients. From these observations the concept of an abnormal serum factor altering cell membrane function to affect ionic transport and secretion by the cell has arisen.

Cystic Fibrosis Serum Factor.

The first indication of an inhibitory serum factor in cystic fibrosis patients came when Spock and co-workers noticed a disorganisation of ciliary beat on rabbit respiratory epithelial cells after exposure to serum from an affected child. This phenomenon was observed in sera from all of 75 patients and in none of the same number of controls. /

controls. The sera of the heterozygous parents showed similar effects after concentration of the euglobulin fractions. Crawfurd in Leeds (1969) using the gills of the fresh water mussel, Dreissensia polymorpha, was able to demonstrate a similar inhibition of the metachronal ciliary beat using sera of 11 of 12 patients. Of 163 parents tested, 150 demonstrated the presence of the inhibitory factor. Crawfurd interpreted the remaining 13 to be "false negatives" in that the positives included a small number in which the original negative finding (i.e. lack of inhibition) was reversed on retesting. There are obvious variations in the effect on the cilia from one mussel to another but more important is the seasonal variation demonstrated in the mussels. During the autumn and winter months the gills produce excessive mucus when any serum is added to them. This results in the adherence of cells eventually to inhibit the cilia. This effect undoubtedly makes interpretation of results more difficult. No inhibitory factor was demonstrable in patients less than five months old even although the diagnosis of cystic fibrosis had been firmly established (Crawfurd, 1970).
The cilia test has proved interesting but for the sake of standardisation a more objective immunological or biochemical assay would be desirable. Further investigations of the nature of the serum factor using immunological and gel filtration techniques are being carried out by this department. It is not yet known if the factor is an abnormal product or a normal metabolite accumulating as a result of an enzyme defect. /

defect.

Sodium Reabsorption Inhibitory Factor.

In 1967 Mangos and colleagues, using perfusion of the parotid duct system of the rat at different rates of flow, demonstrated an inhibition of sodium reabsorption after perfusion with saliva or sweat from cystic fibrosis patients. The primary secretory fluid of the rat parotid has plasma-like osmolarity and sodium and potassium ionic concentrations. As this fluid passes on through the striated ducts its composition is altered by reabsorption of sodium in excess of water so giving rise to a hypotonic secretion which is conveyed to the mouth by excretory ducts. Perfusion of this system with saliva and sweat from affected children resulted in a marked increase in the salivary sodium concentration at all flow rates. Theoretically, factors must be present in this disease to cause inhibition of sodium transport in the striated ducts of the rat parotid. Inhibition of the sodium and potassium adenosine triphosphatase by the cystic fibrosis factor has also been indicated when the activity of rat intestinal mucosal membranes was measured in the presence of saliva from patients (Besley and Patrick, 1969). Marked inhibition was noted at limiting concentrations of magnesium and inhibition was eliminated by calcium. Besley and Patrick suggested that "cystic fibrosis /

fibrosis saliva" might contain a factor responsible for the displacement of divalent cations from specific combining sites on the enzyme to prevent formation of the magnesium-adenosine triphosphate substrate complex of the calcium-adenosine triphosphate inhibitor complex. Since calcium and magnesium are recognised as playing fundamental roles in the maintenance of such membrane properties as stabilisation of structure, permeability, excitation and enzyme activity, it was proposed that the displacement of these ions by a cystic fibrosis factor might be significant in the pathogenesis of the disease.

The Basic Defect.

It is evident from the many apparently disjointed pieces of information in the literature that it is not yet possible to enunciate the nature of the basic defect in cystic fibrosis. Although cystic fibrosis appears to be due to a single recessive gene in the homozygous condition and could therefore be regarded as a typical inborn error of metabolism, all attempts to identify the defect at this level have so far been unsuccessful. Current evidence suggests that a circulating substance (inhibitory factor) plays a role in changing the secretory products by affecting electrolyte transport. From the point of view of the molecular geneticist, it would seem unlikely that /

that mutation in a single gene would cause the production of different factors affecting electrolyte transport. An important consideration might be whether the sodium inhibitory factor and ciliary beat inhibitory serum factor were the same and involved also in the inhibition of the pancreatic response to secretin as found by Hadorn et al. (1968). If a single humoral factor were involved then it might be an abnormal product or normal metabolite produced in abnormal amounts by an enzyme defect.

Marsden (1969) in a reappraisal of cystic fibrosis considered that biochemical observations and the clinical picture could be reconciled in terms of a single generalised inheritable defect in sodium ion uptake such as is seen in the sweat and submaxillary glands and erythrocytes. The secretion of glycoproteins or acid mucopolysaccharides essentially involves the movement of highly negative ions from the cell. To maintain ionic balance, cations (such as sodium-Na^{+}) must leave the cell simultaneously. With a reduced capacity to take up Na^{+}, secretory activity might be impaired by a build up of secretory materials in cells concerned such as in the goblet cells of the gastro-intestinal tract. Other ions such as calcium-Ca^{2+} may serve as an accompanying cation such as is seen in the submaxillary gland (Chernick and Barbero, 1961), or molecules secreted may become less negatively charged as by the substitution of neutral /

neutral fucose for charged neuraminic acids in epithelial glycoproteins (Dische et al. 1959). In cultured skin fibroblasts restriction of acid mucopolysaccharide secretion would seem to occur to result in accumulated secretory materials (Danes and Bearn, 1968; Matalon and Dorfman, 1968). Epithelial glycoprotein secretion can be very rapid and takes place in a predominantly hypotonic environment such as occurs in cystic fibrosis. This feature could aggravate any defect in sodium uptake. This theory of generalised ionic disturbance could also explain the impaired bicarbonate (HCO_3') secretion (Hadorn et al. 1968). Marsden's interpretation of the basic defect was thus a reconciliation of the different aspects due to a shortage of intracellular sodium in secretory cells.

Johansen (1970) has offered the hypothesis that the disturbance of exocrine glands may be related to the structure or porosity of mucopolysaccharide-rich regions in the connective tissue or in the mucoid layer immediately adjacent to the secretory cells. The extracellular regions may exert control over fluid movement in the initial stages of the elaboration of the primary secretion at a time when all electrolytes must first be transported from the extracellular space and probably also when they pass through the mucoid coat immediately external to the plasma membrane. There is evidence that each cell may synthesise /

synthesise at least part of its own mucoid coat thus lending it its individual cell type specificity which seems chemically related to the acid mucopolysaccharides, themselves being important constituents of the adjacent connective tissue. These macromolecules form a complex network within the ground substance and are known to be important in the maintenance of water and electrolyte balance of tissues in the body. Such variations in mucopolysaccharide distribution may partly explain the variations of composition of secretions under carefully controlled conditions of stimulation in normal individuals. Considering evidence for a circulating humoral inhibitory factor, Johansen suggests that in cystic fibrosis a basic substance, probably a polypeptide, interacts with the mucoid layer to modify its porosity. In the pancreas the material may compete with secretin, also a basic polypeptide, and inhibit its optimal effect. Similarly the basic substance may interact with a negatively charged layer on the luminal surface of the duct cells in the salivary and sweat glands and so inhibit movement of sodium to the site of the active process initiating its transport across the duct. The wide spectrum of individual variations may relate to the normal variation in the composition of the extracellular mucopolysaccharide regions in different exocrine tissues. The basic substance interacts with such mucoid substances modifying the porosity of these areas to ions or fluid movements.

Whatever /

Whatever else, the proposed basic defect must account for the following abnormalities seen in the disease:- the high sweat salt due to poor reabsorption, the inhibition of sodium ion reabsorption by a soluble humoral factor, reduced water flow in the pancreas, mucin precipitation in the pancreas and the ciliary beat inhibitory factor of Spock et al..

The evolution of the concepts of cystic fibrosis has been rapid. Indeed, the divergence of investigation since the first thorough clinical description of the disease in 1938 has led to the finding of a bewildering complexity of abnormalities for which it is difficult to conceive a unifying genetic cause. Research for the "basic defect" continues along many avenues. Inquiry has been fraught with non-absolute findings. The two most constant defects are still the raised concentrations of electrolytes in sweat and the recently described failure of development of the male reproductive tract. This latter now deserves detailed study because of its early appearance in the development of the foetus which will become the male "cystic fibrosis child".

CHAPTER TWO

THE MALE GENITAL SYSTEM IN CYSTIC FIBROSIS.

Anatomical and hormonal aspects of the male genital system.

Owing to earlier diagnosis and improved management of cystic fibrosis, patients now have a greater expectation of life. As a result, attention has recently been focussed on their reproductive potential. The adult female is capable of bearing children though fertility is impaired due to abnormality of the cervical mucus. In 1966 Grand et al. observed that of eleven children of ten mothers with cystic fibrosis, none were affected by the disease. This was taken as evidence for the genetic autosomal recessive nature of the disorder. There are, to date, however, very few claims for paternity by men with cystic fibrosis. Marks and Anderson (1960) reported sterility in a 46 year old male with normal testicular tissue but with azoospermia. Analysis of his semen indicated that the prostatic secretions were normal but the seminal fluid component was lacking. This was the harbinger of many reports of sterility in the male with cystic fibrosis.

THE ABNORMALITY OF THE MALE REPRODUCTIVE TRACT IN CYSTIC FIBROSIS.

Denning et al. (1968) reported aspermia in adult males with cystic fibrosis. Nonetheless, there was active spermatogenesis in their 10 cases /

10 cases (1 biopsy; 9 post-mortem). Spermatozoon numbers were, however, reduced and 50 per cent were observed as having abnormal forms. The cytological aberrations occurred at spermatocyte and spermatid stages of spermatogenesis. Early suggested causes for these observations were (1) a genetic abnormality affecting spermatogenesis; (2) deficiencies of vitamins A or E and (3) defects in the transport of spermatozoa owing to diminished water and electrolyte secretion.

The first indication of male infertility due to a failure of the normal development of the Wolffian duct was made in an extensive study of reproductive function in cystic fibrosis by Kaplan and colleagues (1968). Complete absence of spermatozoa was noted in the semen of 25 patients whose ages ranged from 17 to 31 years. Semen volume was reduced and more acidic than controls. Fructose concentrations were very low as compared to the greatly increased concentrations of citric acid and acid phosphatase. The viscosity of the semen was not measured but appeared to be greater than normal. These findings on analysis of the ejaculates from patients with cystic fibrosis have been substantiated by recent studies by Rule et al. (1970). Kaplan et al. (1968) postulated that increase of citric acid and acid phosphatase of prostatic origin might reflect failure of normal dilution with fluid not containing these components. Decreased fructose, of seminal vesicle origin, and reduced semen volume lent evidence for a seminal vesicle /

vesicle abnormality.

The vas deferens could not be identified on gross dissection of 10 necropsy specimens between the ages of 5 and 20 years or in 6 patients at herniorrhaphy. They concluded therefore that the aetiology of sterility was in fact a specific anatomical defect preventing transport of spermatozoa from the testis to the urethra. Valman and France (1969) examined at post-mortem the intra-abdominal vasa deferentia in 10 boys. In one child vasa deferentia were not found and in the other cases they were completely or partially reduced to a fibrous or muscular band. Holsclaw (1969) confirmed this anatomical defect at 23 necropsies and in 12 of 13 surgical explorations for hernia repair or orchiopexy. Pathological studies of 32 males by Landing et al. (1969) supported these findings. Gracey et al. (1969) described an atretic vas deferens in a 15 month old boy with cystic fibrosis and Holsclaw found the abnormality present as early as one month of age.

The defects in the male reproductive tract have been shown to be blind-ending efferent ductules at the head of the epididymis, absent or rudimentary body and tail of the epididymis, absent vas deferens and absent seminal vesicles or if present these latter structures are reduced to a dilated cystic structure. The authors considered these manifestations to be developmental in origin. Since the epididymis, vas deferens and seminal vesicles all derive from the Wolffian or mesonephric /

mesonephric duct in the embryo, defects common to all three systems are not surprising. Valman and France suggested that the mesonephric duct was obliterated during the tenth to twelfth week of foetal life, the critical time for the establishment of the Wolffian duct structures. To the contrary, di Sant' Agnese (1968) and Oppenheimer and Esterly (1969) proposed that the lesions in the male were secondary to the obstruction of the epididymis or vas deferens by abnormally viscous secretions and therefore analogous to the concept of obstructive pancreatic atrophy. However, since vasa deferentia were absent from two males less than three days old and five of less than four months of age in Valman and France's study, and since no inspissation, inflammation or atresia was demonstrated it appears that an intra-uterine maldevelopment of the male Wolffian duct system occurs in cystic fibrosis. Albeit the history of the disease has been one of non-absolute findings, the pathogenesis of the male genital duct may be the most invariable anatomical feature in early life.

NORMAL SEXUAL DIFFERENTIATION OF THE FOETUS.

From their earliest appearance the gonads are intimately associated with the nephric system. The gonadal ridges differentiate about the fourth week of foetal life while the mesonephros is still the dominant excretory organ. These ridge-like thickenings on the ventral border /

border of the mesonephros consist of closely packed cells covered by an epithelium continuous with that covering the mesonephros. Because it was believed to give rise to the primordial sex cells it has long been termed "germinal epithelium". By the sixth week, germ cells can be recognised in the gonad. At this indifferent stage of development, the gonad is composed of a medulla (potentially the definitive testis) and a cortex (potentially the definitive ovary) and a double set of sexual ducts are present. The male (Wolffian) ducts are appropriated from the regressing mesonephros whilst the female (Müllerian) ducts develop independently alongside the mesonephric ducts. The differentiation of the genital tract takes place in the foetus only after the differentiation of the gonads which is often explained on the basis of Witschi's theory of a dual system of intragonadal inductors (Witschi, 1967). A feminising inductor located in the cortex and a masculinising inductor in the medulla compete until one gains prevalence and imposes the development of the appropriate gonad by inhibition of either the cortex or medulla of the primitive gonad. If the differentiated foetus is female then the Wolffian ducts become rudimentary; if male then the Müllerian ducts undergo regression and the appropriated mesonephric ducts and tubules undergo further growth and differentiation (Table I). Some of the mesonephric tubules lying close /

close to the testis are retained as efferent ductules and, together with that part of the mesonephric duct into which they empty, they constitute the epididymis. Caudal to the epididymis, the mesonephric duct is invested in smooth muscle and becomes the vas deferens. A short distance before the vasa deferentia enter the urethral part of the urogenital sinus, local dilations appear destined to become the seminal vesicles. By the twelfth week interstitial cells are recognisable, the Wolffian duct structures differentiating, the Müllerian duct system is regressing and the penile urethra undergoing its initial stages of development. By seventeen weeks, Leydig cells are the most characteristic feature of the testis and the internal and external genitalia have almost completed their development.

Table 1. Chronology of Differentiation of the Genital Tract in the Male Foetus.

Weeks Gestation	Gonadal Characteristics	Wolffian Duct	Müllerian Duct	External Genitalia
4 – 6	Indifferent gonadal ridges	-	-	-
6 – 7	Seminiferous tubules	Present	Present	Undifferentiated.
7 – 12	Leydig cells	Differentiating	Regressing	Penile urethra developing
12 – 18	Leydig cells maximum	Differentiation completed	Regressed	Development completed

Valman and France (1969) believed that the abnormality seen in the male reproductive tract in cystic fibrosis reflected a defect in development soon after the mesonephros was superseded by the metanephros. They believed that the persistence of a recognisable epididymis and the caudal end of the vas deferens, as seen in some cases of cystic fibrosis, might be evidence for normal development until between ten and twelve weeks gestation.

HORMONAL CONTROL OF THE DEVELOPMENT OF THE MALE GENITAL TRACT.

The role of gonadal hormones in the differentiation of the genital tract has been determined largely from animal experimentation and in particular from the classical *in vivo* studies on rabbit foetuses undertaken by Jost (1947, 1953, 1970). In rabbit foetuses castrated *in utero* at a time when the gonads were already histologically sexually differentiated but while the internal and external genitalia were still undifferentiated, the genital tract of the foetuses were feminised regardless of the genetic sex. This result was confirmed by Raynaud at the Institut Pasteur in Paris in 1947 when he used a beam of X-rays to destroy the sex organs of mouse foetuses *in vivo*. Both *in vivo*, (Jost, 1955) and *in vitro*, (Picon, 1971) grafts of testis initiated masculinisation. Humans congenitally deprived of gonads, as /

as may occur in patients with Turner's syndrome and pure gonadal dysgenesis demonstrate feminine trends in development regardless of chromosomal sex. Thus it appears that the foetal testis imposes masculinity on the soma and represses femininity, whereas the ovary has no important role in the sexual differentiation of the genital tract. The testicular influence is to cause retrogression of the Müllerian ducts, to stabilise the Wolffian ducts which regress in the absence of testes, to stimulate the development of the epididymes, vasa deferentia and seminal vesicles, and to impose masculine organogenesis on common undifferentiated anlagen (urogenital sinus and external genitalia). (Fig. 5).

Androgens, such as testosterone, can act in place of foetal testes in their masculinising effects but exogenous androgens fail to inhibit the Müllerian ducts (Jost, 1953). These findings have been taken to suggest that the testis produces a Müllerian duct inhibitory factor and in addition a masculinising hormone probably androgenic in nature. This hypothesis is further strengthened by the noted effects of the anti-androgenic drug, cyproterone acetate, in male rabbit foetuses. This drug opposes the masculinisation of the genital tract but fails to prevent inhibition of the Müllerian ducts (Neumann, 1969; Elger, 1966; Jost, 1967).

In /

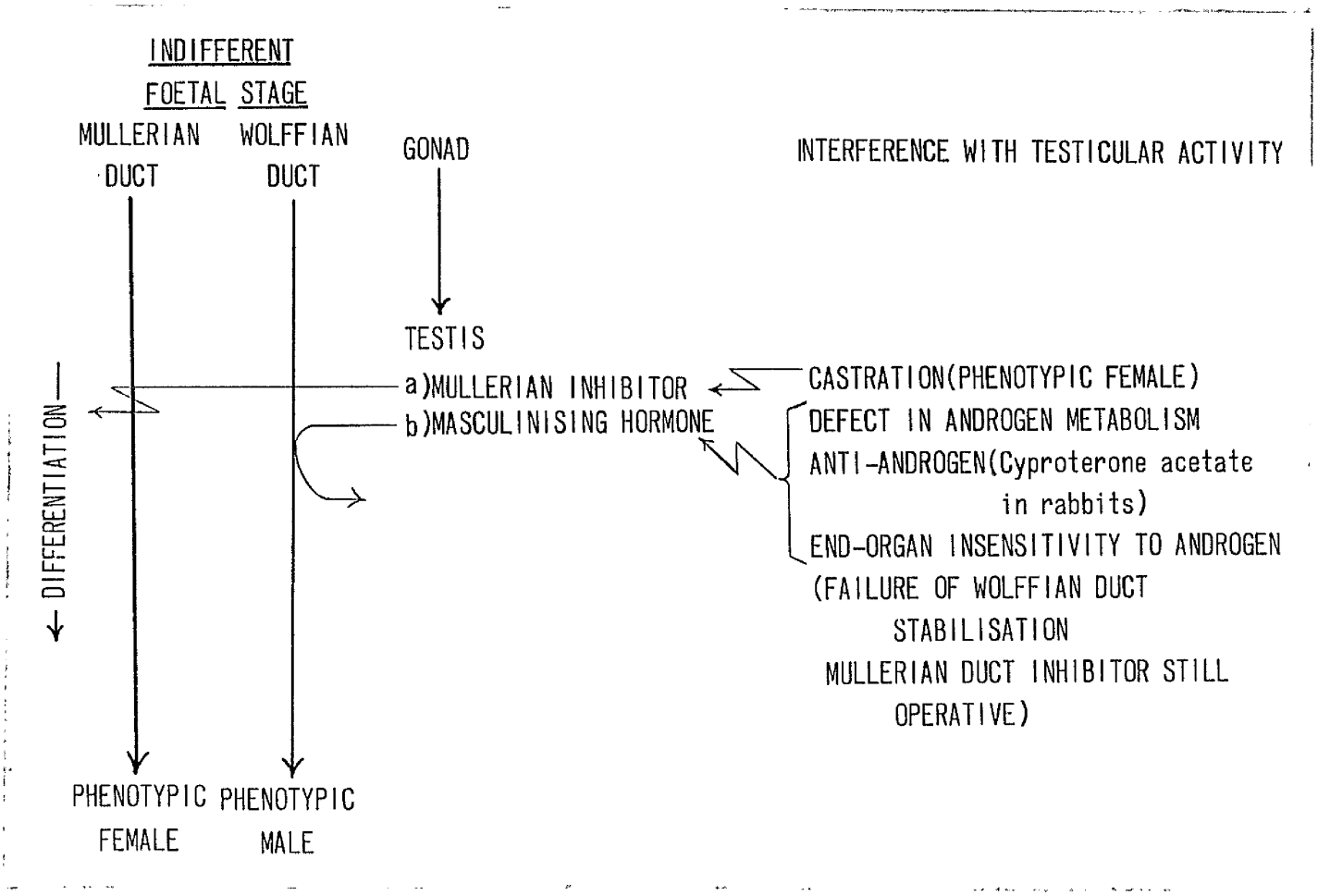


Fig. 5. Diagram indicating the testicular control of sexual differentiation.

In early *in vivo* experiments Greene et al. (1939), Raynaud (1937) and Dantchakoff (1937), using rats, mice and guinea pigs, demonstrated that the degree of masculinisation of female foetuses was related to the dose of androgen (testosterone propionate) administered to the pregnant females. Jost in 1968 linked the differential response to varying concentrations of the same hormone to various thresholds of sensitivity exhibited by the tissues involved. He observed that in rats, masculinisation of the urogenital sinus and increased anogenital distance were obtained with lower hormone dosage than was required for the development of the penis. Still higher concentrations of androgen were required for the stabilisation and development of the Wolffian duct system.

FOETAL ANDROGENS.

Review articles by Jost (1968) and Price and Ortiz (1965) have placed emphasis on the importance of foetal androgens in the normal development of the Wolffian duct system. Androgen biosynthetic pathways have been well established by both *in vitro* testicular tissue incubation findings and *in vivo* studies (Fig. 6). Early human foetal testicular incubations by Bloch (1964) and Acevedo et al. (1963) demonstrated testosterone synthesis from $[4\text{-}^{14}C]$progesterone and $[7\alpha\text{-}^{3}H]$pregnenolone. At this time the quest was for a temporal relationship /

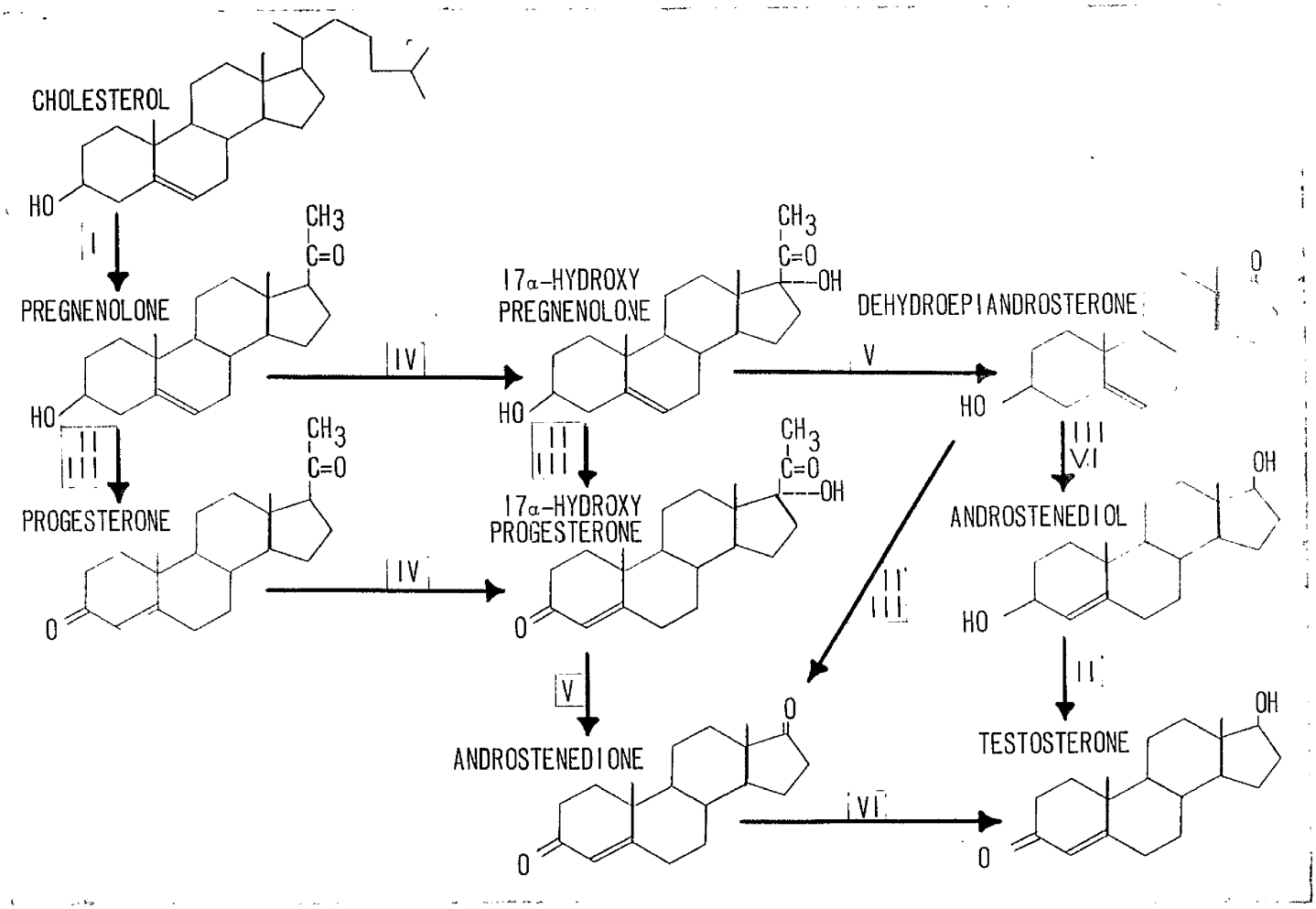


Fig. 6. Classical androgen biosynthetic pathways. Enzyme systems involved are:-

I. C20,22-desmolase

II. Δ^5-3β-hydroxysteroid dehydrogenase

III. Δ^5-Δ^4- isomerase

IV. 17α-hydroxylase

V. C17,20-desmolase

VI. 17β-hydroxysteroid dehydrogenase.

relationship between male reproductive duct differentiation and foetal androgen production. Lipsett and Tullner (1965) were able to demonstrate that the conversion of pregnenolone to testosterone increased rapidly just prior to and during the differentiation of the rabbit foetal Wolffian duct derivatives. The appearance of Leydig cells correlated closely with the development of the male reproductive system, an effect assumed to be due to androgens.

There are, however, inherent difficulties in the experimental approach to such problems in the human. The steroidogenic capacity of the human foetal testis and adrenal at the end of the first trimester of gestation has been established by histochemical (Cavallero and Magrini, 1967) and in vitro studies (Bloch, 1967), and at midpregnancy by in vivo perfusion experiments (Diczfalusy, 1967; Solomon, 1967). Early androgen production by testes and adrenals from 7.5 to 20 week old human foetuses has been investigated in organ culture using rat ventral prostate as a bioindicator (Zaaijer and Price, 1968). In these experiments foetal organs were cultured in contact with the prostatic tissue which is dependent on androgen for maintenance of structure and secretory function. After 9.5 weeks gestation all testes used experimentally displayed androgenic activity. After 12 weeks the adrenals also were androgenic. In the earlier age groups studied, a few adrenal explants demonstrated little or no androgen production. /

production. Interpretation of such results must be made with caution because of the possibility of an adrenal inhibitor being operative within the experimental system.

Testicular incubations with radioactively labelled substrates have demonstrated the importance of the Δ5-3β-hydroxysteroid pathway in early foetal life (Hamilton et al. 1970). Using ^{3}H and ^{14}C in double label techniques for the estimation of competition or "preference" for substrates, testes of less than 12 weeks gestation converted dehydroepiandrosterone and androstenediol to testosterone more readily than androstenedione. Tissue of more than 12 foetal weeks had a greater percentage conversion of androstenedione to testosterone and thus reflected the adult pattern (Lucis et al. 1967). If these investigations truly mirror the <u>in vivo</u> androgenic biosynthetic pathways, then testosterone synthesis from pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone and androstenediol must be important during the formative period since the main differentiating processes of the reproductive tract have begun by the twelfth week of gestation.

<u>Possible Endocrine Aetiology for the Abnormality of the Male Genital Ducts in Cystic Fibrosis</u>.

The anatomical nature of the defect in the male reproductive tract in cystic fibrosis patients has aroused interest. Could there be an abnormality /

abnormality in the differentiation process of the Wolffian duct? The failure in development has been thought to occur soon after the metanephros has superseded the mesonephros as the functional kidney. (Valman and France, 1969). The possibility arises that the arrest in development prior to the twelfth foetal week might be hormonal in aetiology and reflect an impaired biosynthesis of androgen. The external genitalia in male patients are however normal, indicating that testicular androgen production is not completely absent. Jost (1968) observed that in rats the threshold of sensitivity to androgens was lower for external genitalia tissue than for Wolffian duct tissues. Providing the same differential tissue sensitivity applies to humans, the theory of a deficient, though not absent, androgen production can be used to explain the apparent anomalies found in the male patient with cystic fibrosis. There are obvious difficulties in investigating androgen biosynthetic pathways in the affected foetus. Tissue from a foetus which might be affected cannot readily be obtained. However, other features of the disease seen in children with cystic fibrosis might reflect an endocrine involvement.

ENDOCRINE ASPECTS IN CYSTIC FIBROSIS.

It is well recognised that, despite stringent dietary management, underheight /

underheight and underweight are among the characteristic clinical expressions of the disease. Puberty is delayed and the pubertal growth spurt is not only delayed but it is not so marked as in normal adolescents. Aspermia, because of the absence of the vas deferens, is an almost constant finding in the male. Histological examination of post-pubertal testes has revealed a decrease in spermatogenesis and frequent abnormal spermatozoal forms (Kaplan et al. 1968). This arrest of spermatozoal maturation appears to occur at the stage of spermatid formation.

Failure to thrive, a characteristic of several chronic diseases including cystic fibrosis, has been the justification for the use of anabolic hormones in patients with the disease (Dennis and Panos, 1965; Good and Bessman, 1966; Dooley et al. 1969; Harris and Waring, 1970; Hamilton, 1970). Androgens are known to stimulate protein anabolism in many tissues of the body. They are believed to act at the cell nucleus to stimulate the production of ribonucleic acid with resulting increase of protein biosynthesis (Kochakian, 1965). Such anabolic therapy is still very much in the experimental stage. The precise mode of action of many drugs remains obscure and undesirable side-effects, due to the androgenic or oestrogenic nature of the steroid used, may occur in patients undergoing treatment. Moreover, assessment of therapy is in large part dependent on subjective assessment both by patient and physician. However, despite variable reports on the effects

on /

on lung function, all investigators noted an increase in the rate of growth and weight gain following steroid administration. Dooley et al. (1969) treating their patients with norethandrolone (Nilevar) observed that greatest benefit was derived by pre-adolescent females. This finding raised the possibility that pre-adolescent females with cystic fibrosis have a low endogenous androgen production and so are more responsive to administered anabolic steroids.

The features characterising puberty reflect changes of adrenocortical function and are accompanied by a rise in urinary 17-oxosteroids, these being mainly the metabolites of androgenic steroids produced in increasing quantities by the adrenal and the testes during adolescence. Indeed, Rosenfield and Eberlein (1969) found increasing plasma levels of dehydroepiandrosterone and dehydroepiandrosterone sulphate at this time and equated the changing adrenal function with increased secretion of these compounds by the gland. The delay in maturation in patients with cystic fibrosis might, therefore, reflect deficient synthesis or secretion of dehydroepiandrosterone sulphate by the adrenal. On reflection, the failure of development of the male reproductive tract in the foetus occurs at a time when dehydroepiandrosterone is an important intermediate in the testicular biosynthesis of the more androgenic steroid - testosterone.

The aetiology of the anatomical defect found in male patients and /

and the delayed puberty associated with cystic fibrosis could be related to hormonal imbalance and in particular to a deficit of dehydroepiandrosterone and its sulphate ester.

METABOLIC EFFECTS OF DEHYDROEPIANDROSTERONE (DHA).

There is now evidence that DHA exerts an effect over a wide range of cellular processes. The difficulty in following the effects of DHA is in part due to the fact that orally administered DHA may be converted to testosterone and indeed there is *in vitro* evidence that many tissues can elaborate testosterone from such precursors as DHA.

In 1962 Howard postulated that testosterone advanced bone age in humans. Salmon (1963) reported an increased carrier-free radioactively-labelled ^{35}S-sulphate incorporation into cartilage of hypophysectomised male rats following the administration of testosterone and other anabolic steroids. The uptake of radioactive sulphate as ^{35}S in sulphuric acid had been investigated in the growing bones of cockerels treated with cortisone (anti-anabolic) and androgenic anabolic steroids (Kowalewski, 1958). The deposition of radioactive sulphate in tissues is due to the utilisation of sulphate ions in the synthesis of chondroitin sulphate in the connective tissue ground substances and in collagen. The anabolic steroid 17α-ethyl-19-nortestosterone counteracted the anti-anabolic effect of cortisone on growing /

growing bone when both were administered simultaneously. From these observations, Kowalewski proposed that cortisone inhibited and anabolic hormones stimulated the production of certain mucopolysaccharides, essential in collagen fibrillogenesis, which specifically incorporate labelled sulphate. Principè and Bellucci (1952) administered testosterone propionate to guinea pigs with fractures and demonstrated early stabilisation of the callus. Van Wagenen and Hurme (1950) had treated immature rhesus monkeys with the same compound and produced precocious skeletal development and eruption of canine teeth. Androgen treated animals of 2.8 years chronological age had a skeletal development of about 7 years.

The temporal relationship between the sexual and osseous maturation in humans has long been recognised. For instance, girls menstruate more in relation to bone age than to chronological age. In animal experiment histological changes after androgen administration to pre-puberal mice have shown that testosterone and DHA advance the maturation of the proximal epiphyseal plate of the tibia (Howard, 1963). Howard thought that the relationship between skeletal and gonadal maturation could perhaps reflect the influence of a common maturation-rate-control, operating in part via the adrenal cortex, and associated with the increased output of DHA during the human adolescent years.

Recently /

Recently Puche and Romano (1970) investigated the effects of DHA and testosterone on the carbohydrate metabolism of bone. Chick embryo frontal bone explants were used in _in vitro_ studies and _in vivo_ investigations were made of the endochondral ossification of pre-puberal mice. Both experimental systems indicated an increased rate of calcification produced by these hormones and an increase in glucose-6-phosphate dehydrogenase activity. These authors related treatment to an increased proportion of glucose oxidised via the hexose monophosphate shunt. Oertel (1971) has also cast DHA in the role of a possible regulator of human glucose-6-phosphate dehydrogenase.

DHA has been shown to offer some protection against elastase degradation and to reduce metachromatic substances within elastase-affected tissues (Mikulaskova and Linet, 1964). The influence of DHA on the aortic wall was investigated histochemically in rabbits with experimental atheromatosis. After DHA administration a diminished degradation of elastic fibres by elastase was observed and the quantity of metachromatic substances reduced. These findings may have a bearing on the increase of metachromatic substances reported in fibroblasts and white blood cells from some cystic fibrosis patients.

Granick and Kappas (1967) described the effects of steroids on porphyrin synthesis in chick-embryo liver-cell culture. Steroid glucuronosides /

glucuronosides were devoid of porphyrin inducing activity. Sulphates of DHA and oestrone demonstrated weak activity as did the free steroids. Aetiocholanolone, however, was a strong inducer. The investigations suggested that the area of activity was the stimulation of haem biosynthesis. This study induced Goldberg et al. (1969) to undertake an investigation of urinary steroid excretion levels in patients with acute intermittent porphyria. The most consistent finding was that of raised urinary levels of DHA. This steroid introduced intraperitoneally into rats significantly raised the level of δ-aminolaevulic acid synthetase (A.L.A. synthetase) which is the rate limiting enzyme in haem and porphyrin biosynthesis. The enzyme is present in excess in the liver in acute porphyria whether natural or experimentally induced. The steroid is believed to act as a derepressor on the operon controlling A.L.A. synthetase.

Yet another facet of the subject is that steroids are known to bind to sites on spermatozoa (Ericsson et al. 1967). Steroids also suppress oxygen uptake by spermatozoa (Mounib, 1964). Studies on rats have demonstrated the ability of the epididymis, vas deferens, prostate and seminal vesicles to accumulate and retain androgens which are believed to bind to specific androgen 'receptors' in the cellular cytoplasm and nuclei (Hansson and Tveter, 1971; Tveter and Attramadal, 1968; Tveter and Unhjem, 1969). Frankel and Eik-Nes (1970) /

(1970) studying concentration gradients of testosterone and DHA within the epididymis of the rabbit, recorded higher levels of both steroids in the cephalic end of the duct than at the caudal extreme. It is generally accepted that testosterone is able to influence the differentiation of the Wolffian duct and that its stimulatory action in the male reproductive tract, particularly in the seminal vesicles and prostate, is probably mediated by an increased biosynthesis of ribosomal RNA (Villee and Fujii, 1968; Kochakian, 1965). Moreover, the concentration gradient studies of testosterone within the male genital tract in conjunction with the known effects of the steroid on spermatozoa suggest a further role for testosterone in the maturation process of spermatozoa. By preserving endogenous nutritive reserves in the gametes, high levels of testosterone in the epididymis, more particularly the caput epididymis, are believed to inhibit maturation by preventing oxidative metabolism.

The process of spermatogenesis itself has a complex of requirements for its successful completion. An interaction of gonadotrophins, androgens and non-hormonal factors is known to be essential (Steinberger and Steinberger, 1969). The precise hormonal requirements for normal spermatogenesis are still conjectural though some deductions have been made based on experiments with rats. The formation of spermatogonia /

spermatogonia from the germ cells may require testosterone as may also the meiotic division of the primary spermatocytes. The maturation of the spermatids may not require hormones or may require testosterone. The completion of spermatogenesis certainly requires F.S.H..

Androgens are therefore known to be essential for some stages of spermatogenesis and deficiency might explain the abnormal histology of testes in patients with cystic fibrosis where an arrest in development appears to occur at spermatocyte and spermatid stages (Denning et al. 1968).

Metabolism of Dehydroepiandrosterone.

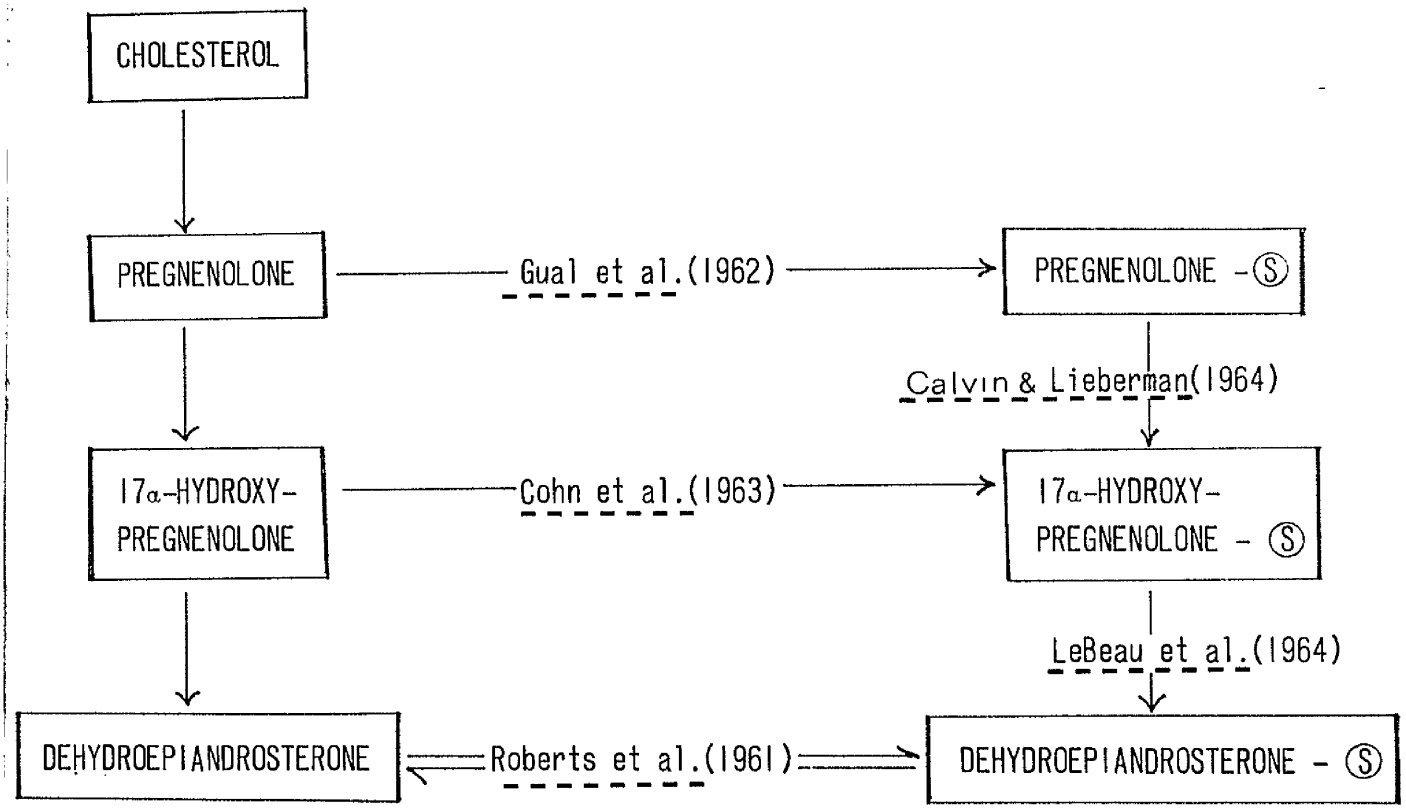
ISOLATION OF DEHYDROEPIANDROSTERONE.

Dehydroepiandrosterone was first isolated from urine in 1933 by Butenandt and though in 1938 Callow postulated its possible adrenal origin this steroid was for long not isolated from adrenal tissue and indeed was not considered as a normal urinary steroid metabolite. In 1944, dehydroepiandrosterone sulphate (DHAS) was isolated from human urine (Munson et al. 1944) and a decade later the occurrence of this ester was detected in peripheral human plasma (Migeon and Plager, 1954). Interest quickened in DHA with the realisation /

realisation that it was secreted by the adrenal cortex and from it the bulk of the urinary 17-oxosteroids are derived (VandeWiele and Lieberman, 1960). Until 1962 DHAS was considered as a catabolite, formed principally in the liver from adrenal-elaborated DHA. In the same year Baulieu (1962) demonstrated that the concentration of the sulphate ester (DHAS) was higher in adrenal venous blood of a patient with an adrenal tumour than in the peripheral circulation. Further evidence for the adrenal secretion of DHAS came from secretory rate studies using radio-isotope labelled steroids (VandeWiele et al. 1962; Gurpide et al. 1963). Indeed an unexpectedly high adrenal secretion rate of $>$ 15 mg per day was recorded (VandeWiele et al. 1963).

ADRENAL SECRETION OF DEHYDROEPIANDROSTERONE SULPHATE.

Secretion of DHAS from the adrenal may be the result of a direct steroid sulphate pathway within the gland. Calvin and Lieberman (1964) unequivocally demonstrated, in incubation studies, the pathway from Δ5-pregnenolone sulphate to 17α-hydroxypregnenolone sulphate using homogenates of hyperplastic adrenal tissue. LeBeau et al. (1964) reported the conversion by human adrenal tumour tissue of 17α-hydroxypregnenolone sulphate to DHAS (Fig. 7). Alternatively sulphation may be a terminal biosynthetic event prior to secretion of /



Colm, G.L., Mulrow, P.J., and Dunne, V.C. (1963). J. clin. Endocr., 23, 671.

Gual, C., Rojo, B., Lemus, A.E., and Rivera, R. (1962). Sociedad Mexicana de Nutricion y Endocrinol., 3rd Meeting, p. 126.

Fig. 7. Diagram of the steps in the adrenal biosynthesis of dehydroepiandrosterone sulphate from cholesterol. Steps in the "sulphate pathway" were confirmed by investigators indicated thus:- _ _ _ _ _ _ _. The symbol - S indicates the 3β-yl sulphate of the steroid.

of the sulphate from the gland. Certainly sulphokinases have been identified in adrenal, testicular and ovarian tissues (Wallace and Lieberman, 1963; Pierrepoint et al. 1965; Wallace and Silberman, 1964).

METABOLISM OF DEHYDROEPIANDROSTERONE.

Only 1 to 12 per cent of DHAS is excreted unchanged after intravenous injection of a tracer amount (Baulieu, 1965). Some of the DHAS undergoes a "direct metabolism" without breakage of the ester link implying a biosynthetic pathway involving steroid sulphates as intermediates. A moiety of DHAS is hydrolysed. Gibian and Bratfish (1956) isolated sulphatase from mammalian liver acting on oestrones and DHAS. Roy (1957) found sulphatase localised in the microsomes of ox and rat liver. Indeed, Warren and French (1965), Burstein and Dorfman (1963) and Kim and Herrman (1968) have demonstrated a widespread distribution of steroid sulphatase in human tissues. A moiety of the DHA so liberated from its sulphate may be resulphated in the liver, while a further part is metabolised to androsterone and aetiocholanolone and then sulphated for excretion, i.e. "indirect metabolism".

As well as being a secretory product from the adrenal, DHAS is also a metabolic conjugate. The liver is probably the most important sulphating organ (Schneider and Lewbart, 1956) though it is /

is not unique in this respect. DHAS and DHA are interconvertible (Roberts et al. 1961) and the DHA escaping further metabolism in the liver is secreted into the gut with the bile and may be resulphated in the intestinal wall as it is reabsorbed into the portal circulation.

"Detoxication" products of liver catabolism such as androsterone and aetiocholanolone, conjugated with glucuronic acid or sulphuric acid so rendering them more water soluble, are quickly excreted in the urine and thus have a high clearance rate. Glucuronosides were generally considered as end products which were completely excreted. However, dehydroepiandrosterone glucuronoside (DHAG) administered orally increased the urinary output of androsterone and aetiocholanolone glucuronosides (Kellie, 1961). Only 15 per cent of an administered tracer dose of [7α-^{3}H] DHAG was recovered as the glucuronoside from the urine, indicating that further metabolism must have taken place. An "indirect metabolism", implicating the splitting of the osidic link, was found after the isolation of urinary [7α-^{3}H] DHAS following intravenous injection of [7α-^{3}H] DHAG. Several [7α-^{3}H] labelled 3β-hydroxysteroids ([7α-^{3}H] 3of [7α-^{3}H] DHAG androstan-17one, [7α-^{3}H] androstane-3β, 17βdiol, Δ5-androstenediol) were recovered as glucuronosides indicating that a "direct metabolism" also probably occurs (Robel et al. 1967).

DHAS does not have a potent androgenic activity and its biological significance /

significance comes from the peculiarity of its metabolism. The biological half-life of DHAS is 8 to 10 hours while that of free DHA is 25 minutes (Baulieu, 1965). It is not known if this long half-life is due to protein-binding, difficulties of membrane penetration or weak sulphatase activity. However, DHAS may be considered as a "privileged hormone substrate" in that it circulates for a long time and seems to resist hepatic catabolism.

Thus the roles of DHA and DHAS are not all identified, nor are they clearly defined. Certainly the facts that DHA and DHAS are precursors of testosterone, that sulphatases are present in many tissues of the body and that, even as a weak androgen, DHA may act as a metabolic stimulant at cell level, have suggested that circulating levels of DHA and DHAS and their metabolism in patients with cystic fibrosis should be investigated.

Enunciation of Thesis and Proposed Mode of Study.

If DHA or DHAS circulated in suboptimal concentrations in patients with cystic fibrosis then the following aspects of the disease might be explained:-

(a) Failure to complete the development of the male reproductive tract

(b) Arrest of spermatogenesis at spermatocyte and spermatid stages of development

(c) /

(c) Delayed puberty

(d) Restricted pubertal growth spurt

(e) Retarded bone age, and

(f) Increased metachromasia on staining with toluidine blue in fibroblasts and white cells.

Such diminished availability of steroid could be the result of:-

(a) reduced secretion of DHAS by the adrenal glands

(b) reduced sulphatase activity for DHAS decreasing the availability of active DHA to the cells of the body, or

(c) reduced sulphokinase for DHA resulting in its rapid excretion.

Thus the investigation of DHA in cystic fibrosis patients was undertaken. Urine was processed to determine the daily excretion of total 17-oxosteroids, 17-oxogenic steroids, and total 17-hydroxycorticosteroids. Urinary DHAS and DHAG were also quantitated and in addition the plasma DHAS representing the circulating "available pool". Attempts were madeto increase available DHA at cellular level and these attempts were monitored by estimating urinary and plasma DHAS levels. Some somatic and cellular effects were observed.

CHAPTER THREE

MATERIALS AND METHODS

Introduction

In order to investigate a possible deficiency of dehydroepiandrosterone (DHA) in patients with cystic fibrosis, methods were sought by which low urinary and plasma levels of the steroid could be detected. Normal urinary excretion of 17-oxosteroids below the age of 6 years is reportedly low (< 1 mg per 24 hours)(Loraine and Bell, 1971). Fractionation employing paper chromatography, demonstrated DHA to constitute 4 per cent of the total 17-oxosteroids in children from 5 to 7 years (Loras et al., 1966). Thereafter excretion of DHA and 17-oxosteroids gradually rises to adult levels between 17 and 20 years. Boys of 14 to 16 years have an output of 600 μg DHA per 24 hours, representing 16 per cent of the excreted 17-oxosteroids. Small quantities of 17-oxosteroids are also found in plasma, the range throughout early childhood and adolescence being from 1 to 160 μg per 100 ml plasma (Rosenfield and Eberlein, 1969).

Since levels of DHA in patients with cystic fibrosis were thought likely to be subnormal, it was necessary that the methodology could detect nanogram (ng) levels of DHA in the biological material processed

(50 - 100 ml urine; 4 - 10 ml plasma). This sensitivity of detection was achieved using g.l.c. and by electron capture detection of the heptafluorobutyrate derivative of the steroid. Utilising this method, rigorous preparative purification of samples was found necessary. Losses were envisaged and consequently radioactive DHA was added initially to samples. On the basis that recovery of labelled DHA represented equivalent recovery of native steroid, correction after g.l.c. quantitation was made according to estimations of the recovery of the labelled steroid.

Materials

RADIOACTIVE-LABELLED STEROIDS

All radioactive labelled steroids used in this investigation were obtained from the Radiochemical Centre, Amersham, Bucks., England. Dehydroepiandrosterone-$[7\alpha\text{-}^{3}H]$ (^{3}H-DHA) and dehydroepiandrosterone sulphate-$[7\alpha\text{-}^{3}H]$ (^{3}H-DHAS) were used in tracer amounts to estimate the recovery rates of the experimental procedures. Testosterone di-heptafluorobutyrate was used as an internal standard for g.l.c. quantitation of biological samples and $[7\alpha\text{-}^{3}H]$ testosterone (^{3}H-T) was used to estimate the efficiency of derivative formation.

Prior to use, all radioactive steroids were purified in thin layer /

layer chromatographic (t.l.c.) systems until a single peak of Gaussian proportions was produced on radiochromatogram scanning. Generally 3 separate systems were required. The purified steroids were then stored at 5°C in 50 per cent benzene in ethanol for free steroids and ethanol for conjugated steroids.

REFERENCE COMPOUNDS - UNLABELLED

Standard steroids were obtained commercially either from Ikapharm (Ramat-Gan, Israel.) or Sigma London Chemical Company Ltd. (London, S.W.6., England.). Small quantities of the glucuronoside and sulphate conjugates of dehydroepiandrosterone (DHA), androsterone (AND) and aetiocholanolone (AET) were obtained from the M.R.C. Steroid Reference Collection (Chemistry Dept., Westfield College, Hampstead, London, N.W.3.).

REAGENTS

Benzene, cyclohexane, acetone and ethyl acetate were supplied by Hopkin and Williams Ltd. (Freshwater Rd., Chadwell Heath, Essex, England.) and were of the M.F.C. (Materials for Chromatography) range of products. Absolute ethanol (A.R. quality) was obtained from James Burrough Limited (Fine Alcohols Division, 60 Montford Place, London, S.E.11, England.). "Peroxide-free" diethyl ether was purchased from May and Baker Ltd. (Dagenham, Essex, England.). Heptafluorobutyric anhydride and toluene ("scintillation /

("scintillation grade") were obtained from Koch-Light Ltd. (Colnbrook, Buckinghamshire, England.). Both PPO and POPOP, the primary and secondary solutes incorporated into toluene to produce liquid scintillator, were obtained from Packard Instrument Co., Inc., (Downers Grove, Illinois, U.S.A.). All other chemicals were purchased from B.D.H. Chemicals Ltd. (Poole, Dorset, England.) and were, when possible, of "Analar" specification.

ENZYME PREPARATIONS

Sulphatase prepared from Helix pomatia was obtained from Koch-Light Ltd.. The enzyme β-glucuronidase used for the hydrolysis of urinary steroid glucuronosides was prepared in this laboratory after the M.R.C. method (1963) and its potency estimated by the method of Talalay et al. (1946).

Limpets (Patella vulgata) were gathered from the rocky shore of the Clyde estuary 2 hours after high tide and immediately brought back to the laboratory in sea water. The molluscs were removed from their shells and the black visceral hump was dissected from the muscular foot. Batches of 100 - 200 g visceral humps were homogenised for 5 minutes with ice-cold water (2 ml per g). After centrifugation the supernatant was treated with acetone to bring the concentration to 60 per cent acetone in water in order to precipitate the enzyme.

The /

The mixture was allowed to stand in the refrigerator (5°C) overnight and the supernatant was then removed after centrifugation at 1,000 r.p.m.. The acetone precipitate was washed by thorough mixing with acetone followed by centrifugation until all the chromogenic material had been removed by the acetone. The precipitate was allowed to dry in air, powdered and assayed for β-glucuronidase activity.

Assay of Potency of β-glucuronidase Preparation

The limpet powder (200 mg) was dissolved in deionised water (40 ml) and 1 ml of the resulting suspension was made up to 12.5 ml with deionised water. 0.5 ml of this dilution, corresponding to 200 μg of enzyme powder, was used for the estimation of β-glucuronidase activity.

0.1 M acetate buffer (4 ml)(5.785 g sodium acetate and 3.25 ml glacial acetic acid per litre of deionised water) was placed in 4 tubes. Phenolphthalein glucuronoside (50 μg) was added to two experimental tubes but not to the two control tubes. All tubes were then placed in a water bath at 38°C and allowed to equilibrate. The enzyme solution (0.5 ml) was introduced to each tube at timed intervals, mixed, stoppered and incubated for exactly 60 minutes. The reaction was arrested and the colour developed in all tubes by adding 0.4 M glycine buffer (5 ml) (16.3 g aminoacetic acid, 12.65 g sodium chloride and 10.9 ml /

10.9 ml concentrated sodium hydroxide 1:1 (wt/v)). Finally phenolphthalein glucuronoside (50 μg) was added to the two control tubes. A Unicam SP. 600 spectrophotometer set at 540 mμ and employing cuvettes of 10 mm light path was used to read the optical densities of experimental and control solutions against standards of phenolphthalein (5-20 μg).

The number of Fishman units per g enzyme preparation is equivalent to the μg phenolphthalein liberated from the substrate (phenolphthalein glucuronoside) by 1 g of the enzyme extract powder.

Optical densities (O.D.) obtained on assaying the potency of the β-glucuronidase used in this study are given below. The spectrophotometer was zeroed against deionised water.

Calculation

$O.D._{540}$	Control	003
$O.D._{540}$	Standard (20 μg phenolphthalein)	153
$O.D._{540}$	Experimental test	160

Therefore

20 μg phenolphthalein ≡ O.D. 150

20.9 μg " ≡ O.D. 157

200 μg enzyme powder liberates 20.9 μg phenolphthalein

1 g enzyme powder liberates $\frac{20.9 \times 10^6}{200}$

104,500 μg phenolphthalein

Thus 1 g enzyme powder contains 104,500 Fishman units.

Basic Methodology

COLUMN CHROMATOGRAPHY

Glass columns (6 cm x 0.5 cm I.D.) were made to the detailed specifications indicated in the text by Thomson, Skinner and Hamilton (12 Cadogan St., Glasgow, Scotland.) and latterly by R.S. Wood (10 Hunter St., Paisley, Renfrewshire, Scotland.). Aluminium oxide "CAMAG", M.F.C., Brockmann activities 1 and 2 (alkaline, 100 to 250 mesh), was obtained from Hopkin and Williams Ltd.. The activity of each batch of alumina prior to use was checked by running a series of radioactive standards through a column and assaying the recovery of the radioactive label in consecutive 10 ml aliquots of eluate.

THIN LAYER CHROMATOGRAPHY

Merck pre-coated thin layer plates of silica gel F_{254} (layer thickness 0.25 mm) were supplied by Anderman and Co. Ltd. (Battlebridge House, 87/95 Tooley St., London, S.E.1., England.). These plates were used without further washing or activation by heat. Following application of samples, the plates were developed in t.l.c. tanks lined with Whatman 3 MM chromatography paper (W. and R. Balston Ltd., Springfield Mill, Maidstone, Kent, England.). Approximately 100 ml of appropriate solvent was used for each development.

Unlabelled /

Unlabelled steroid standards were located on t.l.c. plates after spraying with Rhodamine 6 G (0.1 per cent in ethanol (wt/v)). They were visualised as yellow fluorescent areas on a red fluorescent background using ultraviolet light (2537 A) from a B.T.L. ultraviolet lamp for t.l.c. (Baird and Tatlock (London) Ltd.). Steroids with the Δ^4-3-oxo configuration absorbed ultraviolet light at this wavelength, thus facilitating detection.

Areas of radioactive steroids on t.l.c. plates were determined using a Panax thin layer radiochromatogram scanner, Model RTLS 1a (Panax Equipment Ltd., Redhill, Surrey, England.), in conjunction with a Smith's flatbed recorder (Smith's Industries Ltd., Industrial Instrument Division, Kelvin House, Wembley Park, Middlesex, England.). The detector carrier gas was a mixture of argon (2 per cent) and propane (98 per cent)(British Oxygen Company Ltd., Special Gases Dept., Deer Park Rd., London, S.W. 19, England and Messer Griesheim Ltd., Rare Gases Division, 43-45 Knights Hill, West Norwood, London, S.E.27, England). The gas flow rate was regulated to 50 ml per minute. The Geiger-Müller detector was adjusted to a height of approximately 2 mm above the t.l.c. plate and all plates were scanned using the 15 x 2 mm detector aperture. The detector voltage was set at 1,040 V, detector dead time at 200 μs and discriminator bias at 10 mV. A scanning /

scanning speed of 30 mm per hour and a time constant of 100 seconds were employed. The range of counting varied with the amount of radioactivity to be scanned.

After location, the desired radioactive areas of silica were loosened from the plate using a scalpel blade and collected by suction through a vacuum thimble (Glass Appliances, 488 Holburn St., Aberdeen, Scotland.). The thimbles were then inverted and the steroidal material eluted by filtration through the sintered glass base of the thimble (Fig. 8).

LIQUID SCINTILLATION COUNTING

Radioactivity in liquid samples was measured using a Nuclear Chicago Mark 1 Scintillation computer (Nuclear Chicago Corporation, Inc., Des Plaines, Illinois, U.S.A.). The scintillation counter was calibrated for simultaneous counting of ^{3}H and ^{14}C employing three channels. The scintillator used was toluene containing 4 g per litre PPO (2,5-diphenyloxazole) and 0.05 g per litre POPOP (1,4-di-(2-(5-phenyloxazolyl))-benzene). Radioactive $\left[7\alpha\text{-}^{3}H\right]$ dehydroepiandrosterone sulphate, which is preferentially soluble in aqueous solution, was incorporated into the above toluene-based scintillator by dilution with 20 per cent methanol. All samples were counted in 20 ml scintillator for 20 minutes. Quenched standards (Nuclear Chicago Corporation, Inc.) containing an identical scintillator were used to establish /

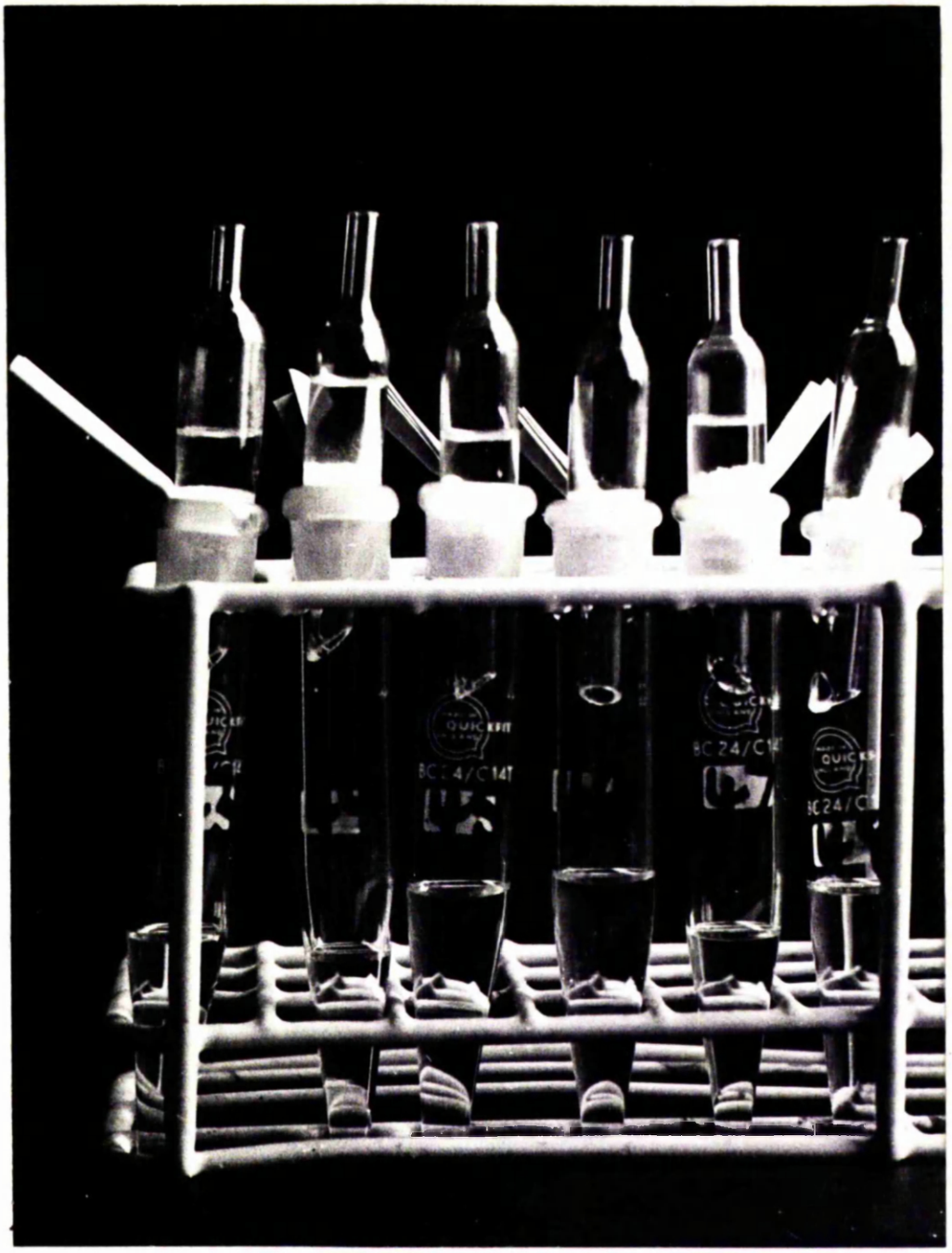


Fig. 8. Extraction thimble used in the collection and elution of silica from t.l.c. plates. Elution of steroids from the silica is facilitated by percolation of solvent through the silica and sintered glass disc of the inverted thimble.

establish quench correction curves using external standard channels ratio techniques and the internal standard of ^{133}Ba incorporated into the scintillation counter. All results of quantitation by such liquid scintillation counting are expressed as disintegrations per minute (d.p.m.) taking account of the channels ratio with an internal standard.

GAS LIQUID CHROMATOGRAPHY

The gas chromatographic system employed was a Pye series 104, Model 84 (W.G. Pye and Co. Ltd., York St., Cambridge, England.). To determine column separating powers, flame ionisation detection (F.I.D.) was used in association with the following gas flow rates:- oxygen-free nitrogen carrier gas - 40 ml per minute, hydrogen - 40 ml per minute, air - 700 ml per minute. All gases were supplied by the British Oxygen Company Ltd..

Electron capture detection (E.C.D.) using a ^{63}Ni source and a pulsating voltage of 150 μs was employed in the final quantitation of extracts from urine and plasma. Column details and running conditions will be indicated in the text.

The recorder was a Leeds and Northrup Speedomax W (Leeds and Northrup, Ltd., Wharfdale Road, Tyseley, Birmingham, 11, England.) and a chart speed of 10 inches per hour was employed throughout the investigation. /

Group Determination of Steroids in Urine

COLLECTION OF SAMPLES

Complete 24-hour urine collections were made from patients either in hospital or at home under parental supervision. Specimens were stored at 5°C over chloroform (5 ml) which was added as a preservative. All urines were tested with "Clinistix" for reducing substances which, if present, were oxidised by incubating the urine samples for 24 hours with yeast (1 g per 20 ml urine).

DETERMINATION OF 17-OXOSTEROIDS IN URINE

After measuring the total urine volume, duplicate 10 ml aliquots of undiluted urine were pipetted into boiling tubes of approximately 30 ml capacity. The tubes were placed, unstoppered, into a vigorously boiling water bath and 1 ml of concentrated hydrochloric acid added to each tube. Boiling was continued for 10 minutes to effect hydrolysis of conjugated 17-oxosteroids.

The tubes were then cooled rapidly in ice prior to extraction of the steroids with 10 ml organic solvent (chloroform). After inversion of the tubes 20 times, the upper aqueous layer was aspirated using a Pasteur pipette attached to a water pump. The chloroform was washed /

washed with sodium hydroxide (2 ml) and then with deionised water (2 ml). Following each wash the aqueous phase was removed by suction. The organic phase was filtered through Whatman No. 1 filter paper and 5 ml of the filtrate transferred to a 10 ml test tube. Evaporation was effected under nitrogen, the tubes standing in a water bath at 40°C. The 17-oxosteroid content of the dried residue was estimated colorimetrically by the Zimmermann reaction.

The Zimmermann Reaction

The James and de Jong (1961) modification of the Zimmermann reaction was employed throughout this study. These authors advocate the use of a reaction mixture of 0.5 per cent dinitrobenzene and concentrated tetramethylammonium hydroxide in the proportions 2:1. The separate reagents were stored at 5°C until used.

The freshly constituted mixture (0.25 ml) was added to the dried urine extracts from the above procedure. Duplicate reagent blank tubes and standard tubes containing DHA (10 μg) plus reaction mixture were set up with each assay. The tubes were stoppered and incubated in the dark at 25°C for 1 hour (Fig. 9). Ethanol (50 per cent)(2 ml) was pipetted into each tube and the Zimmermann colour extracted into diethyl ether (3 ml).

When the phases had separated, the upper ether layer was transferred /

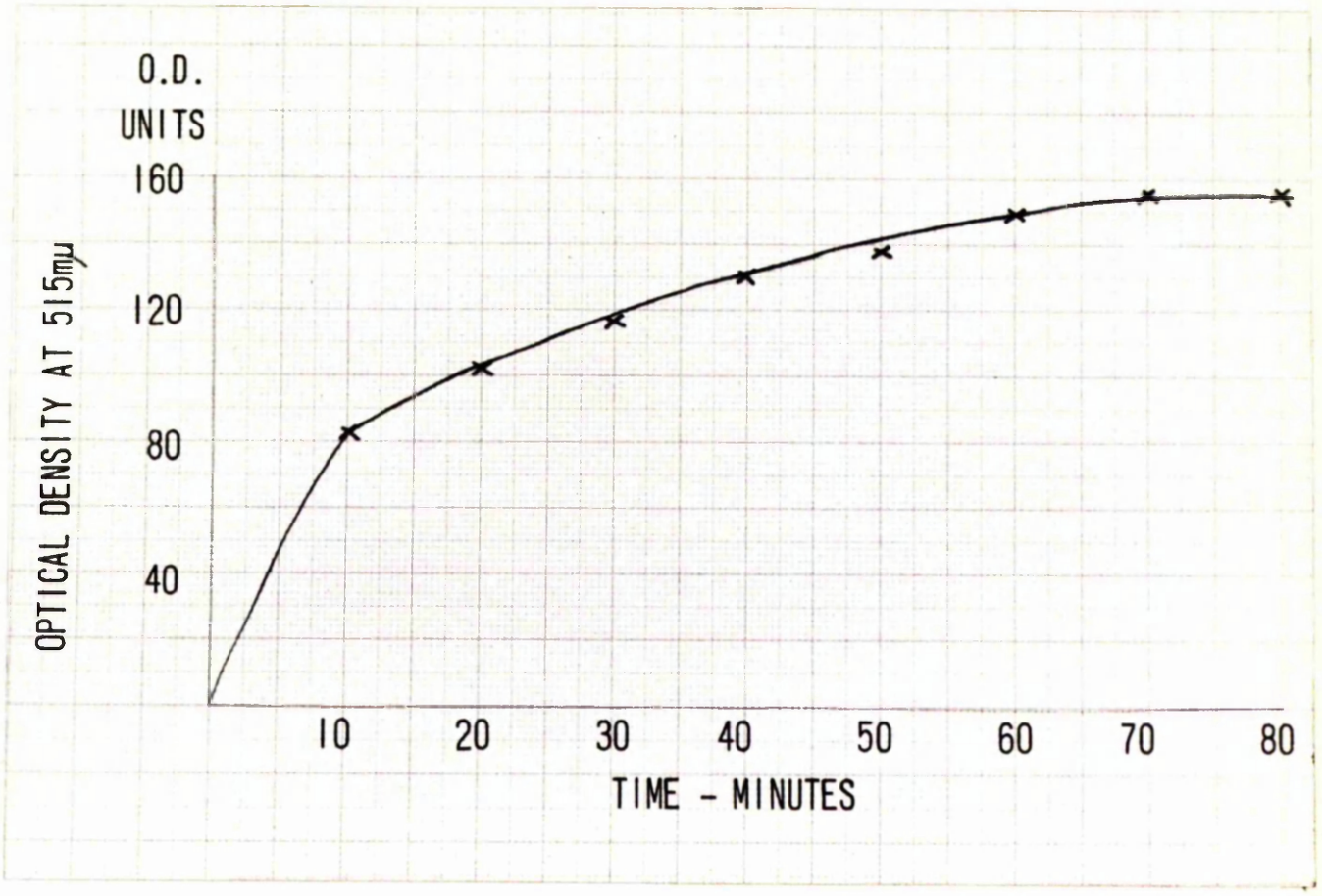


Fig. 9. The increase in optical density of the Zimmermann colour with time. Routinely 1 hour at 25°C in the dark was used. The reaction mixture consisted of tetramethylammonium hydroxide and dinitrobenzene (1:2, v/v)(0.25 ml) added to the dried residue. Standard DHA (10 μg) was used in the above experiment and the Zimmermann positive steroid was extracted into diethyl ether from the aqueous phase.

transferred by Pasteur pipette to an optical cell of 10 mm light path. Optical densities were read at 435, 515 and 595 mμ on a Unicam SP. 600 spectrophotometer zeroed against ether. To correct for interfering chromogens the Allen correction formula (1950) was applied to the optical densities at the wavelengths given above.

The corrected optical density (COD) at 515 mμ is given by:-

$$COD_{515} = OOD_{515} - \frac{OOD_{435} + OOD_{595}}{2}$$

where OOD_{515} is the observed optical density at 515 mμ

OOD_{435} is the observed optical density at 435 mμ

and the OOD_{595} is the observed optical density at 595 mμ.

After subtracting the reagent blank readings from both standard and test readings, the microgram equivalents of the test samples relative to the standard DHA are calculated and, after adjustment for the half volume of chloroform extract taken and for the total daily urine volume, the final result is expressed as mg 17-oxosteroids per 24 hours. Standard DHA obeys the Beer-Lambert law in the diethyl ether phase (Fig. 10).

In Table II typical results from the Zimmermann reaction are given. Frequent recovery experiments were undertaken and the recovery of /

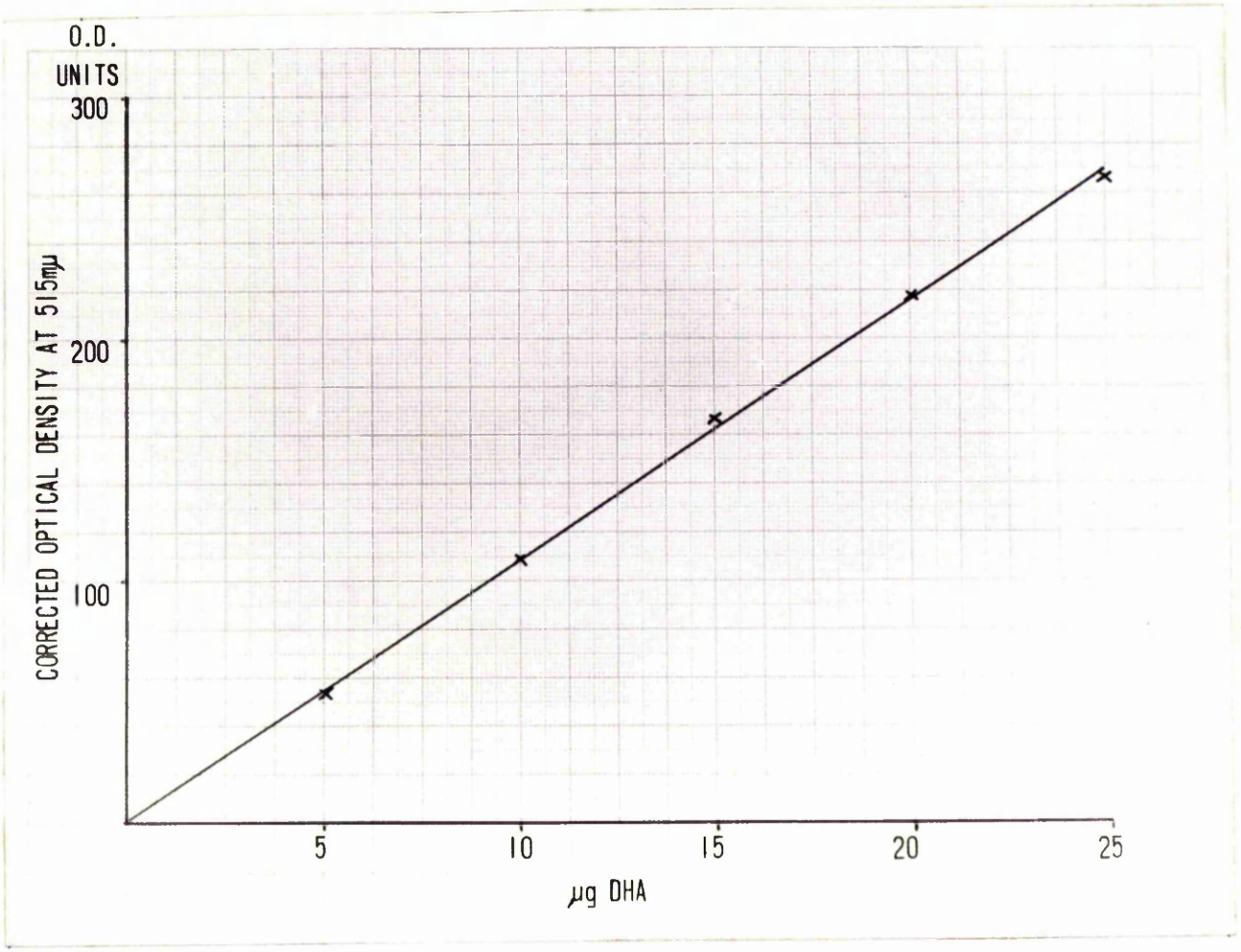


Fig. 10. Optical density of the Zimmermann colour for DHA in the range 5 - 25 μg (corrected for reagent blank). Note the obedience to the Beer-Lambert law.

TABLE II. Recovery rates of DHA from water and urine. The complete method of assay for 17-oxosteroids was used.

Extraction was made with 10 ml chloroform.

Aliquot of 5 ml taken for the Zimmermann reaction.

TEST	WAVELENGTH (mμ)			AC	AC-RB	PERCENTAGE RECOVERY OF DHA
	435	515	595			
REAGENT BLANK	031	033	012	012		
	013	032	007	012		
STANDARD DHA (10 μg)	066	163	065	098	088	
	045	157	065	102		
WATER	029	032	009	013	002	
	023	032	012	015		
WATER PLUS DHA (20 μg)	098	197	094	101	089	99.2
	093	193	091	101		
URINE (ADULT)	242	311	132	124	116	
	245	323	138	132		
URINE PLUS DHA (20 μg)	292	436	177	202	190	84.4
	329	466	200	202		

AC - Allen correction (1950).

RB - Reagent blank.

of DHA added to water and urine was consistently greater than 80 per cent. For each patient the estimation of 17-oxosteroids was made on duplicate samples of urine. When the results of the duplicates differed by plus or minus 5 per cent from the mean the test was repeated.

DETERMINATION OF 17-OXOGENIC STEROIDS AND TOTAL 17-HYDROXY-CORTICOSTEROIDS IN URINE.

17-oxogenic steroids and total 17-hydroxycorticosteroids were estimated only when sufficient urine was available.

Estimation of urinary 17-oxogenic steroids was by Brooks and Norymberski's method (1952, 1953) but using sodium metaperiodate (Few, 1961) rather than sodium bismuthate. This method is essentially the oxidative procedure advocated by the M.R.C. Clinical Endocrinology Committee in 1969 and estimates the 17,20-dihydroxy-21-deoxysteroids (C), and the 17,20,21-trihydroxysteroids (D) (Fig. 11). The 17-oxosteroids (E) are simultaneously measured in the estimation of the 17-oxogenic steroids. Correction for true 17-oxogenic steroid levels is made by subtraction of the 17-oxosteroid concentration determined prior to oxidation.

This method does not estimate the 17,21-dihydroxy-20-oxosteroids (B) or the 17-hydroxy-20-oxo-21-deoxysteroids the latter being increased in the urine when 21-hydroxylation is defective

(e.g. /

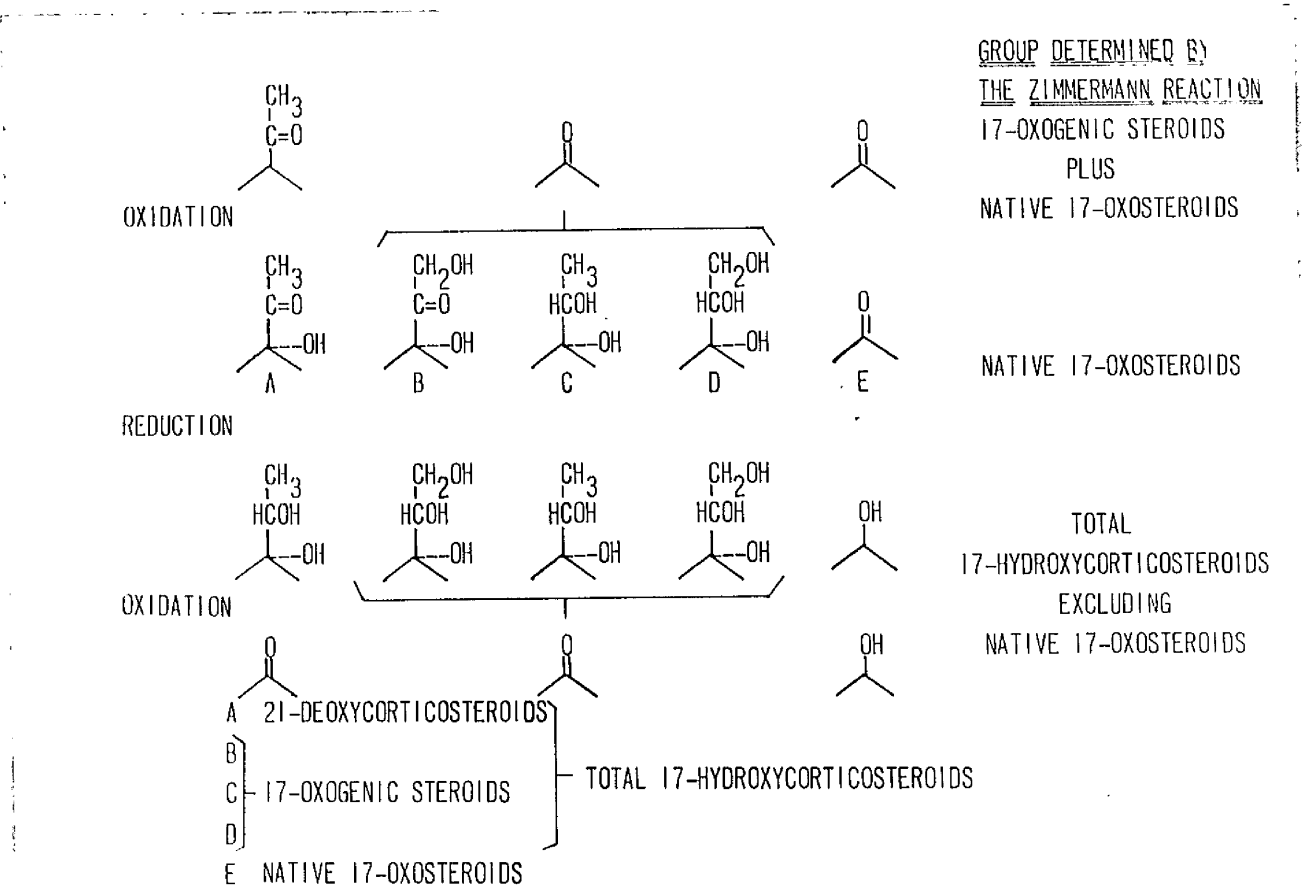


Fig. 11. The effects of chemical oxidation and reduction on urinary steroids. Side chain from C17 shown.

(e.g. congenital adrenal hyperplasia). Treatment of these two compounds with sodium borohydride results in reduction of the C20 ketonic group to a secondary alcohol which after oxidation can then be estimated as 17-oxosteroids by the Zimmermann reaction (Appleby et al. 1955).

Thus, if a urine specimen is firstly reduced by sodium borohydride and subsequently oxidised with sodium metaperiodate the resulting 17-oxosteroids will be derived from the 17-oxogenic steroids and the 17-hydroxy-20-oxo-21-deoxysteroids which together are regarded as the total 17-hydroxycorticosteroids or total 17-oxogenic steroids (M.R.C. Clinical Endocrinology Committee, 1963). Throughout this thesis however total 17-hydroxycorticosteroids will be used to refer to glucocorticosteroids with these characteristics. Native 17-oxosteroids are reduced to alcohols by this regime and so do not interfere with total 17-hydroxycorticosteroid estimations. In health urinary 17-oxogenic steroid levels should approximate those of the total 17-hydroxycorticosteroids.

Estimation of the total 17-hydroxycorticosteroids

Two aliquots of urine (10 ml) were pipetted into separate 30 ml boiling tubes and adjusted to pH7 with 25 per cent acetic acid or 2 N sodium /

sodium hydroxide as necessary. Sodium borohydride (200 mg) was added to each tube and reduction was allowed to proceed overnight or for a minimum of 2 hours. The reaction was stopped with 25 per cent acetic acid (0.5 ml) and the samples were again neutralised.

Oxidation was effected by incubating the urine samples at 37°C with freshly prepared 10 per cent (wt/v) sodium metaperiodate (4 ml) and 1N sodium hydroxide (1 ml). After 1 hour formates were hydrolysed by the addition of 2.5N sodium hydroxide (1 ml) and incubation was continued for a further 15 minutes.

After cooling, chloroform (10 ml) was added to each tube and the steroids extracted into the organic phase by inverting the stoppered tubes twenty times. When the phases had separated the upper aqueous phase was removed by suction. The chloroform was washed with 5 per cent sodium hydroxide (5 ml) containing 2.5 per cent (wt/v) of sodium dithionate. The upper aqueous phase was once more removed by suction and the organic phase filtered through Whatman No. 1 filter paper. Aliquots of 5 ml were taken from each tube and evaporated to dryness in a water bath at 40°C under a stream of nitrogen.

The residues were subjected to the Zimmermann reaction and the 17-oxosteroids produced by reduction and oxidation were estimated against /

against standard dehydroepiandrosterone (10 μg). After adjustment for the 5 ml aliquot of chloroform taken and for the total urine volume, results were expressed as mg total 17-hydroxycorticosteroids per 24 hours. Recovery of 17α-hydroxyprogesterone added initially to urine samples was of the order of 78 per cent (Table III).

Estimation of the 17-oxogenic Steroids.

17-oxogenic steroids in duplicate 10 ml samples of urine were oxidised to 17-oxosteroids by the oxidative procedure described above. Quantitation was by the Zimmermann colorimetric method and results expressed as mg 17-oxogenic steroids per 24 hours.

Occasional interference with 17-oxogenic steroid estimations occurred due to a yellow complex being formed with the Zimmermann reagents. Most patients with cystic fibrosis are currently on full therapeutic doses of cloxacillin (3-o-chlorophenyl-5-methyl-4-isoxazolyl penicillin sodium salt monohydrate) as prophylaxis. Interference due to cloxacillin therapy was reported by Metcalf (1967) when she estimated gross 17-oxogenic steroid levels in patients taking this antibiotic. This falsely high result was attributed to a substance excreted in the urine which mimicked 17-oxogenic steroids by giving a purple complex with alkaline m-dinitrobenzene. When such interference

was /

TABLE III. Recovery of 17α-hydroxyprogesterone from adult urine. After reduction and oxidation the Zimmermann reaction was used.

TEST	WAVELENGTH (mμ)			AC	AC-RB	PERCENTAGE RECOVERY
	435	515	595			
REAGENT BLANK	014	020	007	010		
	013	020	007	010		
STANDARD DHA (15 μg)	135	332	128	201	190.5	
	147	342	137	200		
URINE (ADULT)	279	678	381	348	336.5	
	283	681	389	345		
URINE PLUS 17α-HYDROXY-PROGESTERONE* (20 μg)	276	733	309	441	435	78
	273	739	314	446		

AC - Allen correction (1950).

RB - Reagent blank.

* - Correction for possible chromogenicity differences (between androstenedione and DHA) was not made.

was experienced during this investigation (in urine from 9 patients) quantitation of the 17-oxogenic steroids was not made.

Estimation of Dehydroepiandrosterone in Urine

Since DHA is excreted mainly as the sulphate or glucuronoside ester, an efficient hydrolysis or solvolysis procedure is required to liberate the parent steroid.

EVALUATION OF HYDROLYSIS METHODS

The following schema was devised to test the efficiency of enzymatic hydrolysis and solvolysis of dehydroepiandrosterone sulphate (Fig. 12). Urine samples (50 ml) were adjusted to pH 6.2 with sulphuric acid (40 per cent), sulphatase from Helix pomatia was then added (1,000 u per ml) and hydrolysis effected at 37°C for 24 hours. Extraction of the free DHA was made with ether (3 x 20 ml). The urine was then adjusted to pH 4.5 with sulphuric acid (40 per cent), β-glucuronidase was then added (1,000 F.u. per ml) and the hydrolysis allowed to proceed for 24 hours at 37°C. Free steroids were extracted with ether as before. The same urine aliquot was brought to pH 1 and solvolysis effected according to Burstein and Lieberman (1958). Sodium chloride (10 g) was added to the acidified urine and ethyl acetate (50 ml) layered onto the aqueous phase. Following rigorous shaking /

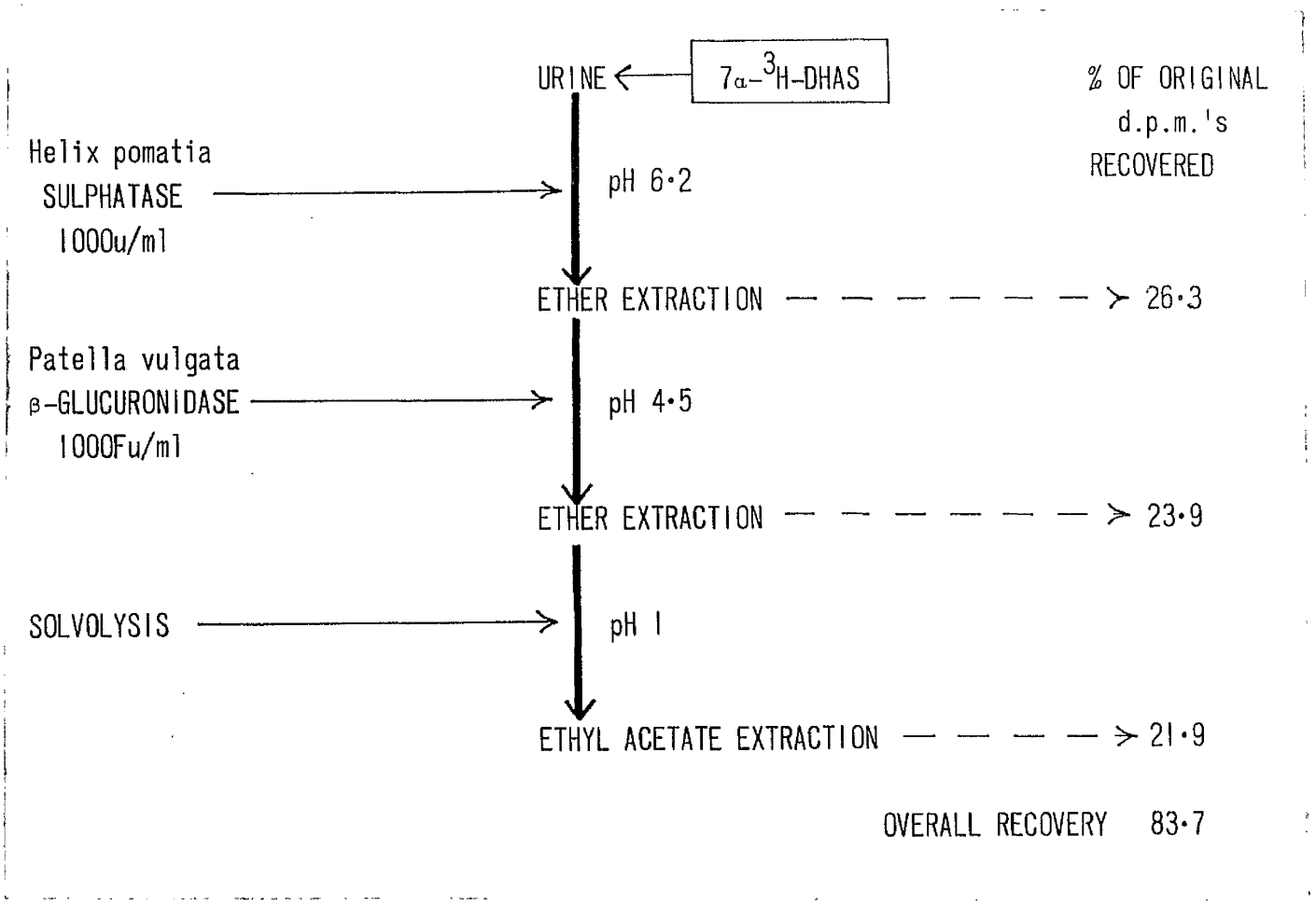


Fig. 12. Flow diagram for the extraction of total DHA (free, glucuronoside and sulphate) from urine.

shaking, the mixture was incubated at 37°C for 24 hours and two further extractions with ethyl acetate (20 ml) were subsequently made. The overall recovery of [7α-^{3}H] DHAS added initially to the urine was 83.7 per cent.

Commercially available sulphatase used under these circumstances did not afford complete hydrolysis of DHAS. Only 26.3 per cent of the original [7α-^{3}H] DHAS was recovered in the ether extract. Moreover β-glucuronidase prepared in this laboratory contains some sulphatase activity (23.9 per cent of radioactivity was recovered following this hydrolytic procedure).

However, hot hydrolysis at neutral pH as described by Fotherby (1959) was found to give good yields of [7α-^{3}H] DHA from ^{3}HDHAS after benzene extraction (73 per cent)(p.160). This method, used throughout this investigation, has not had a widespread clinical application which may in part be due to its specificity for Δ5-3β-hydroxysteroid sulphates.

Glucuronoside conjugates were hydrolysed enzymatically with β-glucuronidase. Sulphatase, found to be present in this preparation, was inhibited by potassium dihydrogen phosphate (Kellie and Wade, 1957).

A flow diagram indicating these hydrolytic procedures, subsequent purification and quantitation is shown in Fig. 13.

METHOD /

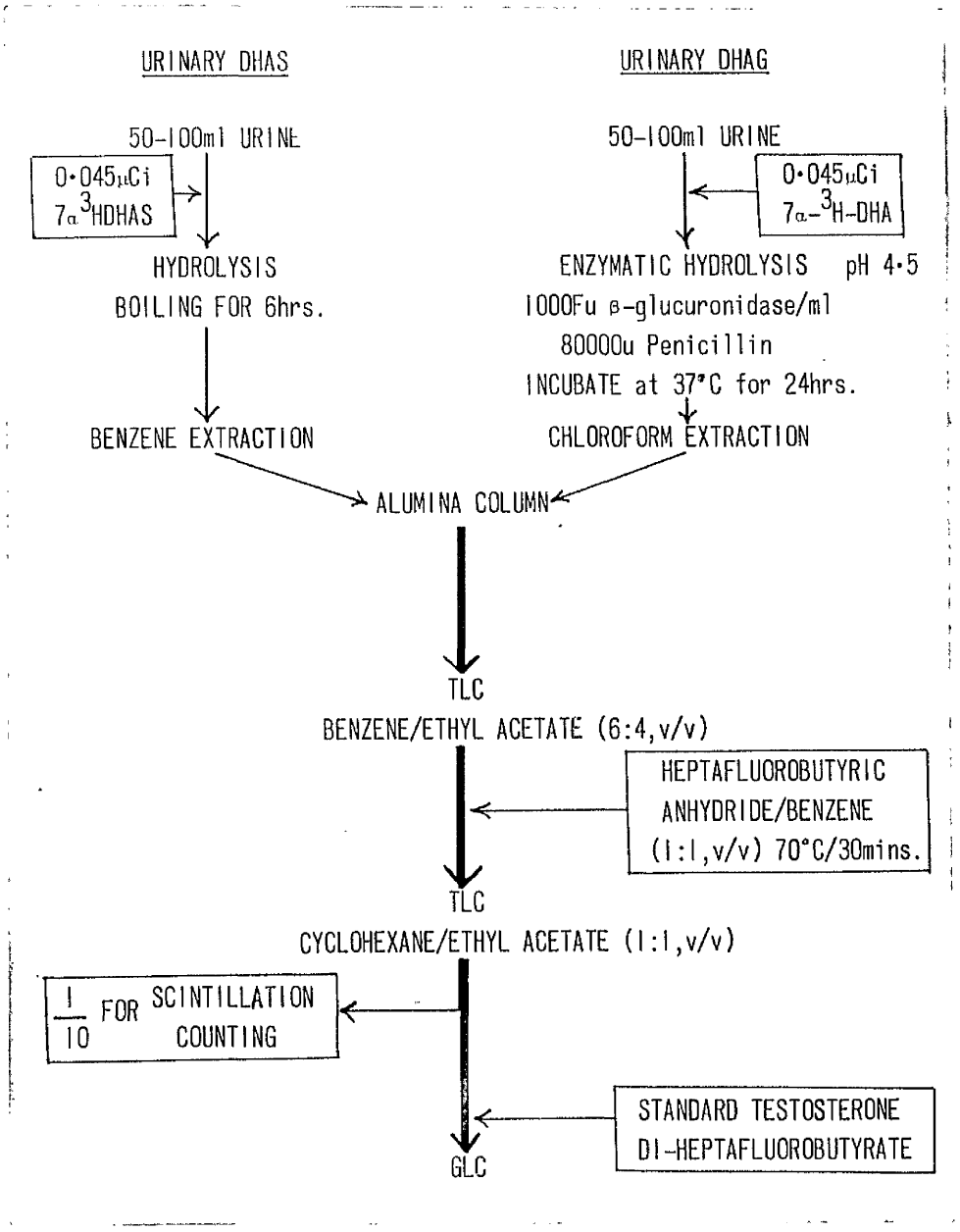


Fig. 13. Flow diagram of procedures used in the estimation of urinary DHA (sulphate and glucuronoside esters).

METHOD FOR THE ESTIMATION OF URINARY DEHYDROEPIANDROSTERONE SULPHATE (DHAS).

Dehydroepiandrosterone sulphate in two aliquots of urine (50 - 100 ml) from each 24-hour collection was hydrolysed according to the method of Fotherby (1959). To each urine sample 0.045 μ Ci [7α-^{3}H] DHAS (S.A. 290 mCi/mM) was added to act as an internal standard for recovery estimations. Hydrolysis was effected by placing the aliquots of urine, without pH adjustment, in boiling water for 6 hours (Fig. 14). A condenser was fitted to each flask. After cooling in ice, the free steroid (DHA) was extracted twice with benzene (20 ml) and the benzene extract washed once with deionised water (20 ml). The extract was reduced to small volume by evaporation in a water bath at 40°C under a stream of oxygen-free nitrogen and then introduced quantitatively with benzene (total 10 ml) onto an alumina column (6 cm x 0.5 cm I.D.:1.7g aluminium oxide). The benzene fraction was discarded and steroids eluted with 0.3 per cent ethanol in benzene. After reduction to small volume the eluate was applied to a silica gel plate and the chromatogram developed twice (1 hour each run) in benzene:ethyl acetate (6:4,v/v). Dehydroepiandrosterone [7α-^{3}H] was located using the Panax radiochromatogram scanner. Silica in the area corresponding to the radioactive peak was taken up by suction onto the sintered glass disc within the extraction thimble. Elution

of /

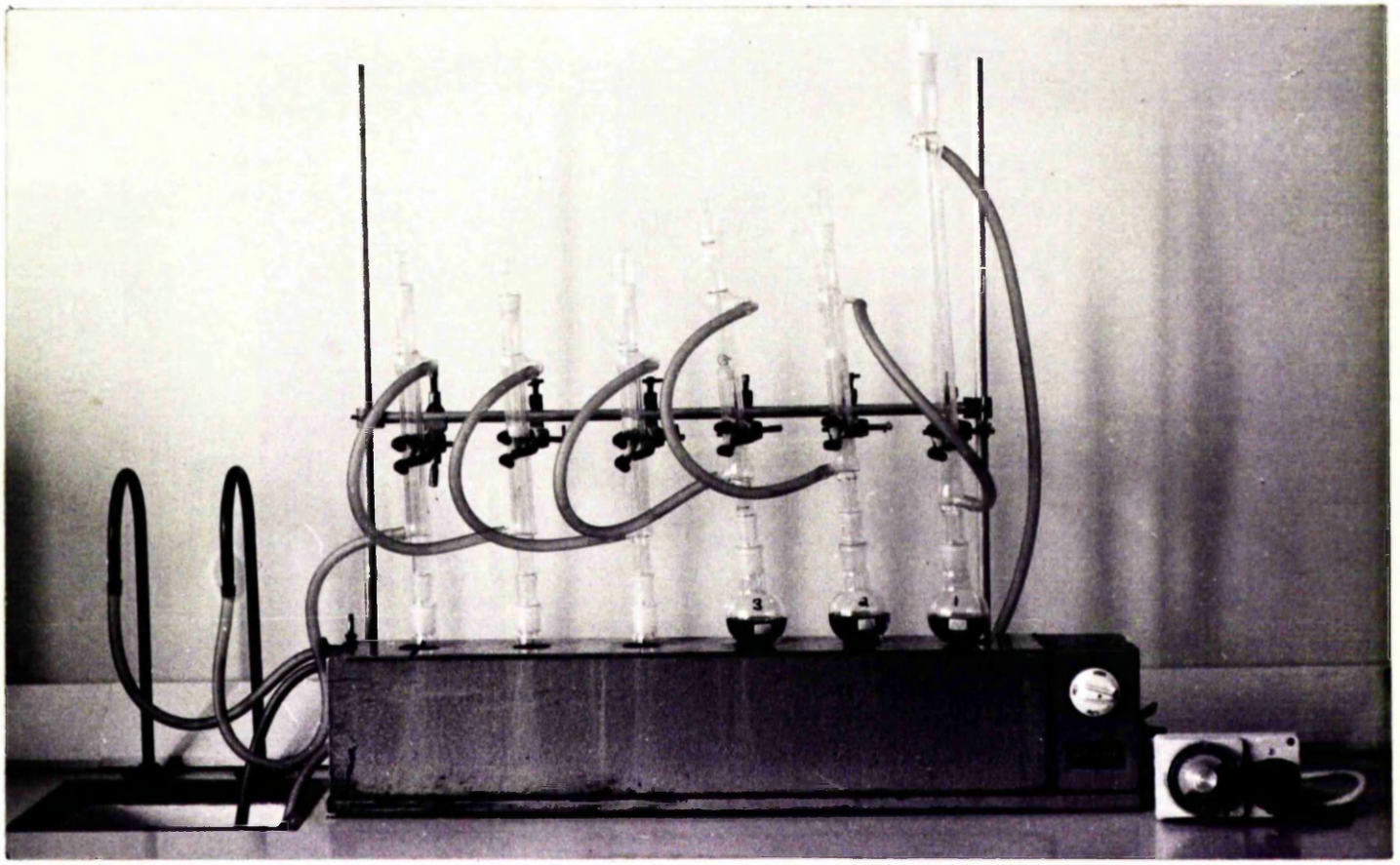


Fig. 14. Apparatus used in the hydrolysis of dehydroepiandrosterone sulphate in urine samples (Fotherby, 1959).
Urine samples in round-bottomed flasks are placed under condensers in a boiling water bath for 6 hours.

of steroid from the silica was effected with ethyl acetate (3 x 2 ml) by inverting the extraction thimble and allowing the solvent to percolate through the silica and sintered glass disc into a pear-shaped flask. After blowing to dryness, the residue was dried completely in a vacuum desiccator for 1 hour and thereafter esterified with dried benzene and heptafluorobutyric anhydride (equal parts, 25 µl). The reaction mixture was heated at 70°C for 30 minutes according to the method of Exley and Chamberlain (1967). Excess reagent was removed under a stream of nitrogen at 40°C. Samples were further purified by t.l.c. in the solvent system cyclohexane:ethyl acetate (1:1,v/v). Prior to quantitation by g.l.c., an aliquot of each sample was taken for radioactive counting by liquid scintillation. Standard testosterone di-heptafluorobutyrate was injected into the g.l.c. column along with each sample. The column employed for quantitation was a 9 foot coiled hybrid column comprising 2 feet of 1 per cent SE 30 (Silicone Gum Rubber E - Applied Science Laboratories, Inc., P.O. Box 440, State College, Pennsylvania, U.S.A.) on Gas Chrom Q, mesh-size 100/120, (Applied Science Laboratories) and 7 feet of 1 per cent NGSebacate (Neopentyl Glycol Sebacate - Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire, Wales) on Gas Chrom Q. Injection was made with an S.G.E. 10 µl syringe having an 11.5 cm needle (Scientific Glass /

Glass Engineering Pty Ltd., 657 North Circular Road, London, England). Final quantitation was by electron capture detection. The nitrogen carrier gas flow was 40 ml per minute. The column oven temperature was 180° C and the detector oven temperature 230° C.

Evaluation of the Fotherby Method for DHAS Hydrolysis.

To test the efficiency of hydrolysis by the Fotherby method (1959) for Δ^5-3β-hydroxysteroid sulphates, recovery of DHAS (20 μg) added to a series of flasks containing water (50 ml) was estimated under exactly similar conditions over a period of 6 hours boiling. Following extraction with benzene (2 x 20 ml), the organic phase was blown dry and the residue subjected to the Zimmermann reaction. Percentage recoveries are seen in Fig. 15. It will be seen that hydrolysis proceeded rapidly for the first two hours followed by a slowing down to give maximum recoveries of 84 per cent after 5 hours boiling. In practise, cohorts of 6 flasks were placed under condensers in a water bath controlled by a time switch. A preheating time of 1 hour was allowed for the water to reach boiling point and 6 hours allowed for the hydrolytic action. The system was timed to start at 02.00 hours so that extraction could be effected at 09.00 hours.

Because of the lack of specificity of the Zimmermann reaction for DHA, g.l.c. analysis of the hydrolysate was undertaken. Further aliquots of /

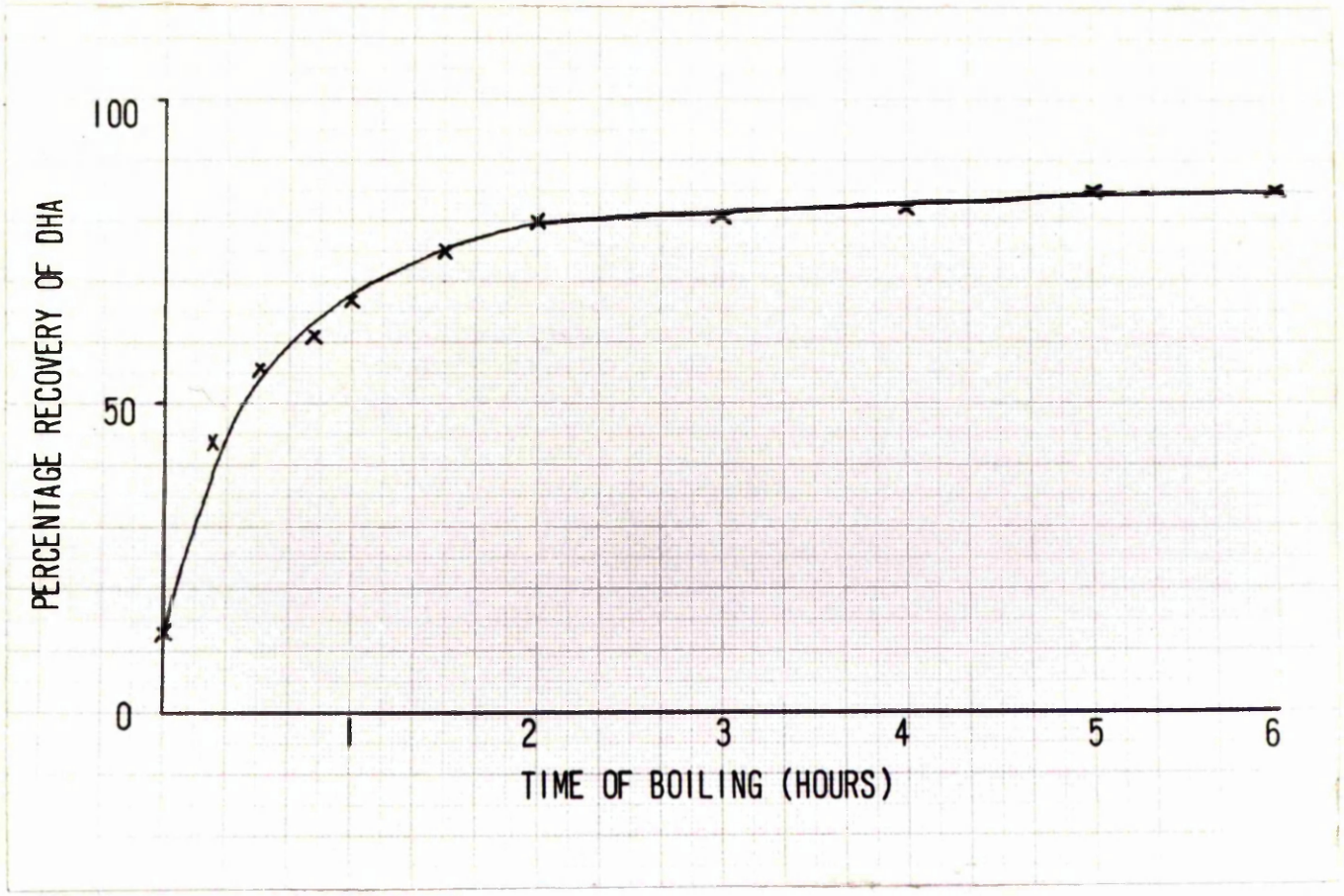


Fig. 15. Percentage recovery of dehydroepiandrosterone from dehydroepiandrosterone sulphate introduced into 50 ml aliquots of water and subjected to boiling (Fotherby, 1959). Recovery from three samples at each time interval was undertaken.

of DHAS (20 μg) and DHA (20 μg) in water (50 ml) were taken through the method. The dried residues were treated with dry benzene (25 μl) and heptafluorobutyric anhydride (25 μl) for 30 minutes at 70°C (p. 120). Excess reagents were removed under nitrogen and the ester taken into solution in benzene:ethyl acetate (9:1, v/v) (2 ml) for g.l.c. quantitation. Each sample gave a single peak corresponding in retention time to standard DHA heptafluorobutyrate (DHA HFB) (Figs. 16 and 17). Comparison of peak heights with that of standard DHA HFB indicated a 100 per cent recovery of the DHA added initially to water and an 83 per cent recovery of DHAS. Recovery of DHAS from water correlated well with recovery of ^{3}H-DHAS added to each urine sample (73 per cent) (Table XV).

Duplicate samples of dehydroepiandrosterone glucuronoside (DHAG) (20 μg) and androsterone sulphate (20 μg) in water (50 ml) were also submitted to the hydrolytic procedure in order to determine the stability of the osidic linkage at boiling point and to assert the specificity of the method for Δ^{5}-3β-hydroxysteroid sulphates. Following extraction with benzene (2 x 20 ml) and washing with deionised water (20 ml), the Zimmermann reaction was applied to the dried steroid residue. No Zimmermann positive compounds were obtained. Thus the method is specific for Δ^{5}-3β-hydroxysteroid sulphates and is reliable for the estimation of DHAS.

HYDROLYSIS /

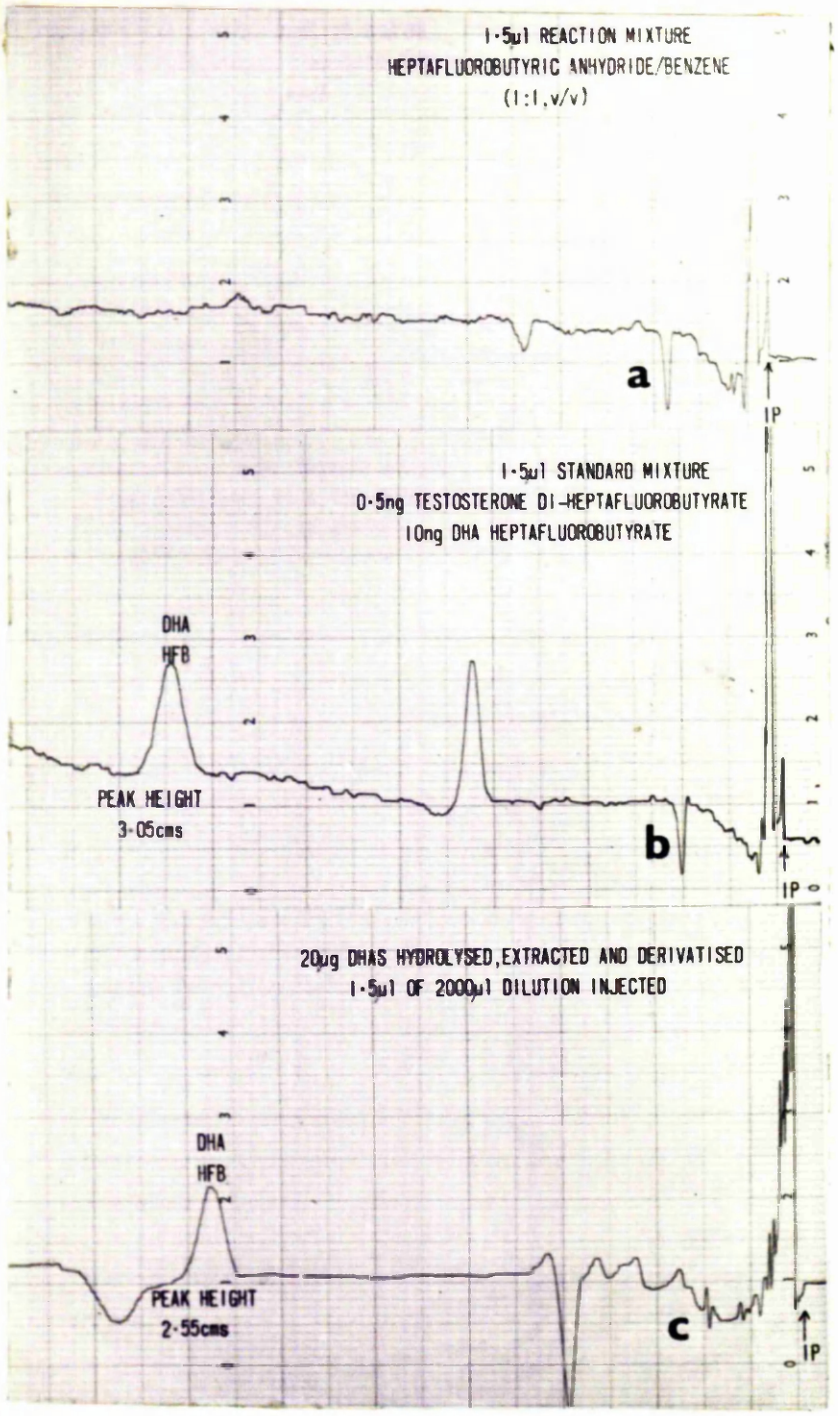


Fig. 16. G.l.c. evaluation of the efficiency of the Fotherby hydrolytic procedure for DHAS (20 µg) in water (50 ml).

Explanation of Fig. 16.

(a) The reaction mixture does not produce a peak in the region of DHA HFB.

10 ng of standard DHA HFB gives a peak of 3.05 cm (b).

Given that 20 µg DHAS ≡ 13.5 µg DHA

and assuming 100 per cent recovery of DHA from DHAS after boiling extraction and esterification

then in 2,000 µl of diluent there should be 13.5 µg DHA

and 2 µl of diluent should contain 13.5 ng DHA.

But 1.5 µl of the sample gives a peak height of 2.55 cm (c).

Therefore 1.5 µl of the sample contains 8.36 ng DHA.

Thus the recovery of DHA by the Fotherby hydrolysis of DHAS is 82.52 per cent.

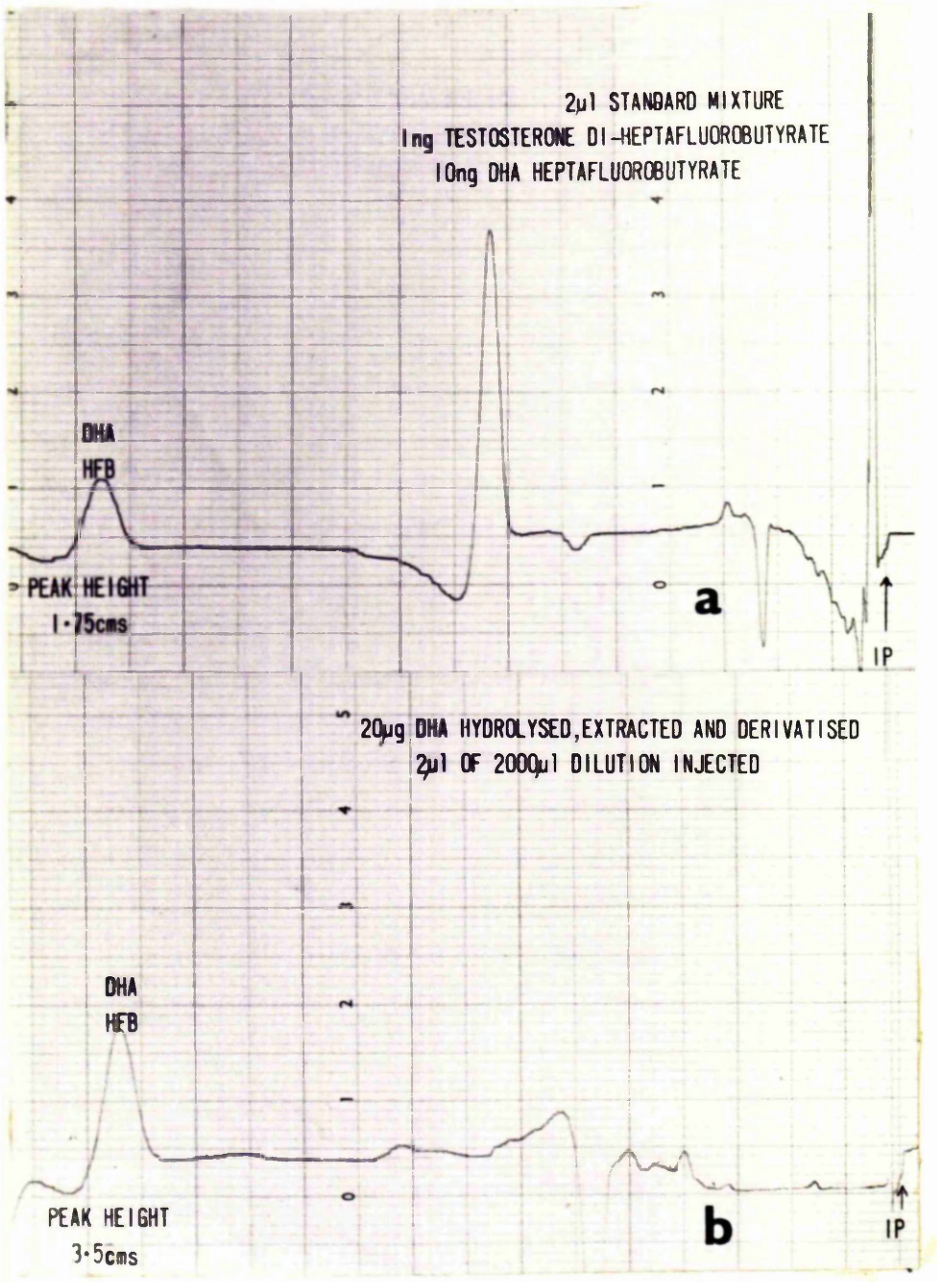


Fig. 17. G.l.c. evaluation of DHA subjected to the Fotherby hydrolytic procedure.

Explanation of Fig. 17.

20 μg of DHA in 50 ml of water was boiled, extracted and esterified.

Assuming a 100 per cent recovery

Then in 2,000 μl of diluent there would be 20 μg DHA

and 2 μl of diluent should contain 20 ng DHA.

10 ng of standard DHA gives a peak height of 1.75 cm (a)

2 μl of sample gives a peak height of 3.5 cm (b).

Therefore 2 μl of sample contains 20 ng DHA.

The recovery of free DHA after boiling according to the Fotherby method (1959) is 100 per cent.

HYDROLYSIS OF URINARY DEHYDROEPIANDROSTERONE GLUCURONOSIDE (DHAG).

The hydrolysis of DHAG in urine (50 - 100 ml) was effected at pH 4.5 by incubation with β-glucuronidase (1,000 F.u. per ml urine) for 24 hours at 37°C. The pH was maintained by the addition of 0.2 M acetate buffer (11.57 g sodium acetate and 7.5 ml glacial acetic acid in 1 l deionised water)($^1/_{10}$ vol.). Penicillin (80,000 units) was added to prevent bacterial growth and potassium dihydrogen phosphate (200 mg) used to inhibit sulphatase activity in the enzyme preparation (Kellie and Wade, 1957). Extraction of free DHA was made with chloroform (3 x 20 ml). The organic phase was washed successively with 0.1 N sodium hydroxide ($^1/_{20}$ vol.) and deionised water ($^1/_{20}$ vol.) and was then dried by filtration through anhydrous sodium sulphate. Recovery of ^{3}H-DHA added initially to urine samples indicated that recovery of free DHA at this stage was 72 per cent. Thereafter samples were chromatographed as for the sulphate fraction.

Evaluation of the Glucuronoside Hydrolysis Method.

The potency of β-glucuronidase used throughout this study was estimated /

estimated using phenolphthalein glucuronoside as substrate (p. 74). The enzyme preparation was also known to have some sulphatase activity (Fig. 12). The addition of potassium dihydrogen phosphate (200 mg) to the incubation mixture inhibited hydrolysis of ^{3}H-DHAS (Fig. 18). The hydrolytic potential of the β-glucuronidase preparation in the presence of potassium dihydrogen phosphate was again tested against phenolphthalein glucuronoside. There was a significant inhibition of β-glucuronidase activity. The potency of the enzyme preparation was reduced from 104,500 Fishman units per gram to 55,803 Fishman units per gram.

Of the 17-oxosteroids excreted by a normal adult male, 84 per cent are conjugated with glucuronic acid (Loras et al., 1966). Normal excretion of 17-oxosteroids for this group is within the range of 6 mg to 25 mg per 24 hours (Mason and Engstrom, 1950).

It was therefore judged that 150 μg DHAG added to 50 ml aliquots of water was in excess of 17-oxosteroid glucuronoside levels likely to be encountered in the urine of children. A series of 12 such flasks were incubated for 24 hours with varying amounts of β-glucuronidase (in the absence of potassium dihydrogen phosphate). As will be seen from Fig. 19, maximum recoveries of DHA were achieved using more than 20,000 F.u. of β-glucuronidase per 50 ml. (400 F.u. per ml). In practice 1,000 F.u. per ml were employed for the /

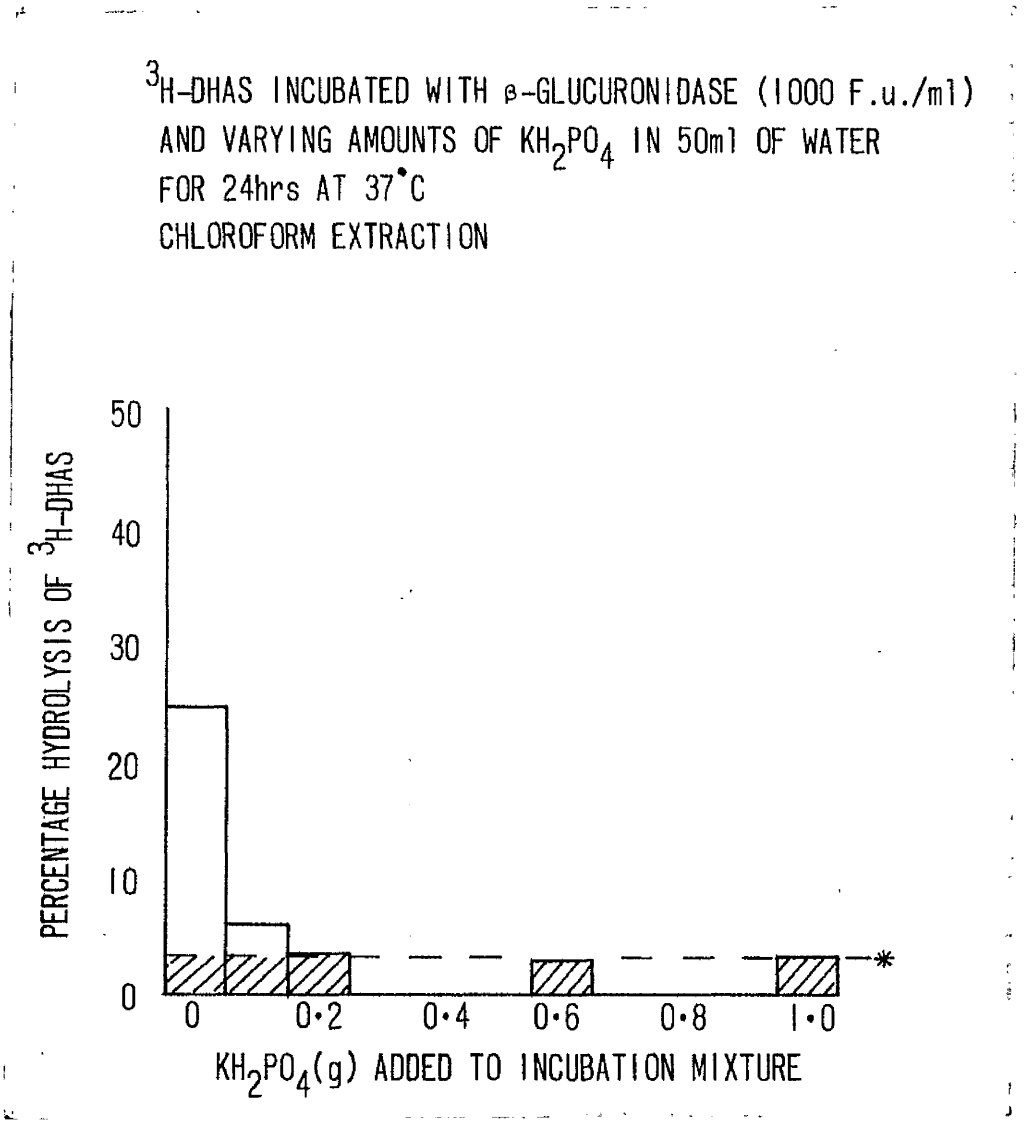


Fig. 18. The sulphatase-inhibiting effects of potassium dihydrogen phosphate on β-glucuronidase powder.

* Partitioning ^{3}H-DHAS between water and chloroform resulted in 3.3 per cent of the radioactivity being recovered from the organic phase.

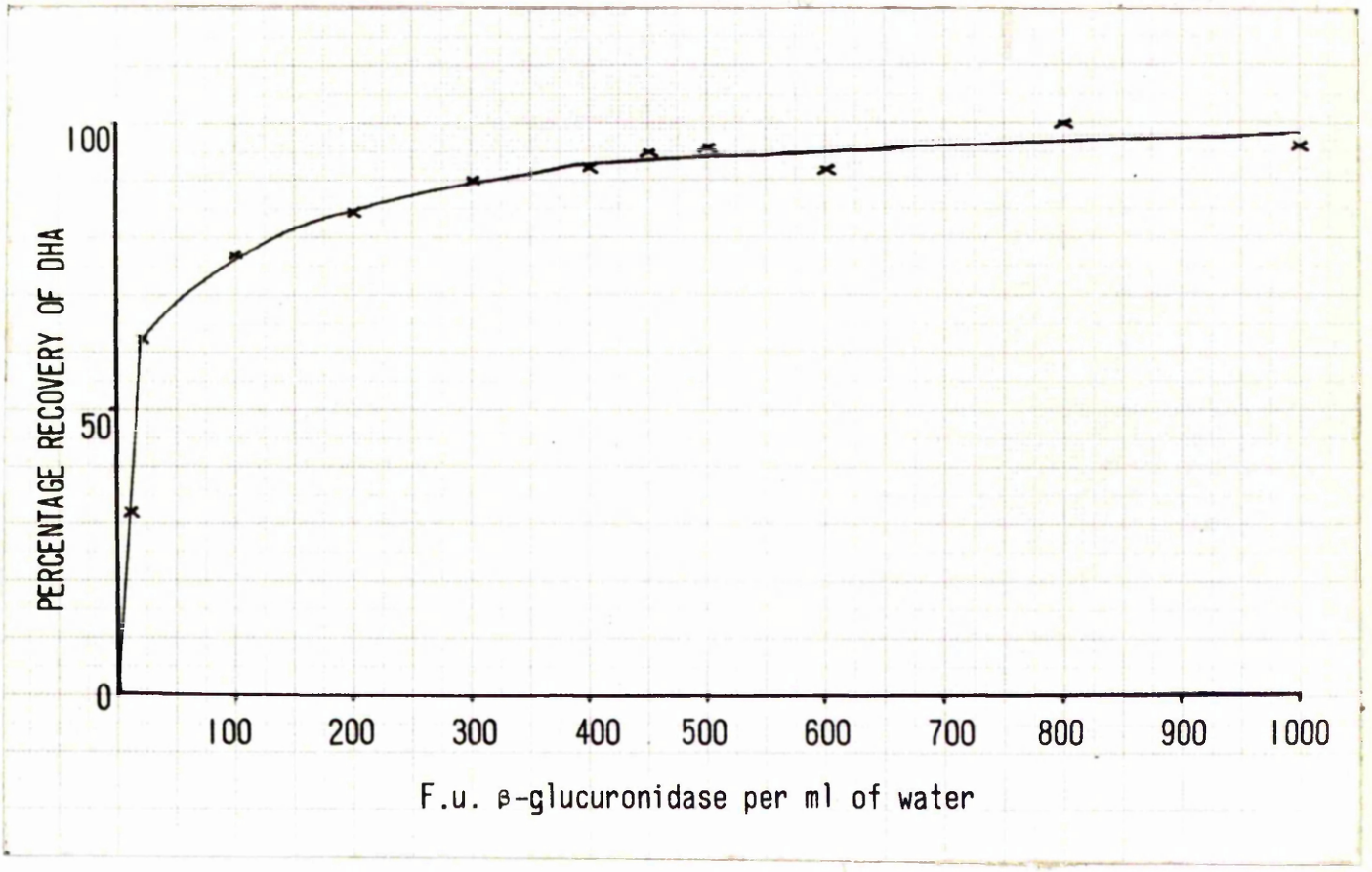


Fig. 19. The recovery of DHA from DHAG (150 μg) added to aliquots of water (50 ml) and incubated with varying concentrations of β-glucuronidase for 24 hours at 37°C. Assay was by the Zimmermann reaction.

the hydrolysis of urine samples. Thus even although enzyme activity is reduced by 53.4 per cent in the presence of potassium dihydrogen phosphate, the quantity added to urine samples is still in excess of the hydrolytic activity required.

EXTRACT PURIFICATION BY COLUMN CHROMATOGRAPHY

Following hydrolysis, urine extracts were applied to alumina columns and the steroid (DHA) eluted with 0.3 per cent ethanol in benzene (v/v).

The properties of the column were tested by introducing ^{3}H-DHA in benzene onto the alumina and eluting it with 20 ml, 40 ml and 20 ml of the above solvent. The eluate was collected in 10 ml aliquots and the radioactivity recovered (d.p.m.'s) in each sample was calculated from liquid scintillation spectrophotometric counting. The overall recovery from the column was 96.1 per cent and 95 per cent of the introduced radioactivity was recovered from the second, third and fourth aliquots (Fig. 20).

The standard principal 17-oxosteroids were applied to and eluted from the column using the same techniques. The Zimmermann reaction was performed on the dried residue in each of the 10 ml aliquots of eluate. Those aliquots giving a Zimmermann positive reaction are shown /

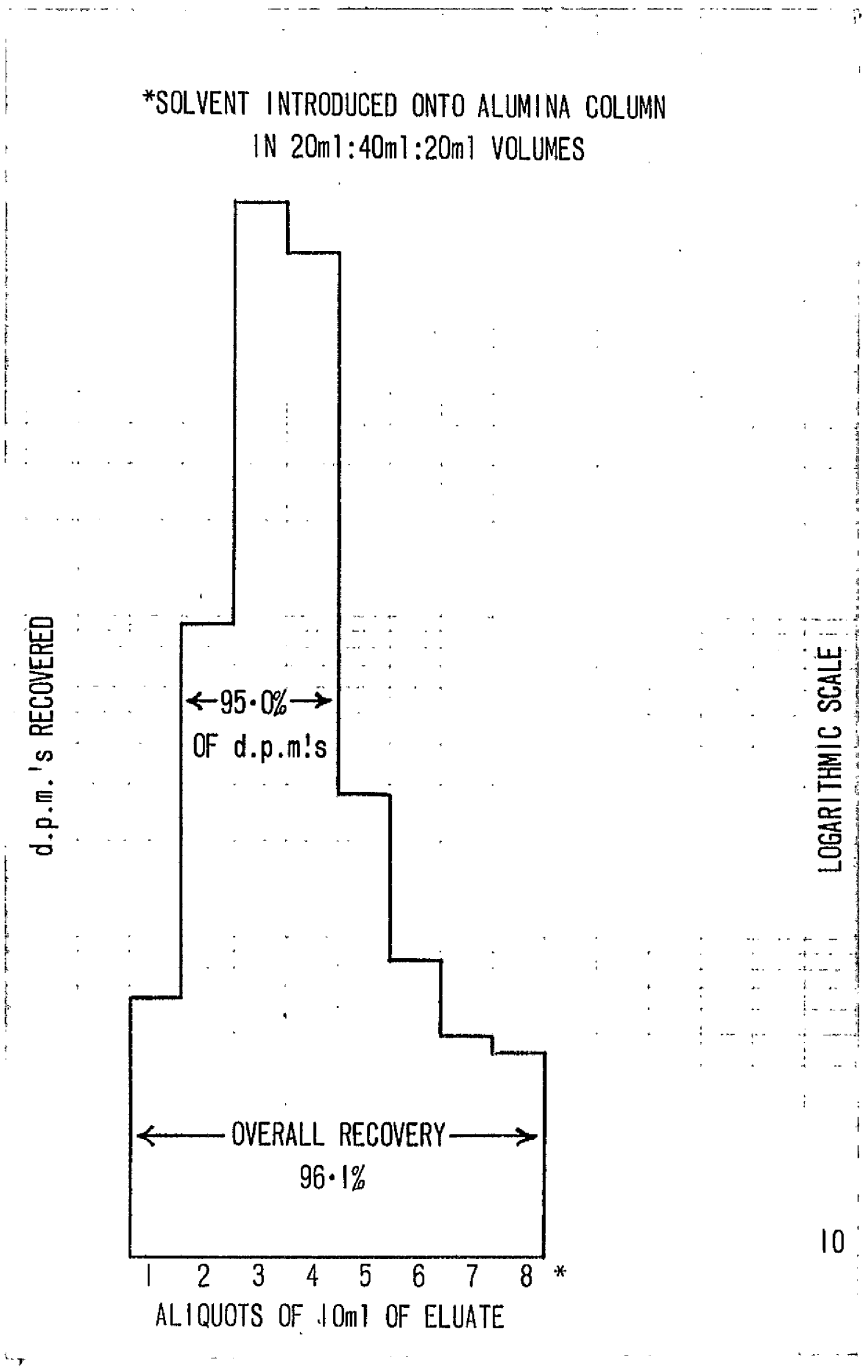


Fig. 20. Recovery of $[7\alpha\text{-}^{3}H]$ dehydroepiandrosterone from a Fotherby-type column (6 cm x 0.5 cm I.D.) containing 1.7 g aluminium oxide. Eight 10 ml aliquots of mobile phase (0.3 per cent ethanol in benzene) were collected.

shown in Table IV. The retention of ^{3}H-T was also determined by submitting the 10 ml aliquots to liquid scintillation counting. It will be seen from Table IV that androsterone, aetiocholanolone and testosterone are also eluted with the DHA.

Urine extracts were applied to the column and 0.3 per cent ethanol in benzene (v/v) was introduced in two fractions (20 ml followed by 40 ml). The salient 30 ml aliquot (representing the second, third and fourth 10 ml aliquots) was collected and reduced to small volume pending further purification by t.l.c..

THIN LAYER CHROMATOGRAPHY OF FREE STEROIDS

Residues were taken up in ethyl acetate (6 x 10 µl) and transferred to silica gel plates using micro-capillary pipettes. Each sample was applied as a spot 2 cm from the edge of the t.l.c. plate. The chromatogram was developed in the solvent system benzene:ethyl acetate (6:4, v/v) for two 1 hour periods, allowing the plate to dry between runs.

After spraying the plates with 0.1 per cent Rhodamine 6 G in ethanol, cold standards, developed in the above manner, were visualised under U.V. light. The resolution of the system was related to standard testosterone and characteristic running times are given in Table V.

Since /

TABLE IV. Retention of some C19 steroids on an aluminium oxide column (6 x 0.5 cm I.D.; 1.7 g alumina).

Solvent (0.3 % EtOH in Benzene)	20 ml		40 ml			
ALIQUOTS (10 ml)	1	2	3	4	5	6
DEHYDROEPIANDROSTERONE	-	+	+	+	-	-
ANDROSTERONE	-	+	+	+	+	-
AETIOCHOLANOLONE	-	+	+	+	+	+
*11-OXO-AETIOCHOLANOLONE	-	-	-	-	+	+
TESTOSTERONE	-	+	+	+	+	+

* AS REPRESENTATIVE OF THE 11-OXY-17-OXOSTEROIDS.

Solvent (0.3 per cent ethanol in benzene) was applied as 20 ml followed by 40 ml.

10 ml aliquots of eluate were collected.

Zimmermann positivity in aliquots are indicated thus:- +.

TABLE V. Separation of steroids by t.l.c. in benzene:ethyl acetate (6:4, v/v).

FREE STEROID	R_S VALUE *
PREGNANETRIOL	0.15
PREGNANEDIOL	0.56
11β-HYDROXYAETIOCHOLANOLONE	0.56
11-KETOAETIOCHOLANOLONE +	0.57
11β-HYDROXYANDROSTERONE	0.71
11-KETOANDROSTERONE ++	0.71
TESTOSTERONE	1.00
17α-HYDROXYPREGNENOLONE	1.14
17α-HYDROXYPROGESTERONE	1.28
EPIANDROSTERONE	1.31
DEHYDROEPIANDROSTERONE	1.36
ANDROSTERONE	1.43
AETIOCHOLANOLONE	1.47
PREGNENOLONE	1.52
ANDROSTENEDIONE	1.54
PROGESTERONE	1.77

DEVELOPMENT 2 x 1 HOUR

* s - TESTOSTERONE

\+ 11-OXO-AETIOCHOLANOLONE

++ 11-OXO-ANDROSTERONE

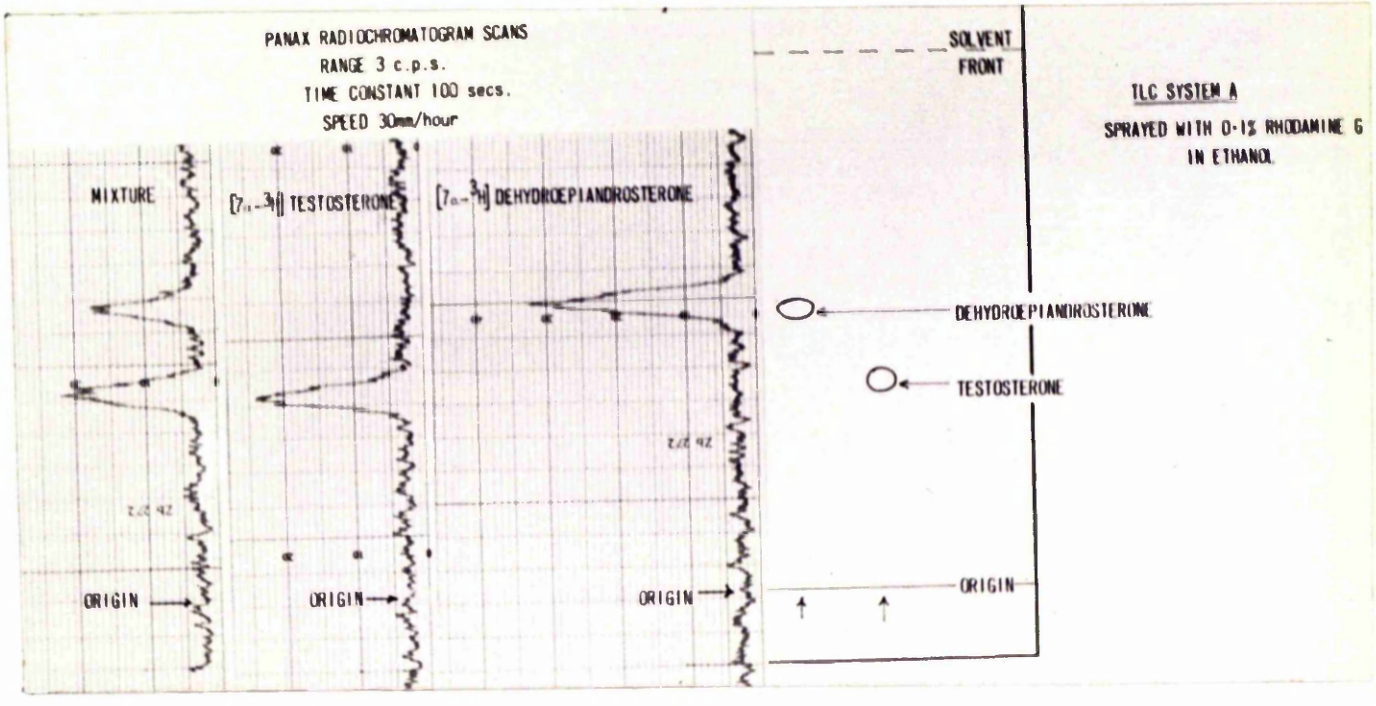


Fig. 21. Separation of DHA from testosterone in benzene:ethyl acetate (6:4, v/v). The position of standards sprayed with Rhodamine 6 G and visualised under U.V. light correlates well with ^{3}H-DHA and ^{3}H-T located by radiochromatogram scanning.

Since radioactivity had initially been added to each urine sample, t.l.c. plates carrying urine extracts were scanned and the area corresponding to the radioactive peak of ^{3}H-DHA was removed and eluted. The t.l.c. system used gives good separation of DHA and testosterone (Fig. 21). Thus testosterone heptafluorobutyrate may be employed as a standard for g.l.c. estimations since testosterone native to urine samples is removed during this purification step.

ESTERIFICATION OF STEROIDS

The low levels of steroids in childrens' urine and plasma demand sensitive methods of detection. Electron capture detection of steroid derivatives with a high electron affinity allows nanogram (ng) and picogram (pg) quantities to be detected (Exley, 1966). The heptafluorobutyrate esters, first used by Clark and Wotiz (1963), have strong electron capturing characteristics due to the introduction of 7 fluorine atoms into the steroid molecule. Urine extracts and steroid standards were therefore treated with heptafluorobutyric anhydride according to the method of Exley and Chamberlain (1967).

Preparation of standard dehydroepiandrosterone heptafluorobutyrate (DHA HFB).

Dehydroepiandrosterone (1 mg) was esterified with dry benzene (50 µl) and /

and heptafluorobutyric anhydride (50 µl) for 30 minutes at 70°C. Excess reagent was removed at 40°C under a stream of nitrogen. A small amount of ^{3}H-DHA (10,000 d.p.m.'s:S.A. 290 mCi per mM) was added to the standard prior to esterification. Partitioning ^{3}H-DHA between 70 per cent methanol in water and n-hexane (1:1, v/v) resulted in a quantitative recovery of radioactivity from the methanol phase. However, following esterification and partition between the above solvents, a near quantitative yield (99.9 per cent) of radioactivity was recovered from the hexane fraction. These results indicate quantitative esterification of the 3β-hydroxysteroid.

The hexane fraction was reduced to small volume and applied to a t.l.c. plate. This was then developed in cyclohexane:ethyl acetate (1:1, v/v) for 1 hour. Following radiochromatogram scanning and elution of the appropriate area, 99 per cent recoveries of radioactivity were consistently achieved. Subsequent dilution in benzene:ethyl acetate (9:1, v/v) was made to standard concentration thus giving stock solutions of 1 µg per 1 µl (for F.I.D.) and 10 ng per 1 µl (for E.C.D.). Gas liquid chromatography of standards esterified in this way gave a single peak (Fig. 22) (p.118).

Preparation of Standard Testosterone Di-heptafluorobutyrate.

Testosterone /

Testosterone heptafluorobutyrate was prepared under exactly similar conditions as DHA HFB. Subsequent partition, as previously, resulted in 94.6 per cent of the initially added ^{3}H-T being recovered from the hexane phase (i.e. 94.6 per cent esterification had been effected).

Esterification of testosterone may occur at C17 and at C3. The products of the above esterification method are the 17 mono-heptafluorobutyrate and the 3,17 di-heptafluorobutyrate. This latter derivative reputedly decomposes when dry on silica gel to form stable 17 mono-heptafluorobutyrate. Following partition, the residue from the hexane phase was applied to a t.l.c. plate in benzene:ethyl acetate (9:1, v/v) (6 x 10 µl) and the chromatogram developed in benzene:ethyl acetate (4:1, v/v) before the application solvent had dried. A two-dimensional development consisting of two 45 minute runs was made. At no time was the t.l.c. plate allowed to dry. Fig. 23 indicates the location of U.V. absorbing steroids. The least polar U.V. region (testosterone di-heptafluorobutyrate) was scraped from the plate and eluted with benzene:ethyl acetate (9:1, v/v) (3 x 2 ml) and acetone (3 x 2 ml). An aliquot, taken for liquid scintillation counting, indicated percentage conversion to the di-ester. A conversion of 72 per cent of testosterone to testosterone di-heptafluorobutyrate may be achieved by this method. According to /

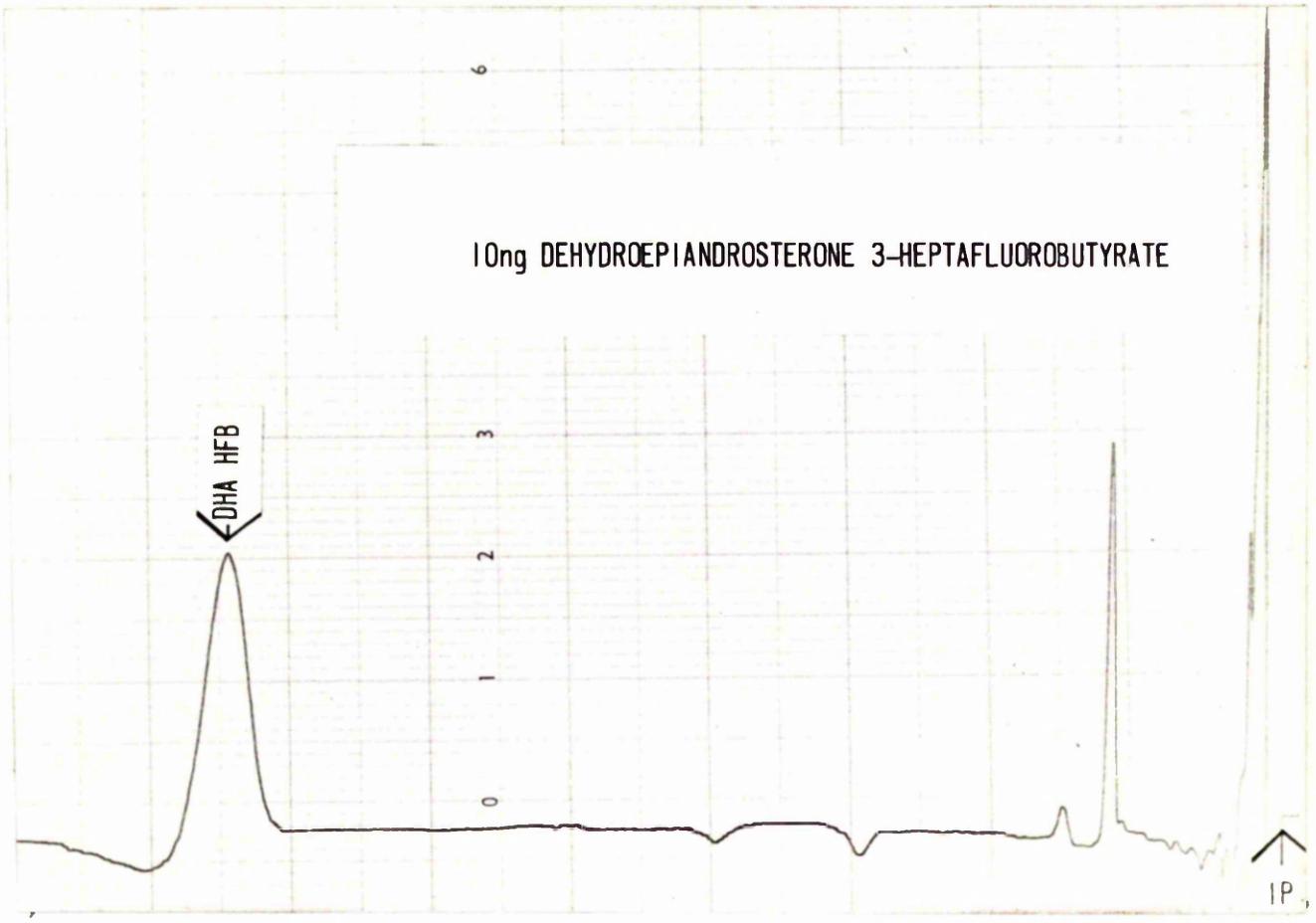


Fig. 22. Tracing obtained on gas liquid chromatography of standard dehydroepiandrosterone heptafluorobutyrate after purification by t.l.c..

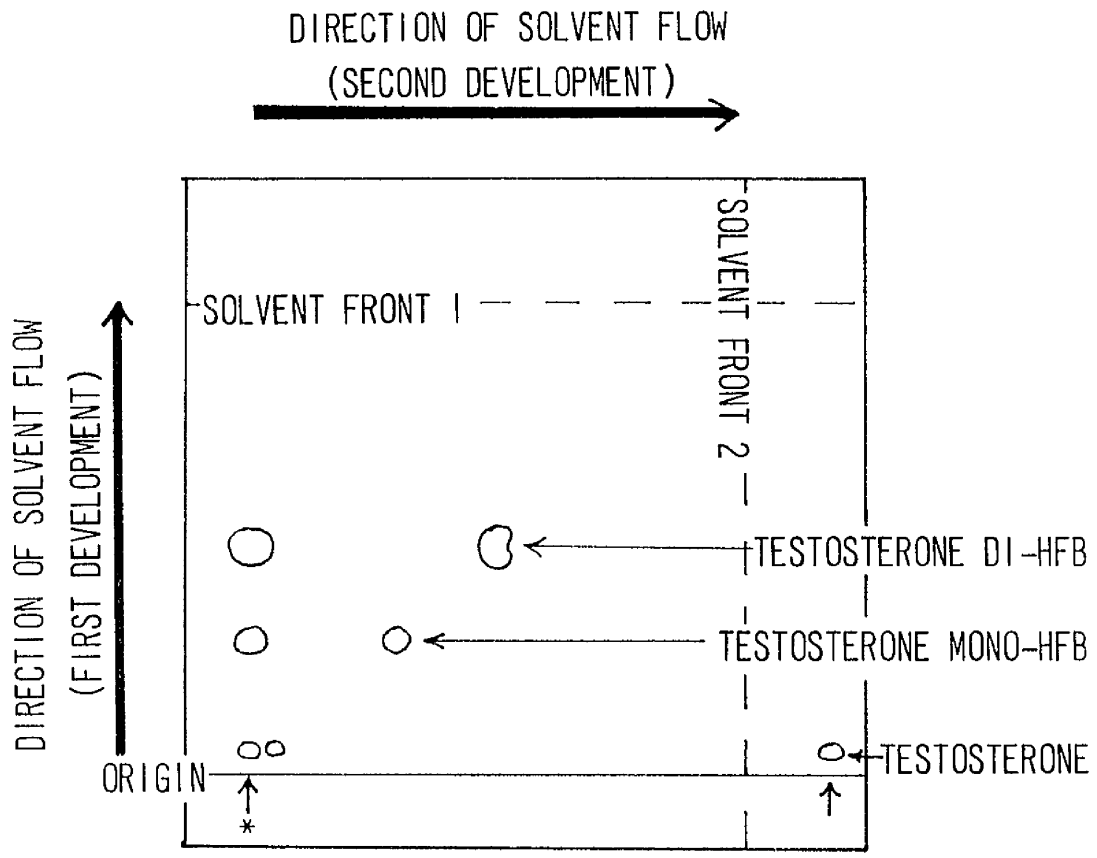


Fig. 23. Two dimensional t.l.c. separation of testosterone heptafluorobutyrates in benzene:ethyl acetate (4:1, v/v). Development for 2 x 45 minutes.

to recoveries, dilutions were made of 1 μg per 1 μl (for F.I.D.) and 1 ng per 1 μl (for E.C.D.). Prior to t.l.c. purification, g.l.c. analysis of the esterified residue indicated two products of heptafluorobutyrate derivatisation (testosterone mono- and di-heptafluorobutyrates). After t.l.c., a single peak (testosterone di-heptafluorobutyrate) was recorded on g.l.c. analysis (Fig. 24).

Esterification of urine extracts

Residues obtained from t.l.c. plates developed in benzene: ethyl acetate (6:4, v/v) were dried in a vacuum desiccator for 1 hour. Esterification was by reaction with dry benzene (25 μl) and heptafluorobutyric anhydride (25 μl) at 70 ° C for 30 minutes. Derivatives were further purified by t.l.c. in the solvent system cyclohexane:ethyl acetate (1:1, v/v) for 1 hour. Running times of standard free and esterified steroids relative to the solvent front are given in Table VI. Plates carrying urine samples were scanned and the area of the peak of esterified ^{3}H-DHA (corresponding in R_f with that of standard DHA HFB) was eluted with benzene:ethyl acetate (9:1, v/v) (3 x 2 ml) and then acetone (3 x 2 ml).

GAS LIQUID CHROMATOGRAPHY

Whereas Fotherby's hydrolysis method is specific for Δ^5-3β-hydroxysteroid sulphates, hydrolysis by β-glucuronidase lacks comparable /

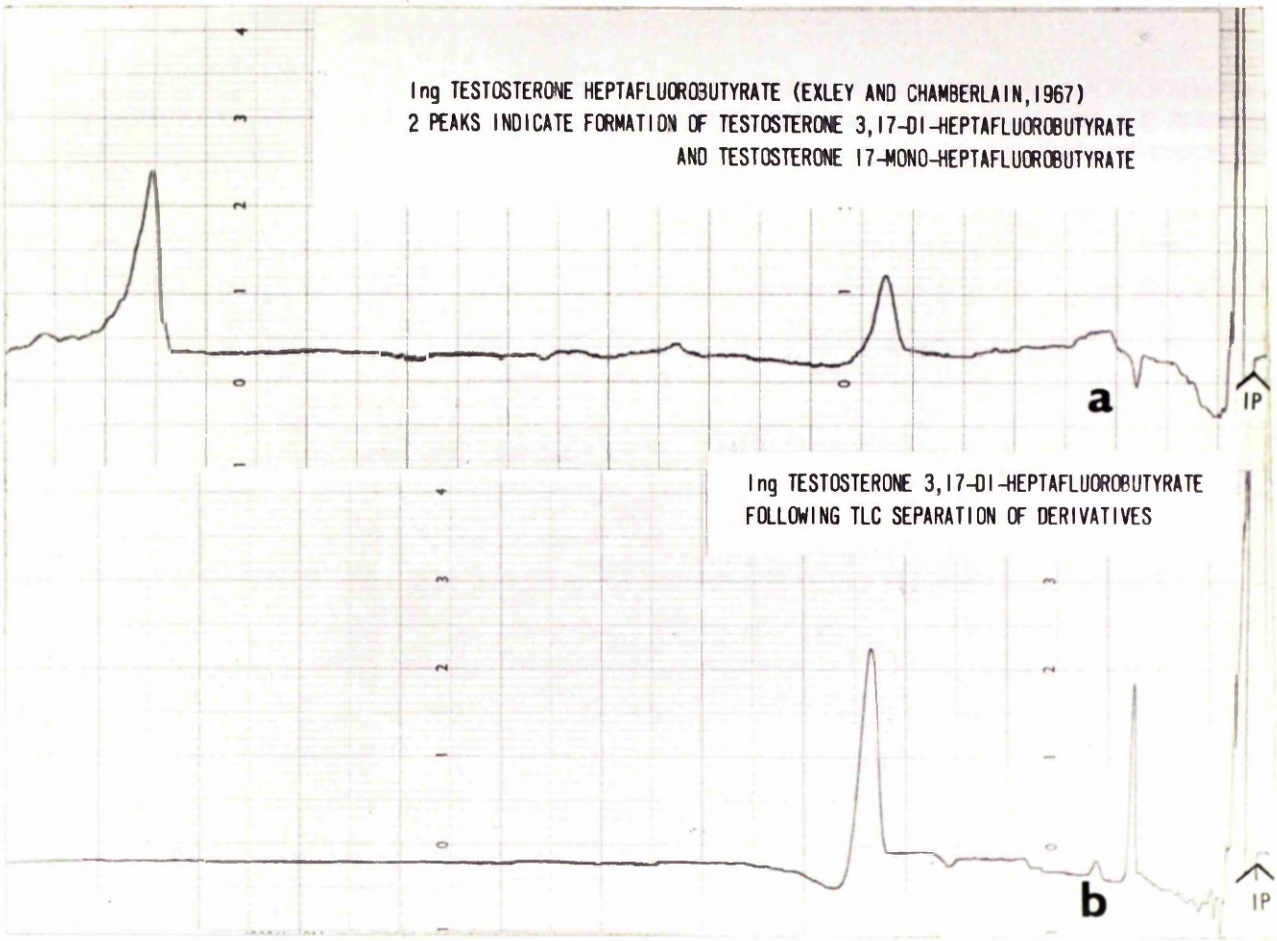


Fig. 24. G.l.c. analysis of testosterone heptafluorobutyrates prior to t.l.c. (a) and of testosterone di-heptafluorobutyrate after a two-dimensional t.l.c. separation in benzene: ethyl acetate (4:1, v/v) (b).

TABLE VI. Separation of standard steroids (free and heptafluorobutyrate derivatives) in cyclohexane:ethyl acetate (1:1, v/v).

FREE STEROID	R_f	HEPTAFLUOROBUTYRATE DERIVATIVE (HFB)	R_f
DEHYDROEPIANDROSTERONE	0.27	3β-HYDROXYL HFB	0.53
ANDROSTERONE	0.28	3α-HYDROXYL HFB	0.51
AETIOCHOLANOLONE	0.31	3α-HYDROXYL HFB	0.55
EPIANDROSTERONE	0.25	3β-HYDROXYL HFB	0.47
17α-HYDROXYPROGESTERONE	0.19		
TESTOSTERONE	0.19	3-ENOL, 17-HYDROXYL HFB	0.44
		17β-HYDROXYL HFB	0.20

Development for 1 hour (single run).

comparable specificity. Moreover, the t.l.c. systems described do not afford complete separation of either free androsterone, aetiocholanolone and DHA or of their heptafluorobutyrate esters (Tables V and VI). Liquid phase and operating conditions for g.l.c. were thus selected with a view to separating these 17-oxosteroids. An internal standard, which was not endogenous to the sample, was also required for g.l.c. analyses.

Haahti et al. (1961) reported that androsterone, aetiocholanolone and DHA could be separated by the use of two columns: one of methyl silicone polymer (SE 30) and the other of neopentyl glycol succinate (NGS). A 9 foot hybrid column incorporating these two phases proved to be successful in separating these three compounds (Prof. K. Griffiths - personal communication). Minor modifications to this column are stated below.

Preliminary silanisation of the column

Prior to packing, the empty glass column (6 mm I.D.) was rinsed with concentrated hydrochloric acid, washed with deionised water and silanised with 5 per cent (v/v) dimethyldichlorosilane in toluene for 15 minutes. The column was ready for packing after a methanol wash and thorough drying.

Coating /

Coating the Support with Liquid Phase

Gas Chrom Q, 100 - 120 mesh, was used as the support. The packing of SE 30 was prepared by dissolving the phase (0.5 g) in toluene (100 ml) (i.e. 0.5 per cent solution of SE 30, wt/v). Likewise a 0.5 per cent solution of NGS was prepared by dissolving the phase (0.5 g) in chloroform (100 ml). The relationship between concentration of phase in solution to percentage coating was that suggested by Dr. T. Simpson of Torry Research Station, Aberdeen. A 0.5 per cent solution of phase was routinely used to give a 1 per cent coating of support. Each phase was applied to 15 g of support by the filtration method. The phase was slowly introduced to the support *in vacuo* and the coating process aided by a constant swirling of the mixture. Once the vacuum was broken, the round-bottomed flask containing the mixture was quickly inverted over a Buchner filter and suction applied by a water pump. In this way the solvent drained evenly through the bed of the support which was then gently transferred, without washing, onto a 200 mm diameter watch glass. The coated support was allowed to dry at room temperature.

Packing and Conditioning the Column

Packing the column was aided by suction from an oil pump applied

to /

to the detector-end of the column and by the use of a hand-vibrator (Phase Separations Ltd.). The first 2 feet of the column was packed with 1 per cent SE 30. This phase has a higher maximum operating temperature than NGS and so was conditioned before packing with NGS. A slow flow of nitrogen carrier gas (oxygen-free) was maintained through the column as the temperature of the column oven was raised slowly to 220°C. Conditioning at this temperature was allowed to continue overnight. In this way excess phase was "bled" from the column. When the remaining 7 feet of the column had been packed with 1 per cent NGS a silanised glass wool plug was inserted into the injection end of the column and conditioning carried out again utilising a slow carrier gas flow rate but at the lower temperature of 200°C. The carrier gas flow rate was increased slowly to 40 ml per minute and the temperature dropped to 195°C. These conditions were maintained for a further 12 hours before coupling the column to the detector.

Operating Conditions

The separating powers of the column were determined by F.I.D.. Urine and plasma extracts were quantitatively analysed using E.C.D. together with the recovery rates of radioactivity which had been added to the biological material initially.

The /

The 9 foot column prepared as described did not give a baseline separation of androsterone heptafluorobutyrate, aetiocholanolone heptafluorobutyrate and dehydroepiandrosterone heptafluorobutyrate when 0.5 μg of each ester was injected into the column (Fig. 25). Neopentyl glycol succinate was replaced by neopentyl glycol sebacate (1 per cent loading) and a lowered operating temperature of 180°C was used to accommodate the phase requirements (maximum stable operating conditions - 180°C). Resolution of these steroid derivatives was satisfactorily achieved (Fig. 26). This latter column was therefore used throughout the investigation. The column oven temperature was held at 180° C and the detector oven operated at 230°C when using the E.C.D. system. Samples were injected onto the column using an S.G.E. 10 μl syringe fitted with an 11.5 cm needle.

Linearity of the Detector

The detector gave a linear response for dehydroepiandrosterone heptafluorobutyrate over the range required for analysis (Fig. 27b). Likewise the response to testosterone di-heptafluorobutyrate was proportional to the amount injected (Fig. 27a). Linearity of detector response to varying amounts of the steroid derivative was determined /

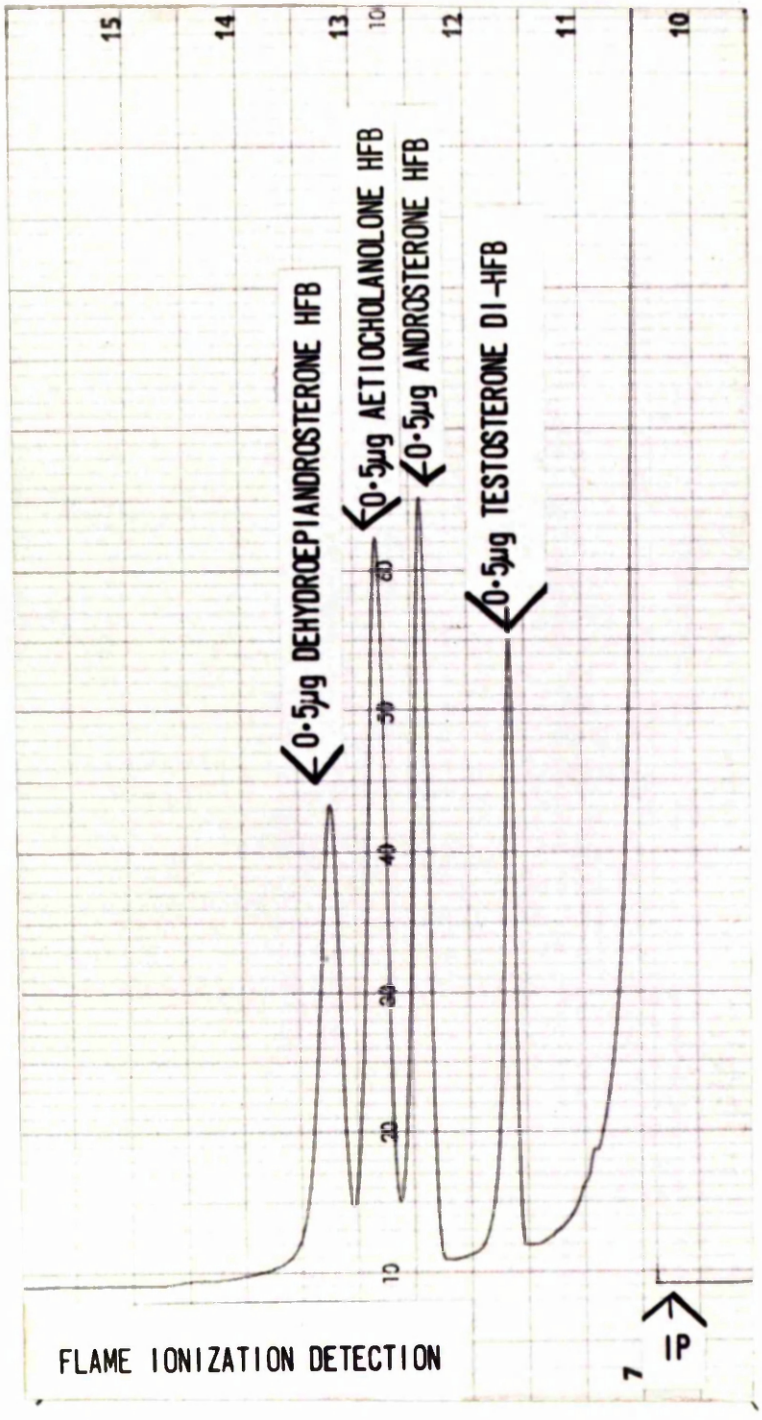


Fig. 25. Separation of the heptafluorobutyrate derivatives of dehydroepiandrosterone, aetiocholanolone and androsterone using a 9 foot hybrid column comprising 2 feet of 1 per cent SE 30 and 7 feet of 1 per cent NGS (succinate).

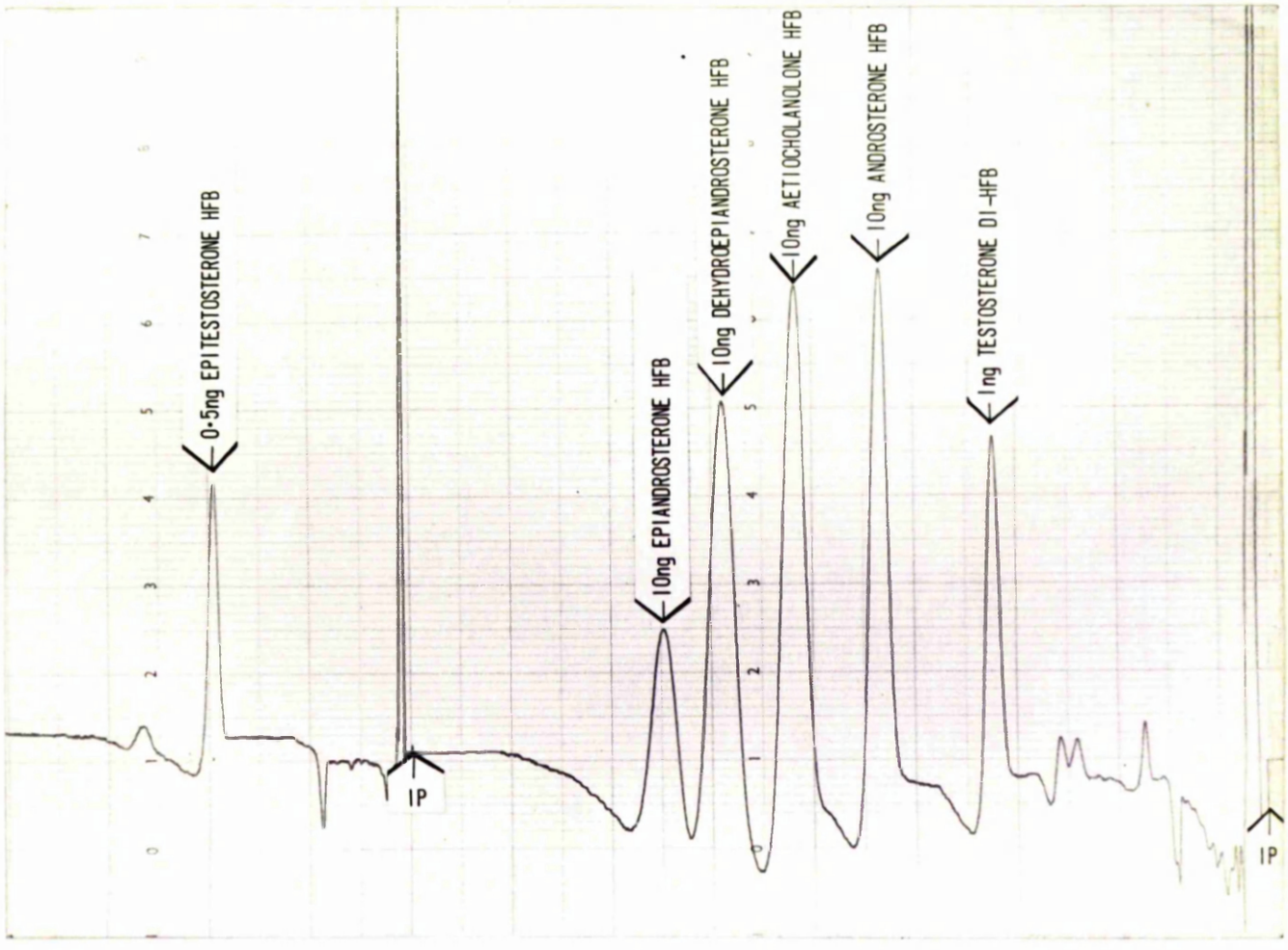


Fig. 26. Separation of the heptafluorobutyrate derivatives of epitestosterone, testosterone (di-HFB), androsterone, aetiocholanolone, dehydroepiandrosterone and epiandrosterone using a 9 foot hybrid column of 1 per cent SE 30 (2 feet) and 1 per cent NGSebacate (7 feet).

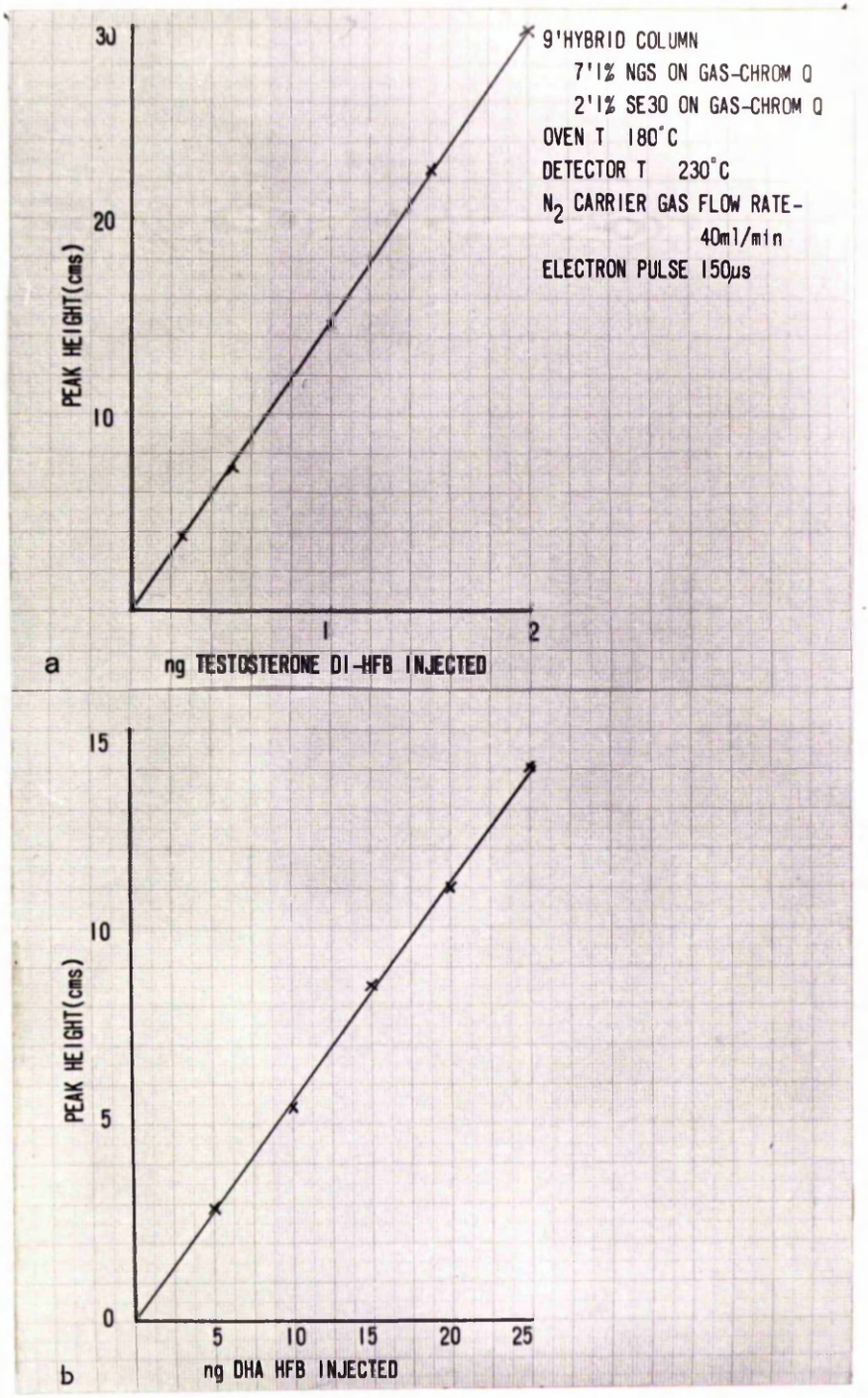


Fig. 27. Electron capture detector response (peak heights) to varying amounts of testosterone di-heptafluorobutyrate (a) and dehydroepiandrosterone heptafluorobutyrate (b).

determined

a) by using peak height only

b) by using peak area determined from the width of the peak at 50 per cent of the height x height

c) by peak area extrapolated by triangulation where the area is determined by halving the product of the peak width on the baseline and the peak height of a triangle superimposed on the signal peak.

The linearity of detection using all three modes of quantitation is shown in Fig. 28. Quantitation using peak height was chosen since fewer errors were likely to occur in the measurement of peak heights and since operating conditions were to be maintained constant as outlined.

An internal standard - testosterone di-heptafluorobutyrate - was injected with each sample. Exley and Chamberlain (1967) had determined that the molar response of testosterone di-heptafluorobutyrate was twenty to fifty times greater than the response of the hydroxyl-heptafluorobutyrates such as dehydroepiandrosterone heptafluorobutyrate. Using the conditions and column described, 0.5 ng of testosterone di-heptafluorobutyrate gave a peak height of a similar magnitude to that given by 10 ng of DHA HFB (Fig. 16b). Standard amounts of DHA HFB /

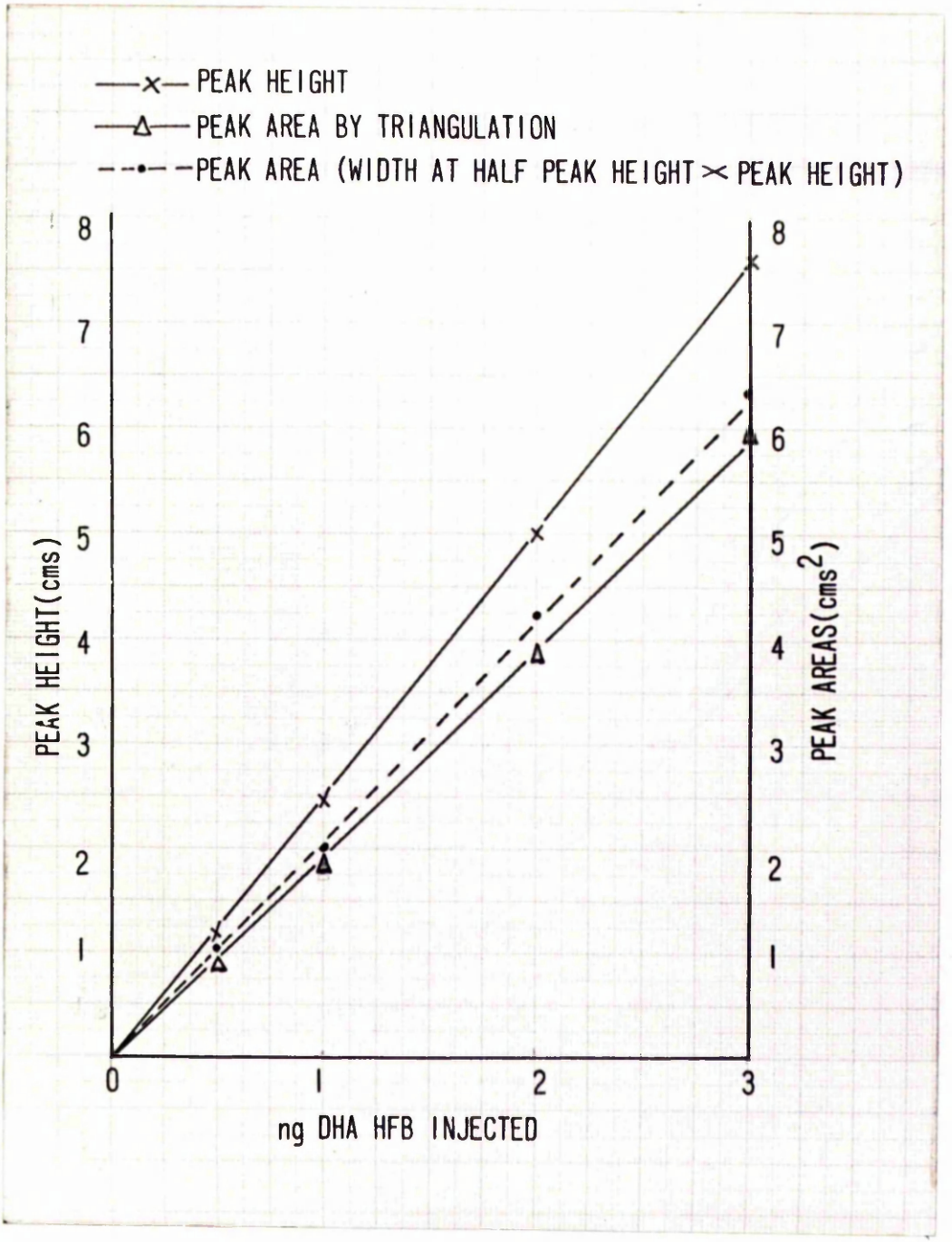


Fig. 28. Linearity of detector response (ECD) determined from peak height and peak area measurements.

DHA HFB and testosterone di-heptafluorobutyrate were injected daily prior to sample analysis. Quantitation was made by a comparison of peak height ratios of native DHA HFB against internal standard testosterone di-heptafluorobutyrate and the standard trace of a known amount of DHA HFB against standard testosterone di-heptafluorobutyrate (p. 138).

Plasma dehydroepiandrosterone sulphate estimation.

Venous blood (10 - 20 ml) was withdrawn from patients with cystic fibrosis and from a series of children who were being investigated for non-endocrine disease. The blood was heparinised and the plasma separated. The plasma was thereafter processed immediately or stored at -15°C until assayed.

Determination of plasma DHAS was according to the method of Brownsey et al. (1972). This method had been recommended to this laboratory (W.H.) by Prof. K. Griffiths, Tenovus Institute, Cardiff, prior to its publication. A flow diagram of procedures used is shown in Fig. 29. Dehydroepiandrosterone sulphate-$[7\alpha\text{-}^{3}H]$ (0.045 μCi per mM:S.A. 290 mCi per mM) was added to each plasma sample for recovery estimations. Samples were extracted twice with four volumes of ice-cold acetone. Following extraction the tubes were placed in ice for 20 minutes and then centrifuged at 1,000 r.p.m. at /

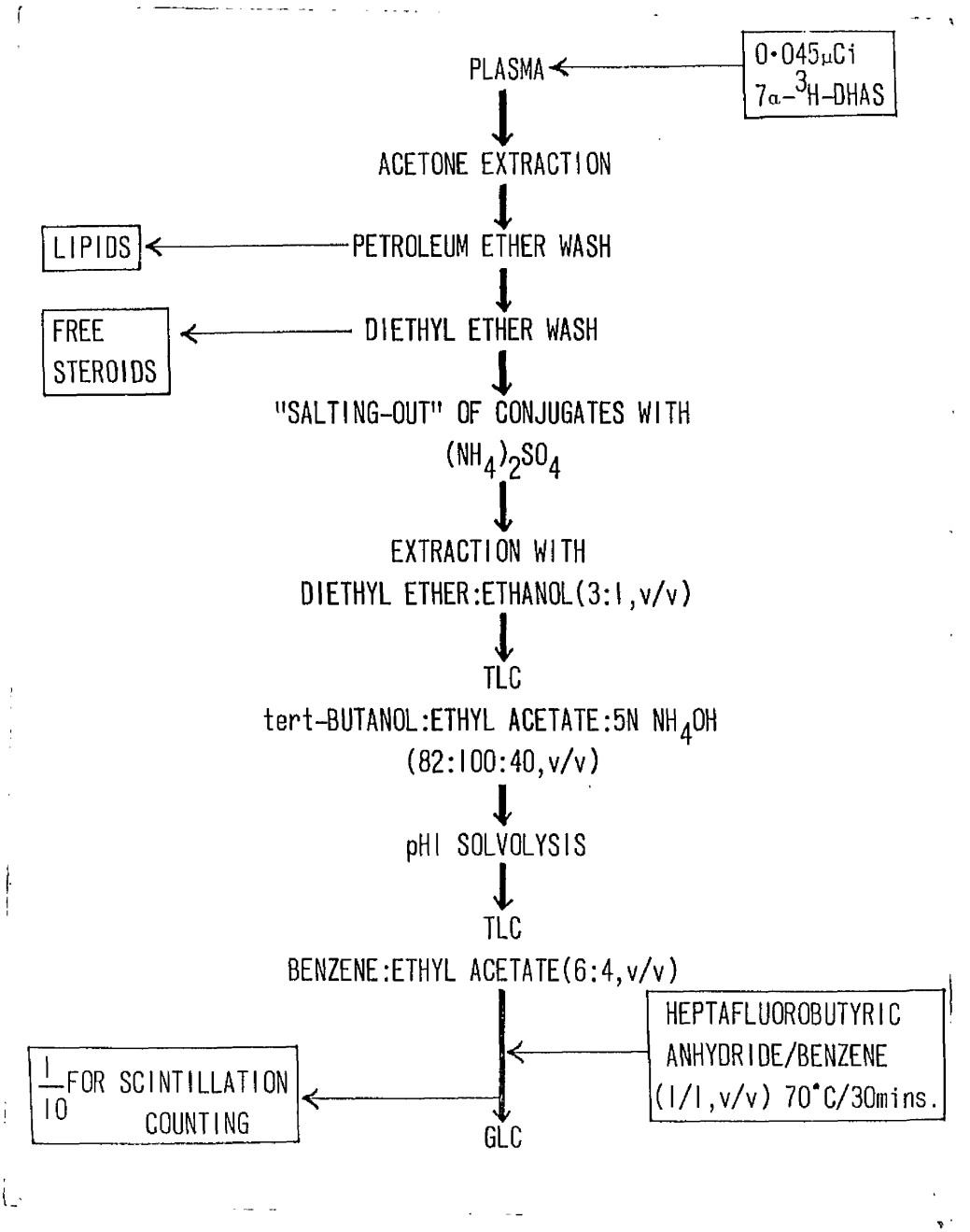


Fig. 29. Flow diagram of the procedures used in the estimation of plasma dehydroepiandrosterone sulphate.

at 4°C using an MSE 2L centrifuge. The acetone layers were removed, pooled and reduced to approximately 2 ml volume at 40°C under a stream of nitrogen. Methanol (20 ml) was added and the lipid material removed by washing with petroleum ether (80 - 100°C) (20 ml petroleum/). The ether phase was discarded. The methanol layer was once more reduced to small volume (2 ml), diluted with deionised water (10 ml) and extracted with diethyl ether (2 x 30 ml) to remove unconjugated steroids. The ether phase was again discarded.

Ammonium sulphate (6 g) was dissolved in the remaining aqueous phase and the conjugated steroid (DHAS) extracted with diethyl ether: ethanol (3:1, v/v) (3 x 30 ml). Quantitative recovery of ^{3}H-DHAS was obtained using this concentration of ammonium sulphate (50 per cent, wt/v). This finding is in agreement with results of extraction procedures reported by Edwards et al. (1953). Pooled extracts of diethyl ether:ethanol were reduced to small volume at 40°C under nitrogen and the residue was applied as a 2 cm streak to a t.l.c. plate. The chromatogram was developed for 3 hours in the solvent system tert-butanol:ethyl acetate:5N ammonia (82:100:40, v/v).

R_f values of standard steroids and their sulphate and glucuronoside esters are given in Table VII. The radioactive area, representing ^{3}H-DHAS added initially to the plasma, was located by radiochromatogram scanning /

TABLE VII. Separation of standard steroids and their sulphate and glucuronoside esters by t.l.c. in tert-butanol: ethyl acetate:5N ammonia solution (82:100:40, v/v).

STEROID	R_f
DEHYDROEPIANDROSTERONE	0.78
ANDROSTERONE	0.78
AETIOCHOLANOLONE	0.79
TESTOSTERONE	0.76
ANDROSTENEDIONE	0.77
DEHYDROEPIANDROSTERONE SULPHATE	0.28
ANDROSTERONE SULPHATE	0.30
AETIOCHOLANOLONE SULPHATE	0.31
DEHYDROEPIANDROSTERONE GLUCURONOSIDE	0.07
ANDROSTERONE GLUCURONOSIDE	0.08
AETIOCHOLANOLONE GLUCURONOSIDE	0.07

Development for 3 hours (single run).

Location by U.V. visualisation using 0.1 per cent Rhodamine 6 G in ethanol.

scanning and was eluted with diethyl ether (3 x 2 ml) and ethanol (3 x 2 ml). The eluate was collected in a boiling tube (50 ml capacity) and blown to dryness under nitrogen. Ethyl acetate (10 ml), deionised water (10 ml), sodium chloride (2 g) and 40 per cent sulphuric acid (0.8 ml:reaction mixture pH 1) were added to the tube. Solvolysis was carried out by shaking the mixture and incubating at 50°C for 4 hours (Burstein and Lieberman, 1958). From Fig. 30 it will be seen that at 50°C the solvolysis of ^{3}H-DHAS in water proceeds rapidly for the first four hours. Thereafter the rate of solvolysis reaches a plateau level at 80 per cent solvolysis. After four hours samples were again shaken and the ethyl acetate layer removed. A further extraction with ethyl acetate (10 ml) was made. The pooled ethyl acetate extracts were neutralised by washing with 5 per cent sodium bicarbonate (2 x 5 ml) and subsequently washed with deionised water (2 x 5 ml) prior to reduction to small volume. The residue was applied to a silica plate in ethyl acetate (3 x 10 µl). Each sample was applied as a single spot and the plate was developed /

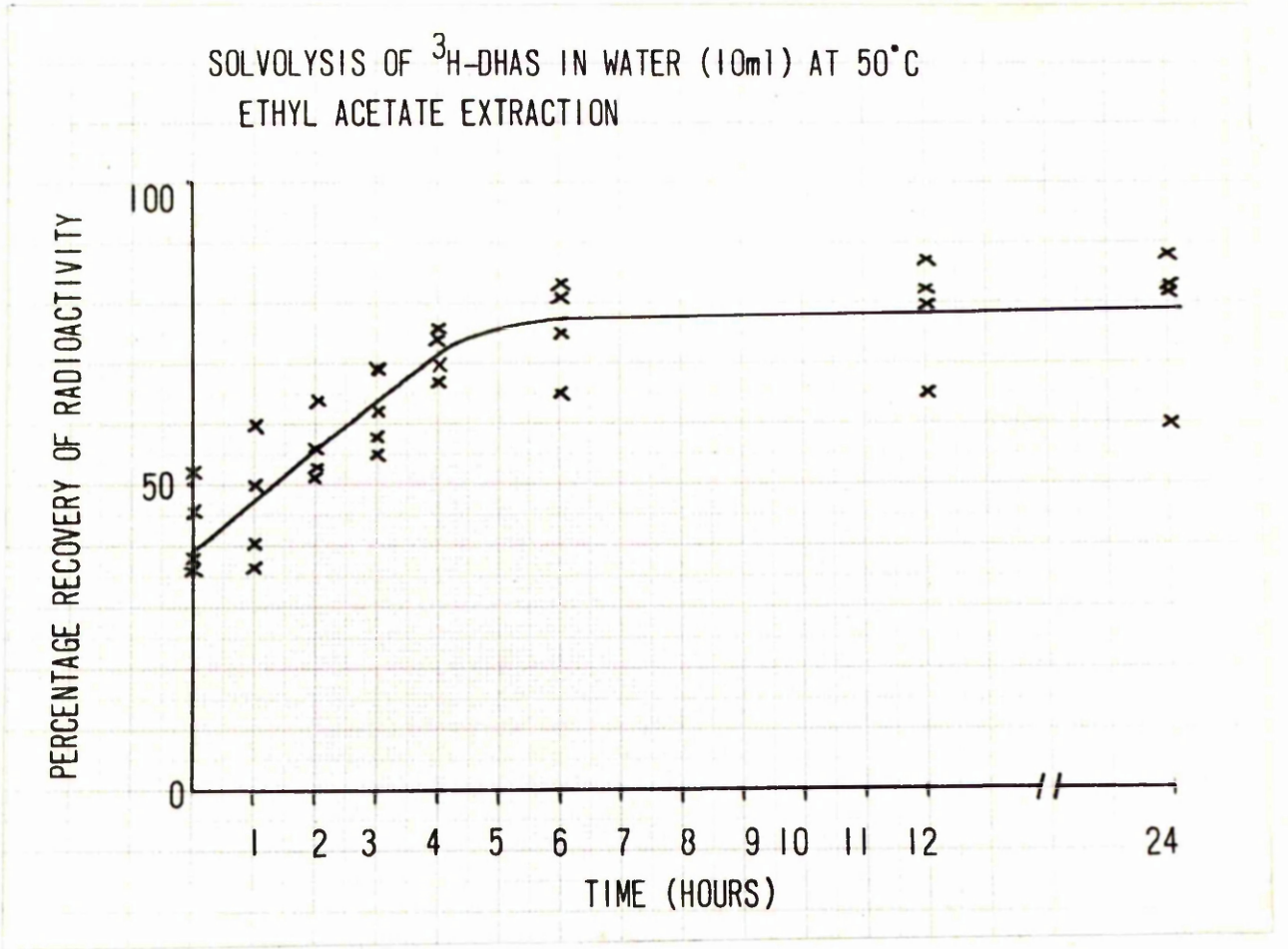


Fig. 30. The recovery of ^{3}H-DHA from ^{3}H-DHAS solvolysed at 50°C according to Burstein and Lieberman (1958). Recovery was estimated from four samples at each time interval.

developed in benzene:ethyl acetate (6:4, v/v) as before (p.111). After scanning, the area corresponding to the radioactive peak ^{3}H-DHA was removed and the steroid eluted with ethyl acetate (6 x 2 ml). The residue, after blowing to dryness, was further dried for 1 hour in a vacuum desiccator . Heptafluorobutyrates were prepared according to the method of Exley and Chamberlain (1967). Excess reagent was removed under nitrogen and the residue redissolved in benzene: ethyl acetate (9:1, v/v) (50 µl). An aliquot (5 µl) was removed for liquid scintillation counting. Following injection of standard DHA HFB and testosterone di-heptafluorobutyrate into the g.l.c. system previously described, each plasma extract was injected along with a standard amount of testosterone di-heptafluorobutyrate.

Quantitation of Dehydroepiandrosterone in Extracts of Biological Samples.

Standard solutions of DHA HFB and testosterone di-HFB were injected into the g.l.c. system daily prior to sample analyses. A known amount of testosterone di-HFB was injected as a standard together with the esterified urine or plasma extract. Quantitation was then made by a comparison of the ratios of peak height of standard DHA HFB to peak height of standard testosterone di-HFB with the peak height of DHA HFB in the biological sample to the peak height of testosterone /

testosterone di-HFB added as internal standard. Correction was made for the dilution of the final residue relative to the amount injected, for the total 24-hour urine volume or for 100 ml plasma, and for the percentage recovery as estimated by the recovery of ^{3}H-DHAS which had been added initially to samples. The mass of the ^{3}H-DHA HFB injected into the g.l.c. system along with the biological extracts was deducted when significant.

Urinary DHAS and DHAG and plasma DHAS are measured as free DHA but results are expressed as μg DHAS or μg DHAG per 24 hours and μg DHAS per 100 ml of plasma.

An example of the estimation of DHA in a 24-hour urine sample and a plasma sample is given in Figs. 31 and 32 and the appropriate explanatory texts. High levels of DHA were found in the biological fluids from this patient who had a virilising adrenal tumour. These results indicate that the methodology used is valid for determining elevated levels of DHA as well as the low levels which occur in samples from cystic fibrosis patients and which are now to be reported.

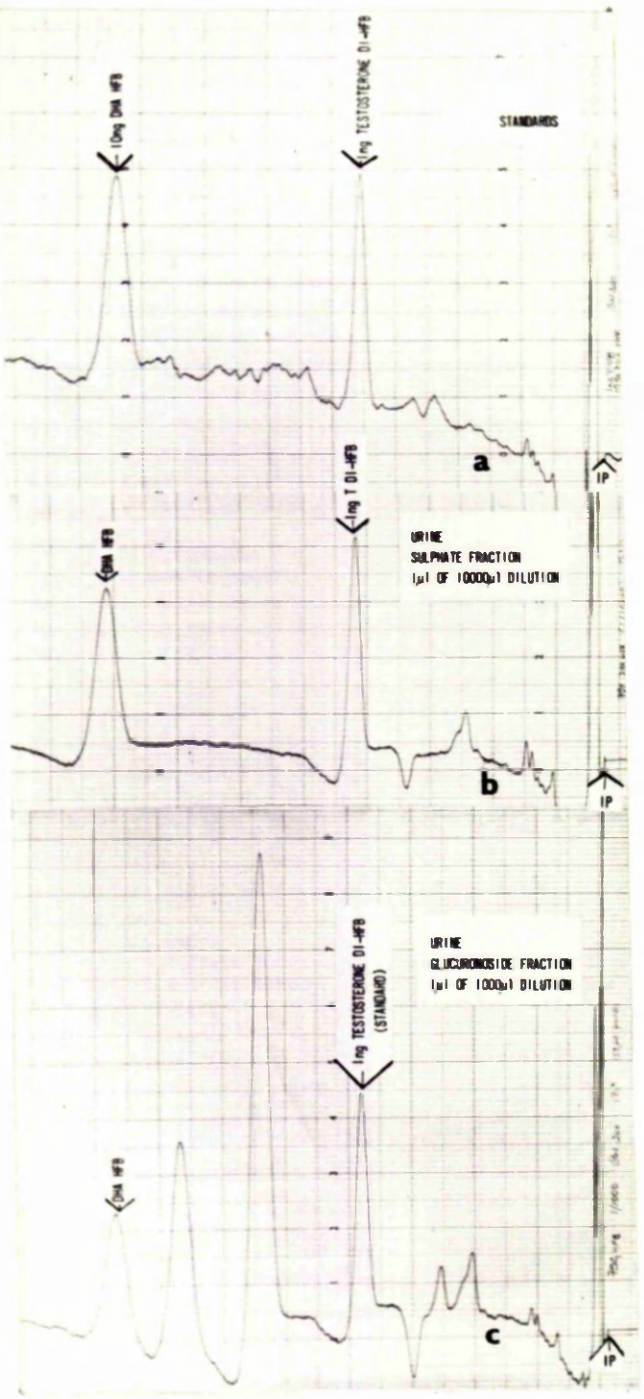


Fig. 31. Tracing of a urine extract from a patient with a virilising adrenal tumour.

a) Standard trace

b) Urine extract - sulphate fraction

c) Urine extract - glucuronoside fraction.

Explanation of Fig. 31. (Work-out of DHA in urine)

Standard trace (Fig. 31a)

1 ng T di-HFB gives a peak height of 9.4 cm

10 ng DHA HFB gives a peak height of 7.8 cm

Urine - Sulphate fraction (Fig. 31b).

1 ng of standard T di-HFB gives a peak height of 9.2 cm Since this peak height differs from that obtained in the standard trace (Fig. 31a) the corresponding peak height for 10 ng DHA HFB in the second trace was calculated thus:-

Let the peak height for 10 ng DHA HFB in the second trace be x cm. Then by comparison of peak height ratios:-

$$\frac{x}{9.2} = \frac{7.8}{9.4} \text{ cm}$$

and $$x = \frac{9.2 \times 7.8}{9.4} \text{ cm}$$

But the peak height of native DHA HFB in the second trace is 6.7 cm

Therefore there are $\frac{10 \times 9.4 \times 6.7}{9.2 \times 7.8}$ ng DHA HFB in the sample injected (1 µl of 10,000 µl)

and $\frac{10 \times 9.4 \times 6.7 \times 10,000}{9.2 \times 7.8}$ ng in the urine residue

$^1/_{10}$ of the residue contains 2,545.27 d.p.m.'s = 2.396 per cent of that added initially (106,193.7 d.p.m.'s).

Thus /

Thus if the recovery of the native steroid is equivalent to the recovery of ^{3}H-DHA from ^{3}H-DHAS added to the urine, then there is 23.96 per cent of native steroid present in the final residue. Therefore in the aliquot of urine processed (50 ml) there are

$$\frac{10 \times 9.4 \times 6.7 \times 10{,}000 \times 100}{9.2 \times 7.8 \times 23.96} \text{ ng of DHA}$$

The total 24-hour urine volume was 440 ml.

Therefore $\frac{10 \times 6.7 \times 9.4 \times 10{,}000 \times 100 \times 440}{9.2 \times 7.8 \times 23.96 \times 50}$ ng of DHA are excreted per 24 hours

3,223,413 ng of DHA are excreted per 24 hours

3.2 mg of DHA are excreted as the sulphate moiety per 24 hours

i.e. 3.2 mg DHAS per 24 hours.

Urine-Glucuronoside fraction (Fig. 31c).

1 ng of standard T di-HFB gives a peak height of 9.4 cm corresponding to the peak height of 1 ng in the standard trace (Fig. 31a)

Therefore by a comparison of peak height ratios with Fig. 31a

10 ng of DHA HFB would give a peak height of 7.8 cm in the third trace.

But the peak height of native DHA HFB in the third trace is 5.4 cm

Therefore there are $\frac{10 \times 5.4}{7.8}$ ng of DHA HFB in the sample injected (1 µl of 1,000 µl)

And $\frac{10 \times 5.4 \times 1{,}000}{7.8}$ ng in the urine residue

$^{1}/_{10}$ /

$^{1}/_{10}$ of the residue prior to g.l.c. contains 4,125.55 d.p.m.'s = 3.745 per cent of the ^{3}H-DHA added initially (110,173 d.p.m.'s). Thus 37.45 per cent of the native steroid is present in the final residue.

Therefore in the 50 ml aliquot of urine processed there are

$$\frac{10 \times 5.4 \times 1{,}000 \times 100}{7.8 \times 37.45} \text{ ng of DHA}$$

In the total urine volume (440 ml) there are

$$\frac{10 \times 5.4 \times 1{,}000 \times 100 \times 440}{7.8 \times 37.45 \times 50} \text{ ng of DHA}$$

i.e. 162,684 ng of DHA per 24 hours

Therefore 162.7 µg of DHA per 24 hours are excreted as the glucuronoside conjugate

i.e. 162.7 µg DHAG excreted per 24 hours.

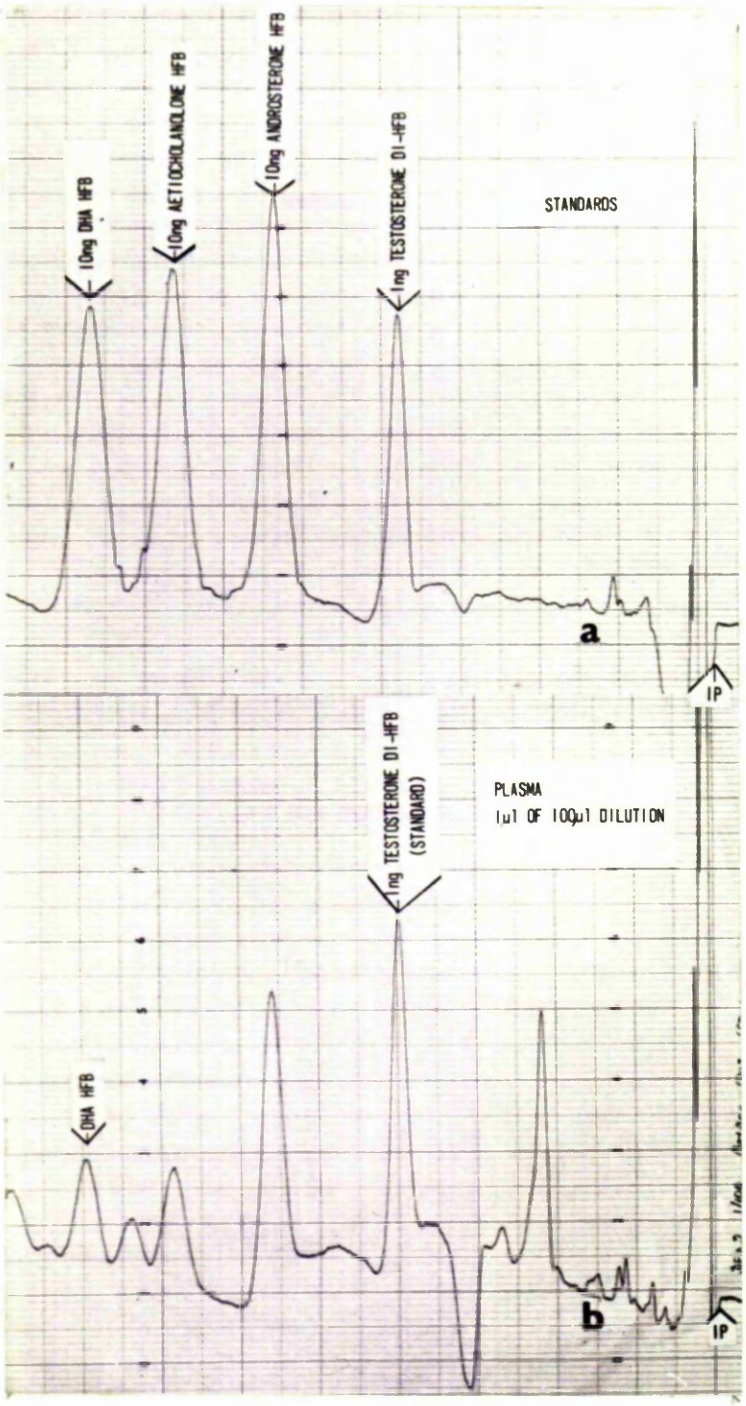


Fig. 32. Tracing of a plasma extract from a patient with a virilising adrenal tumour.

a) Standard trace

b) Plasma extract

Explanation of Fig. 32 (Work-out of DHA in plasma)

Standard Trace (Fig. 32a)

1 ng T di-HFB gives a peak height of 9.45 cm

10 ng DHA HFB gives a peak height of 9.7 cm

Plasma extract (Fig. 32b)

1 ng of T di-HFB injected as internal standard gives a peak height of 10.6 cm.

Since this peak height differs from that obtained in the standard trace (Fig. 32a) the corresponding peak height for 10 ng DHA HFB in the second trace was calculated thus:-

Let the peak height for 10 ng DHA HFB in the second trace be x cm.

Then by comparison of peak height ratios:-

$$\frac{x}{10.6} \equiv \frac{9.7}{9.45}$$

and $$x = \frac{9.7 \times 10.6}{9.45} \text{ cm}$$

But native DHA HFB in the second trace gives a peak height of 3.2 cm.

Therefore there are $\frac{10 \times 9.45 \times 3.2}{10.6 \times 9.7}$ ng DHA HFB in the sample injected (1 µl of 100 µl)

and $\frac{10 \times 9.45 \times 3.2 \times 100}{10.6 \times 9.7}$ ng in the plasma residue

Recovery of ^{3}H-DHA prior to g.l.c. injection is 32.59 per cent

Therefore /

Therefore there are $\frac{10 \times 9.45 \times 3.2 \times 100 \times 100}{10.6 \times 9.7 \times 32.59}$ ng of DHA in the plasma processed (4 ml)

Thus there are $\frac{10 \times 9.45 \times 3.2 \times 100^3}{10.6 \times 9.7 \times 32.59 \times 4}$ ng of DHA per 100 ml of plasma

i.e. 22,565.1 ng DHA per 100 ml plasma (sulphate ester)

22.57 μg DHAS per 100 ml plasma.

CHAPTER FOUR

RESULTS

INTRODUCTION

Since the earliest recorded diagnosis of cystic fibrosis in this hospital in 1948, 196 children have been diagnosed as having the disease. Of these, 60 were alive on the 31st December, 1971, the oldest being 19 years. The investigations reported here were carried out on 48 patients of this group. The majority of urine collections were made at home under parental supervision and some children were too young for complete samples to be collected reliably.

PATIENTS UNDER STUDY

The male children with cystic fibrosis ranged from 4 to 20 years and the girls from 1 to 15 years. The patients investigated are listed in Table VIII. An identification number, age and clinical status is also shown. The protean nature of the disease has already been noted and in 1958 Shwachman and Kulczycki reported a system for the clinical evaluation of the child with cystic fibrosis. This scoring system is shown in Table IX and it was applied to all patients at the time of urine collection and blood sampling. A rating of "excellent" was achieved for 5 of the 48 patients - all being /

TABLE VIII. TOTAL NUMBER OF PATIENTS INVESTIGATED BY AGE AND SEX WITH THEIR CLINICAL RATING OF DISEASE SEVERITY.

Male Children with Cystic Fibrosis				Female Children with Cystic Fibrosis			
Patient	Patient Number	Age Years	Clinical Status *	Patient	Patient Number	Age Years	Clinical Status *
E.B.	1	4	MILD	S.S.	25	1	EXCELLENT
C.B.	2	4	EXCELLENT	B.G.	26	2	MODERATE
S.C.	3	5	GOOD	S.B.	27	3	EXCELLENT
T.McA.	4	6	MODERATE	E.R.	28	3	EXCELLENT
D.G.	5	7	MILD	R.McI.	29	4	GOOD
D.S.	6	7	EXCELLENT	S.McD.	30	4	SEVERE
R.S.	7	7	SEVERE	E.W.	31	4	MILD
T.R.	8	8	SEVERE	J.L.	32	4	GOOD
A.McG.	9	8	GOOD	W.F.	33	5	GOOD
J.S.	10	9	MODERATE	C.N.	34	5	SEVERE
J.R.	11	9	MILD	A.G.	35	5	MODERATE
R.R.	12	9	GOOD	M.D.	36	5	MODERATE
R.G.	13	10	MODERATE	C.C.	37	6	SEVERE
F.B.	14	10	SEVERE	A.K.	38	7	MODERATE
S.C.	15	11	MODERATE	L.C.	39	8	MODERATE
J.H.	16	12	MODERATE	C.H.	40	8	MILD
G.F.McM.	17	14	MILD	A.J.	41	9	SEVERE
I.D.	18	14	SEVERE	L.C.	42	9	GOOD
O.B.	19	14	MODERATE	H.H.	43	11	MILD
D.McG.	20	15	GOOD	A.McM.	44	12	SEVERE
M.C.	21	15	MODERATE	V.C.	45	13	MILD
P.S.	22	16	GOOD	L.McK.	46	14	MODERATE
R.W.	23	18	SEVERE	M.McK	47	14	MILD
D.J.	24	20	GOOD	S.C.	48	15	MODERATE

* Shwachman System of Clinical Evaluation (Shwachman and Kulczycki, 1958).

TABLE IX. SYSTEM OF CLINICAL EVALUATION OF THE PATIENTS WITH CYSTIC FIBROSIS

Grading	Points	General Activity	Physical Examination	Nutrition	X-Ray Findings
Excellent (86-100)	25	Full normal activity; plays ball, goes to school regularly, etc.	Normal; no cough; pulse and respirations normal; clear lungs; good posture	Maintains weight and height at above 25th percentile; well-formed stools, almost normal; good muscle mass and tone	Clear lung fields
Good (71-85)	20	Lacks endurance and tires at end of day; good school attendance	Resting pulse and respirations normal; rare coughing or clearing of throat; no clubbing; clear lungs; minimal emphysema	Weight and height at approximately 15th to 20th percentile; stools slightly abnormal; fair muscle tone and mass	Minimal accentuation of bronchovascular markings; early emphysema
Mild (56-70)	15	May rest voluntarily during the day; tires easily after exertion; fair school attendance	Occasional cough, perhaps in morning upon rising; respirations slightly elevated; mild emphysema; coarse breath sounds; rarely localized râles; early clubbing	Weight and height above 3rd percentile; stools usually abnormal, large and poorly formed; very little, if any, abdominal distention; poor muscle tone with reduced muscle mass	Mild emphysema with patchy atelectasis; increased bronchovascular markings

Moderate (41-55)	10	Home teacher; dyspneic after short walk; rests a great deal	Frequent cough, usually productive; chest retraction; moderate emphysema; may have chest deformity; râles usually present; clubbing 2 to 3+	Weight and height below 3rd percentile; poorly formed, bulky fatty, offensive stools; flabby muscles and reduced mass; abdominal distention mild to moderate	Moderate emphysema; widespread areas of atelectasis with superimposed areas of infection; minimal bronchial ectasia
Severe (40 or below)	5	Orthopneic, confined to bed or chair	Severe coughing spells; tachypnea with tachycardia and extensive pulmonary changes; may show signs of right-sided cardiac failure; clubbing 3 to 4+	Malnutrition marked; large protuberant abdomen; rectal prolapse, large, foul, frequent, fatty movements	Extensive changes with pulmonary obstructive phenomena and infection lobar atelectasis and bronchiectasis

Shwachman and Kulczycki (1958)
(Reproduced by courtesy of the authors).
A.M.A. J. DIS. CHILD., 96, p. 8.

being younger than 8 years. The severity of the disease is not necessarily associated with advancing age since patients rated as "severe" ranged from 4 to 18 years. More boys were available for study in the pubertal and post-pubertal age ranges (10 boys of 11 years and older were alive as opposed to 6 girls) and of these 3 had a "good" clinical rating. None of the surviving girls achieved this grading.

URINE ANALYSES

All the results of the steroid analyses on 24-hour urine samples are shown in Tables X and XI. The 17-oxosteroid, 17-oxogenic steroid and total 17-hydroxycorticosteroid results are shown in columns D, E and F. Those patients undergoing treatment with anabolic hormones are indicated in the tables.

Estimation of 17-oxosteroid excretion was undertaken on all urine samples. When urine volume was adequate the 17-oxogenic steroids and total 17-hydroxycorticosteroids were also determined. A yellow chromogen was found to interfere with colorimetric steroid determinations in 9 of the 59 urine samples assayed. This was attributed to cloxacillin (p. 91) which is normally prescribed as prophylaxis to these patients. Under these circumstances, it seemed incongruous that only occasional interference should be experienced. /

TABLE X. URINARY 17-OXOSTEROIDS, 17-OXOGENIC STEROIDS, TOTAL 17-HYDROXYCORTICOSTEROIDS AND DEHYDROEPIANDROSTERONE IN MALE PATIENTS WITH CYSTIC FIBROSIS

Patient	Patient Number	Age Years	mg per 24 hours			DHA µg per 24 hours		
			17-Oxo-steroids	17-Oxo-genic Steroids	Total 17-hydroxy-cortico-steroids	Glucurono-side fraction	Sulphate fraction	Total
A	B	C	D	E	F	G	H	I
E.B.	1	4	0.32	-	-	<1.8	<1.3	-
C.B.	2	4	CLOXACILLIN INTERFERENCE			2.5	3.6	6.1
S.C.	3	5	0.57	-	-	<2.1	<1.3	-
T.McA.	4	6	0.13	-	-	<3.0	<1.8	-
D.G.	5	7	0.99	2.32	2.35	87.0	10.7	97.7
D.S.	6	7	0.57	-	-	5.1	7.6	12.7
			0.61	0.63	0.88	13.5	16.2	29.7
R.S.	7	7	0.44	0.11	0.31	13.6	2.6	16.2
T.R.	8	8	1.0	-	0.38	<1.0	4.9	4.9
A.McG	9	8	1.46	-	-	46.9	162	208.9
J.S.	10	9	CLOXACILLIN INTERFERENCE			<2.0	2.0	2.0
J.R.	11	9	0.56	-	0.7	0.4	1.6	2.0
R.R.	12	9	0.68	-	0.24	11.7	15.5	27.2
A.McG.	9	9	1.82	-	-	33.8	170.7	204.5
R.G.	13	10	1.13	1.75	4.49	10.6	23.0	33.6
F.B.	14	10	* 0.12	0.01	0.02	5.55	15.8	21.35
			0.29	0.11	0.99	5.9	12.5	18.4
			** 0.44	3.95	3.86	6.4	27.0	33.4
			CLOXACILLIN INTERFERENCE			25.8	7.8	33.6

A	B	C	D	E	F	G	H	I
S.C.	15	11	0.39	2.16	1.46	1.8	0.3	2.1
F.B.	14	11	0.26	-	0.93	1.2	6.7	7.9
J.H.	16	13	1.3	-	4.62	1.9	3.4	5.3
G.F.McM.	17	14	CLOXACILLIN INTERFERENCE			10.8	9.1	19.9
I.D.	18	14	CLOXACILLIN INTERFERENCE			8.8	22.6	31.4
O.B.	19	14	1.42	0.59	2.75	79.8	731.4	811.2
D.McG.	20	15	2.89	-	-	54.7	11.3	66.0
M.C.	21	15	0.71	0.85	1.8	81.7	8.8	90.5
P.S.	22	16	* 1.96	1.54	1.72	37.6	453.0	490.6
			**1.24	1.29	1.84	267.0	13.7	280.7
			**2.00			63.4	179.4	242.8
R.W.	23	18	2.5	0.5	2.0	29.9	118.5	148.4
D.J.	24	20	4.7	-	12.73	186.6	500.4	687.0

Under treatment with:- * Ethyl Estrenol
** Oxymetholone

TABLE XI. URINARY 17-OXOSTEROIDS, 17-OXOGENIC STEROIDS, TOTAL 17-HYDROXYCORTICOSTEROIDS AND DEHYDROEPIANDROSTERONE IN FEMALE PATIENTS WITH CYSTIC FIBROSIS

Patient	Patient Number	Age Years	mg per 24 hours			DHA µg per 24 hours		
			17-Oxo-steroids	17-Oxo-genic steroids	Total 17-hydroxy-cortico-steroids	Glucurono-side fraction	Sulphate fraction	Total
A	B	C	D	E	F	G	H	I
S.S.	25	1	0.05	-	-	-	<2.0	-
B.G.	26	2	0.08	-	-	2.7	3.9	6.6
S.B.	27	3	0.08	0.45	0.6	-	0.4	-
E.R.	28	3	CLOXACILLIN INTERFERENCE			0.5	1.9	2.4
R.McI.	29	4	0.19	-	-	0.3	0.4	0.7
S.McD.	30	4	CLOXACILLIN INTERFERENCE			1.0	4.0	5.0
E.R.	28	4	**0.31	-	-	<0.6	<0.4	-
N.W.	31	4	0.2	0.2	0.37	-	0.1	-
J.L.	32	4	0.38	-	-	<1.8	0.3	0.3
W.F.	33	5	0.56	-	-	<0.4	<0.4	-
C.N.	34	5	CLOXACILLIN INTERFERENCE			0.7	<0.3	0.7
A.G.	35	5	0.28	0.73	0.70	1.4	<0.3	1.4
J.L.	32	5	**0.93	-	2.94	9.2	9.3	18.5
M.D.	36	5	0.1	1.22	1.45	2.0	5.3	7.3

A	B	C	D	E	F	G	H	I
C.C.	37	6	0.13	-	-	4.0	7.8	11.8
A.K.	38	7	0.18	-	-	1.0	<0.5	1.0
L.C.	39	8	0.8	0.63	0.99	0.7	2.9	3.6
C.H.	40	8	0.11	-	0.62	<0.4	7.8	7.8
A.J.	41	9	0.57	-	-	<2.0	<1.4	-
L.C.	42	9	0.09	1.41	1.62	0.3	7.0	7.3
H.H.	43	11	0.70	1.9	2.64	44.6	8.7	53.3
		12	2.44	-	-	5.1	9.0	14.1
A.McM.	44	12	* 1.39	0.79	2.86	126.2	102	228.2
V.C.	45	13	1.01	-	-	4.6	90.6	95.2
L.McK.	46	14	3.22	-	-	24.9	34.7	59.6
M.McK.	47	14	CLOXACILLIN INTERFERENCE			16.8	75.8	92.6
S.C.	48	15	5.32	-	2.11	4.5	0.2	4.7

Under treatment with:- * Ethyl Estrenol
** Oxymetholone

experienced. A more subjective approach to the results indicated that these difficulties were experienced in urine samples collected in hospital and from children with reliable parents who insisted on full therapy at home. Thus it would seem probable that many of the children with cystic fibrosis under study were not having regular administration of antibiotic therapy.

Total dehydroepiandrosterone (μg per 24 hours) is recorded in column I of Tables X and XI. The breakdown of this total value into glucuronoside and sulphate fractions is given in columns G and H. Where no DHA was detected on g.l.c. analysis, recovery of $\left[7\alpha\text{-}^{3}H\right]$ dehydroepiandrosterone along with total urine volume was used to estimate the possible maximum level present in each sample. Results are thus expressed as being less than this maximum possible level (columns G and H). If DHA was not measured in the urine glucuronoside and sulphate fractions, then total DHA could not be recorded (column I).

TOTAL 17-HYDROXYCORTICOSTEROIDS IN CHILDREN WITH CYSTIC FIBROSIS

Statistical comparison of the total 17-hydroxycorticosteroid levels in urine from children with cystic fibrosis and in urines from normal children, as assayed by this laboratory, revealed no significant difference. /

TABLE XII. A COMPARISON OF TOTAL 17-HYDROXYCORTICOSTEROID AND 17-OXOSTEROID EXCRETION IN NORMAL CHILDREN AND IN CHILDREN WITH CYSTIC FIBROSIS

Steroid Group Estimation	Age Range Years	Cystic Fibrosis Patients	Laboratory Normal Values	Normal Values (Reference)
Total 17-OHCS (mg per 24 hours)	2-17	1.97 ± 2.3 (n = 32)	1.39 ± 0.71 (n = 11)	1.5 ± 0.16 (SEM) (n = 52) (Chodos et al. 1965)
		NOT SIGNIFICANTLY DIFFERENT		NOT SIGNIFICANTLY DIFFERENT
17-OS (mg per 24 hours)	6-10	0.63 ± 0.48 (n = 19)	0.54 ± 0.28 (n = 12)	0.89 ± 0.61 (n = 11) (Prout and Snaith, 1958, Method 4.)
		NOT SIGNIFICANTLY DIFFERENT		NOT SIGNIFICANTLY DIFFERENT

difference. Likewise, neither normal values from this laboratory nor the levels found in cystic fibrosis patients differed significantly from control values reported by Chodos et al. (1965) (Table XII).

17-OXOSTEROID EXCRETION BY PATIENTS WITH CYSTIC FIBROSIS

Estimations of 17-oxosteroids were undertaken on urine of patients with non-endocrine disease in the 6 to 10 years age range. These values (0.54 $\pm$ 0.28 mg per 24 hours) did not differ significantly from values reported by Prout and Snaith (1958) (Table XII). The method closest to that used in this study was used for comparison (i.e. method 4 of Prout and Snaith employing spectrophotometric readings at three wavelengths and application of the Allen correction). Urine samples from normal children of 11 to 17 years proved difficult to obtain. Since the 6 to 10 year old subjects had urinary 17-oxosteroid levels approximating those reported by Prout and Snaith (1958), the normal values reported by these authors were used for comparison with results obtained from children with cystic fibrosis. No significant differences from normal were found in the age ranges 1 to 5 years and 6 to 10 years but the affected children between 11 and 17 years had a significantly lower excretion of 17-oxosteroids than normals of the same age range (1.95 $\pm$ 1.625 mg per /

TABLE XIII. A COMPARISON OF URINARY 17-OXOSTEROIDS IN NORMAL CHILDREN AND CHILDREN WITH CYSTIC FIBROSIS

Age Range (Years)	* 17-Oxosteroids mg per 24 hours	
	CYSTIC FIBROSIS PATIENTS	NORMAL PATIENTS +
1 - 5	0.26 ± 0.18 (n = 12)	0.41 ± 0.58 (n = 12)
	NO SIGNIFICANT DIFFERENCE	
6 - 10	0.63 ± 0.48 (n = 19)	0.89 ± 0.61 (n = 11)
	NO SIGNIFICANT DIFFERENCE	
11 - 17	1.95 ± 1.625 (n = 14)	3.71 ± 1.76 (n = 31)
	SIGNIFICANT DIFFERENCE	

* Mean $\pm$ 1 Standard Deviation

\+ Prout, M., and Snaith, A.H., (1958).

TABLE XIV. TOTAL DAILY EXCRETION OF DEHYDROEPIANDROSTERONE IN NORMAL CHILDREN (mg per 24 hours)
(A REVIEW OF THE LITERATURE)

MALE			FEMALE		
Age Range	µg DHA per 24 hours	Author(s); Method of Quantitation	Age Range	µg DHA per 24 hours	Author(s); Method of Quantitation
5- 7	11.25	Loras et al. 1966 (Colorimetry)	5- 7	11.25	Loras et al. 1966 (Colorimetry)
8-10	97.5		8- 9	77.9	
14-16	614.0		13-15	350.5	
2- 5	10	Uozumi et al. 1969 (Colorimetry)	2- 5	10	Uozumi et al. 1969 (Colorimetry)
7-12	40		7-12	10	
15-16	60		15-16	110	
5-15	220-1900	Pal and Teller, 1972 Sulphate and Free (Colorimetry)	6	50	Vestergaard, 1965 (Colorimetry)
			9	50	
			10-14	200	
			15-16	510	
5- 6	30	Vestergaard, 1965 (Colorimetry)	4- 7	29	Berger et al. 1970 (G.l.c.)
8- 9	110		8-10	22	
10-14	225		11-14	172	
15-17	1150		15-17	273	
4- 7	28	Berger et al. 1970 (G.l.c.)	7-14	300-1200	Pal and Teller, 1972 Sulphate and Free (Colorimetry)
8-10	45				
11-14	126				
15-17	351				
MALE AND FEMALE CHILDREN					
4- 6	15	Blunck, 1968 (Colorimetry)	9-10	16	Paulsen et al. 1966 (Colorimetry)
7-10	47		14	160	
14-17	218				

per 24 hours as opposed to 3.71 $\pm$ 1.76 mg per 24 hours) (Table XIII).

URINARY DEHYDROEPIANDROSTERONE

Several authors have reported normal values for the daily excretion of dehydroepiandrosterone in children. Table XIV gives a series of normal values obtained from a review of the literature. To compile a list of normal values of steroid excretion for all age ranges would be a massive undertaking. For the purposes of this investigation comparison was made with normal values determined by a methodology comparable to the one used in this thesis. For this reason the results of Berger et al. (1970) obtained by g.l.c. analysis of urine samples were utilised. However, only Loras et al. (1966) differentiate between DHA present as the glucuronoside conjugate and as the sulphate.

Many authors have disputed the presence of DHA in the urine of pre-pubertal children. Paulsen et al. (1966) could not detect DHA in the urine of any child below the age of 8 years. Likewise, Beas et al. (1962) found no DHA in the urine of 5 boys and 7 girls aged 5 to 7 years. However, Loras et al. (1966) were able to isolate 21 μg of DHA from 6 pooled 24-hour urine samples from children of 5,6 and 7 years respectively. Similarly,

Vestergaard /

Vestergaard (1965) found DHA present in pooled urine samples from 5 to 7 year old boys and girls. Also an extract of the urine from a 6 year old girl gave a distinct, though small, peak in the area of DHA on g.l.c. analysis. Berger et al. (1970) reported excretion of DHA in 6 boys of 4 years to be in the range 0 to 80 μg per 24 hours.

In the g.l.c. traces which follow, the quality of the peak representing DHA from urine samples processed as described can be seen. The first tracing (Fig. 33) from a boy of 14 years (patient number 19) demonstrates DHA to be present in both glucuronoside and sulphate fractions though a preponderance is excreted as the sulphate. Figure 34 shows g.l.c. tracings from a 9 year old girl (patient number 41) where no peak corresponding to DHA was present in either urine fraction. On the other hand, a small peak in the area of DHA could be detected on analysis of urine from a 5 year old healthy girl (Fig. 35) and also from a 4 year old patient with cystic fibrosis (patient number 30) (Fig. 36). Using the reported methodology, DHA is measurable in urine from 4 year old subjects and upwards. When not detected DHA must be presumed to be either absent or less than the tolerance of the method.

Quantitation was made on the basis of the peak height corresponding to DHA in relation to standard injected with each sample. To correct /

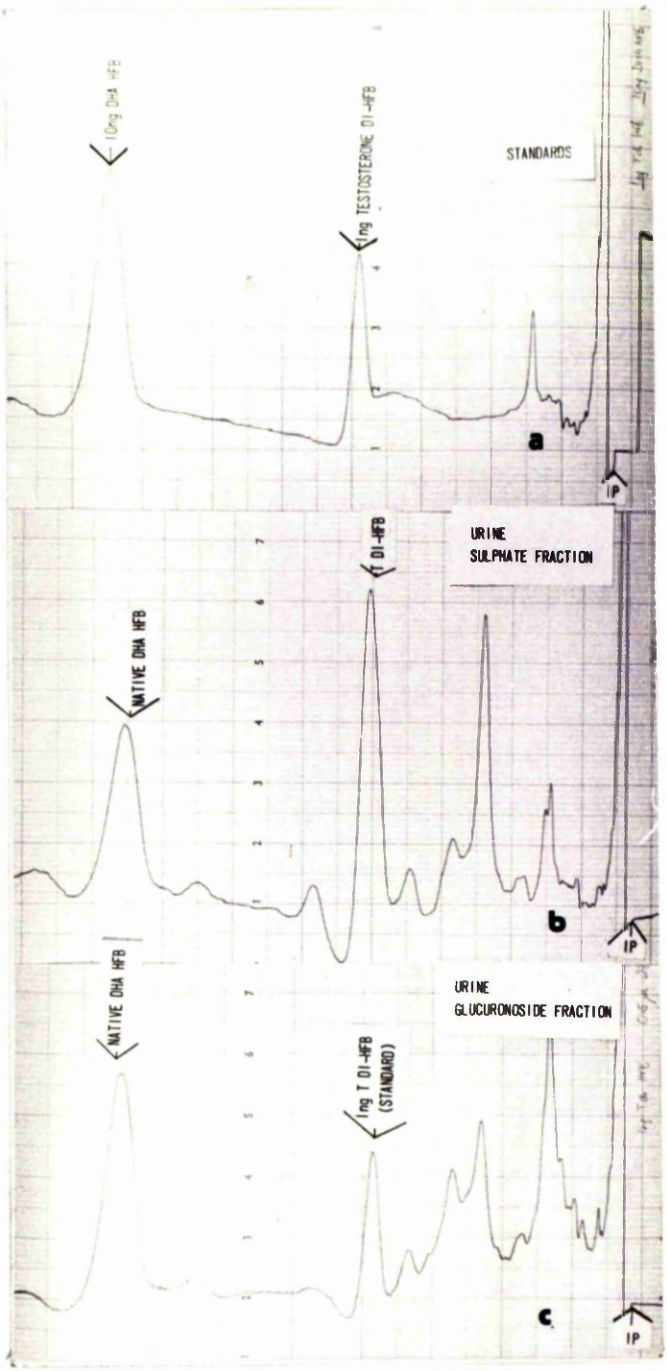


Fig. 33. Tracing obtained on g.l.c. analysis of urine extracts from patient 19, (male; 14 years).

Total urine volume - 820 ml; volume analysed - 50 ml

Sulphate fraction - 0.5 µl of 500 µl injected

Glucuronoside fraction - 0.5 µl of 50 µl injected

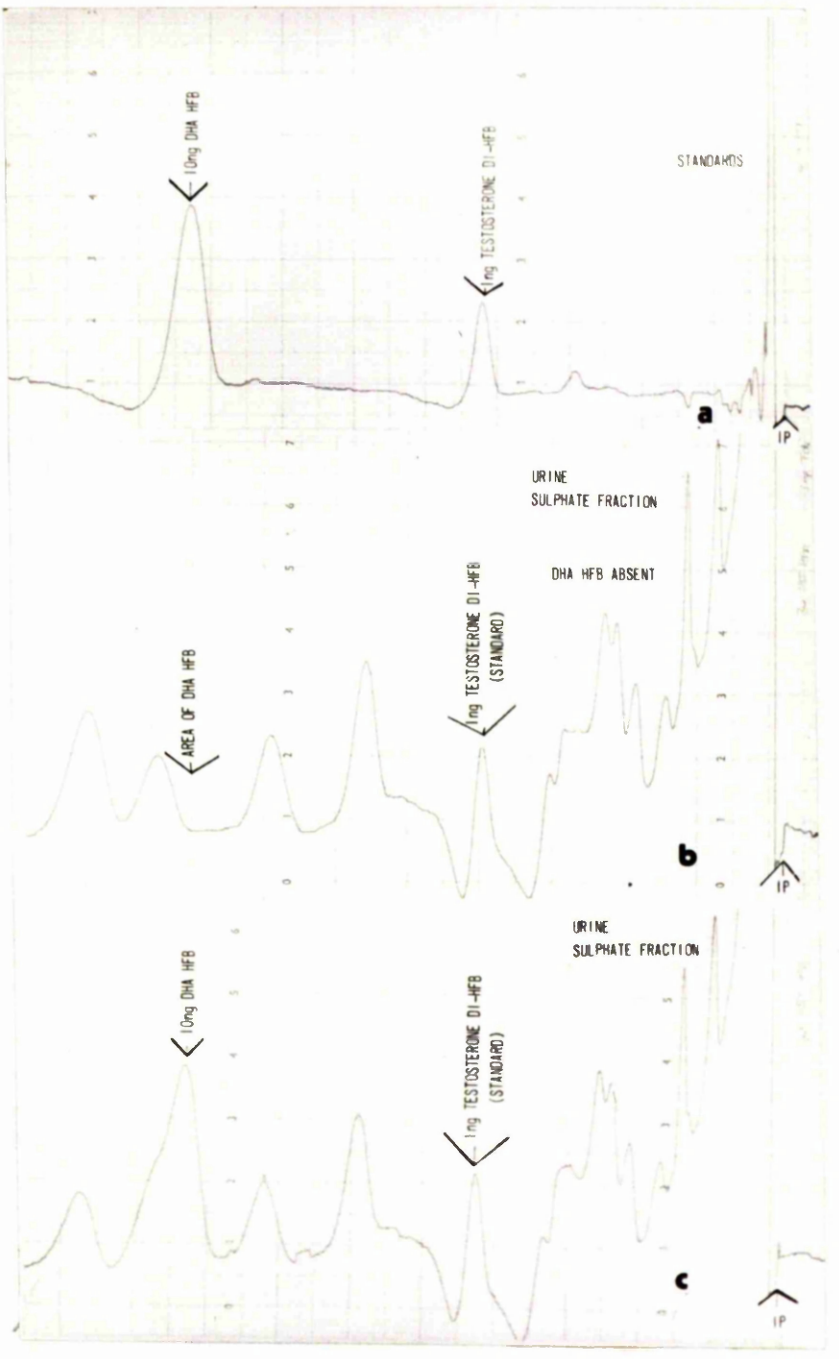


Fig. 34. Tracing obtained on g.l.c. analysis of urine extracts from patient 41, (female; 9 years).

Total urine volume - 1,060 ml; volume analysed - 100 ml

Sulphate fraction - 4 µl of 50 µl injected

Glucuronoside fraction - 5 µl of 50 µl injected

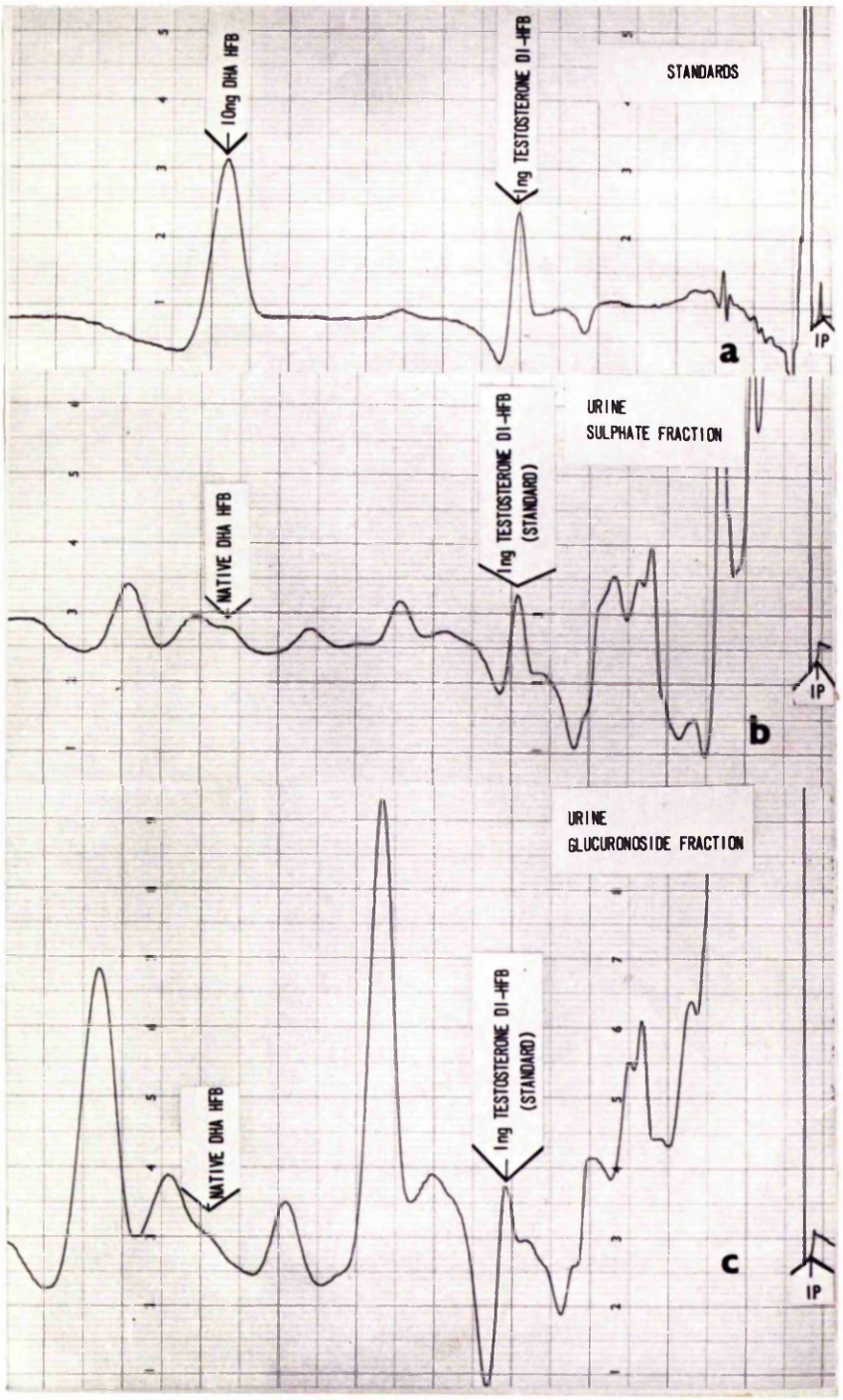


Fig. 35. Tracing obtained on g.l.c. analysis of urine extracts from a 5 year old normal girl (upper respiratory infection). Total urine volume - 455 ml; volume processed - 100 ml
Sulphate fraction - 2 µl of 50 µl injected
Glucuronoside fraction - 1.5 µl of 50 µl injected

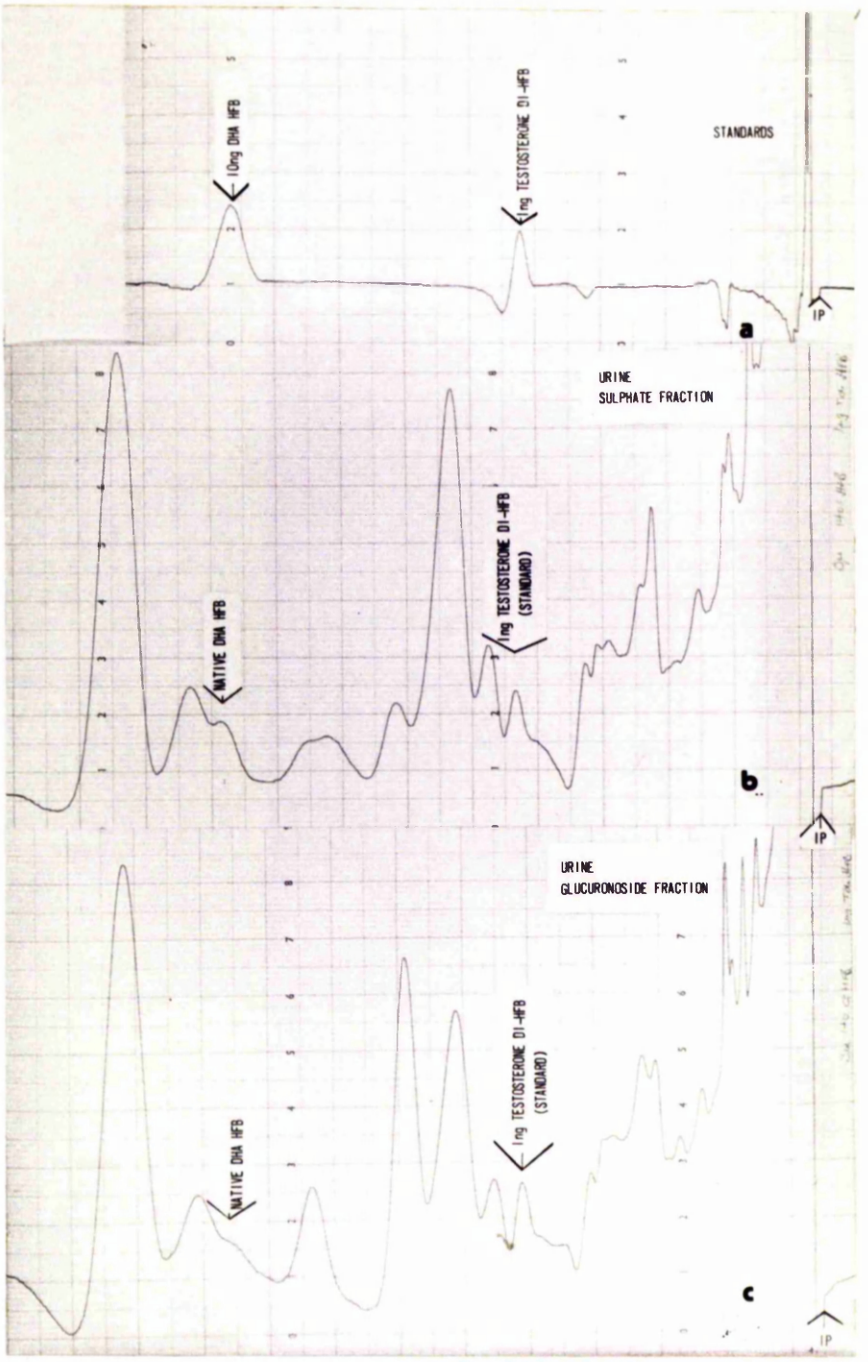


Fig. 36. Tracing obtained on g.l.c. analysis of urine extracts from patient 30 (female; 4 years).

Total urine volume - 400 ml; volume processed - 100 ml

Sulphate fraction 3 µl of 50 µl injected

Glucuronoside fraction - 3 µl of 50 µl injected

TABLE XV. PERCENTAGE RECOVERY OF $[7\alpha\text{-}^{3}H]$ DHA IN 15 RANDOM URINE SAMPLES AT VARIOUS STAGES IN THE ANALYSIS.

	% Recovery from Sulphate Fraction Stage of Analytical Procedure				% Recovery from Glucuronoside Fraction Stage of Analytical Procedure			
Urine Number	3	9	15	19	3	9	15	19
1	78.85	60.25	44.65	26.50	82.19	78.80	55.77	21.05
2	55.20	53.72	50.60	33.57	74.12	73.99	63.57	39.96
3	74.09	67.29	57.51	44.02	68.47	58.50	52.89	37.96
4	50.77	42.59	38.22	20.92	71.44	70.17	59.15	37.64
5	73.03	66.55	56.62	40.55	76.26	64.81	51.76	42.47
6	68.16	62.63	48.48	31.20	77.41	77.31	56.85	28.67
7	69.59	68.65	43.26	24.58	78.15	70.84	64.24	41.06
8	84.12	73.80	66.00	40.35	74.63	70.70	60.77	36.14
9	75.36	68.32	54.93	29.87	77.76	71.23	54.71	27.93
10	76.36	74.46	59.21	32.55	73.89	74.16	61.70	38.80
11	70.50	58.20	51.69	24.98	72.78	69.58	64.95	37.60
12	81.03	69.50	69.00	49.60	83.50	65.90	57.70	21.60
13	79.90	68.00	69.60	50.40	71.20	60.00	59.20	45.20
14	82.10	70.00	68.00	52.00	66.50	48.80	49.00	45.80
15	81.30	63.50	62.00	51.30	80.00	67.50	61.60	40.10
Mean ± 1 Standard Deviation	73.36 ±9.60	64.5 ±8.26	55.98 ±9.86	36.8 ±10.75	72.22 ±4.8	68.15 ±7.78	58.26 ±4.76	36.13 ±7.78

Stage 3: Extraction following Hydrolysis
Stage 9: After Column Chromatography
Stage 15: After thin layer Chromatography (Benzene:Ethyl Acetate, 6:4, v/v)
Stage 19: After t.l.c. system 2 (Cyclohexane:Ethyl Acetate, 1:1, v/v)
i.e. prior to g.l.c. injection.

correct for methodological losses the recovery of the ^{3}H-DHA added initially to samples was estimated. The percentage recovery of ^{3}H-DHA from 15 random urine samples at various stages in the analyses will be seen in Table XV. Correction was made for all aliquots removed for liquid scintillation counting. The recovery of [4-^{14}C] DHA and of cold standard amounts of DHA were of the same order as that for [7α-^{3}H] DHA. The reported tendency for the 7α-tritium label to become detached was therefore not a significant problem in these experiments.

The total urinary dehydroepiandrosterone and dehydroepiandrosterone sulphate estimated by these means are given in Tables XVI, XVII and XVIII. Normal values by Berger et al. (1970) and Loras et al. (1966) are given for comparison and the clinical rating of each child with cystic fibrosis is recorded. In Table XVI high levels of total DHA are found in patients 5 and 9 with mild and good clinical ratings respectively. However patients with the same rating (patients 11 and 12) did not have a correspondingly high excretion of DHA. The unexpectedly high urinary DHA level in both patient 5 and patient 9 does not correlate with an elevated plasma level of DHAS (Table XIX). DHA from urine from patient 5 with hypospadias is excreted mainly as the glucuronoside conjugate. The significance of /

TABLE XVI. TOTAL URINARY DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULPHATE IN NORMAL MALE CHILDREN AND IN CHILDREN WITH CYSTIC FIBROSIS (4 - 10 years)

Patient	Patient Number	Age Years	Total DHA µg per 24 hours		DHAS µg per 24 hours		Clinical Rating
			C.F. Children	Normals*	C.F. Children	Normals+	
E.B.	1	4	-		<1.3		MILD
C.B.	2	4	6.1		3.6		EXCELLENT
S.C.	3	5	-		<1.3		GOOD
T.McA.	4	6	-		<1.8		MODERATE
D.G.	5	7	97.7++		10.7		MILD
D.S.	6	7	12.7	28	7.6	6	EXCELLENT
			29.7		16.2		
R.S.	7	7	16.2		2.6		SEVERE
T.R.	8	8	4.9		4.9		SEVERE
A.McG.	9	8	208.9		162		GOOD
J.S.	10	9	2.0		2.0		MODERATE
J.R.	11	9	2.0		1.6		MILD
R.R.	12	9	27.2	45	15.5	43	GOOD
A.McG.	9	9	204.5		170.7		GOOD
R.G.	13	10	33.6		23.0		MODERATE
F.B.	14	10	21.35		15.8		SEVERE
			18.4		12.5		
			33.4		27.0		
			33.6		7.8		

*Berger et al. (1970).

+Loras et al. (1966).

++ Hypospadias.

TABLE XVII. TOTAL URINARY DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULPHATE IN NORMAL MALE CHILDREN AND IN CHILDREN WITH CYSTIC FIBROSIS (11 - 20 years)

Patient	Patient Number	Age Years	Total DHA µg per 24 hours		DHAS µg per 24 hours		Clinical Rating
			C.F. Children	Normals*	C.F. Children	Normals+	
S.C.	15	11	2.1		0.3		MODERATE
F.B.	14	11	7.9		6.7	255	SEVERE
J.H.	16	13	5.3		3.4		MODERATE
G.F.McM.	17	14	19.9	126	9.1		MILD
I.D.	18	14	31.4		22.6		SEVERE
O.B.	19	14	811.2		731.4		MODERATE
D.McG.	20	15	66.0		11.3	468	GOOD
M.C.	21	15	90.5		8.8		MODERATE
P.S.	22	16	490.6	351	453.0		GOOD
			280.7		13.7		
			242.8		179.4		
R.W.	23	18	148.4		118.5	392	SEVERE
D.J.	24	20	687.0		500.4		GOOD

* Berger et al. (1970)

\+ Loras et al. (1966)

TABLE XVIII. TOTAL URINARY DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULPHATE IN NORMAL FEMALE CHILDREN AND IN CHILDREN WITH CYSTIC FIBROSIS (1 - 15 years)

Patient	Patient Number	Age Years	Total DHA µg per 24 hours		DHAS µg per 24 hours		Clinical Rating
			C.F. Children	Normals*	C.F. Children	Normals+	
A	B	C	D	E	F	G	H
S.S.	25	1	-		<2.0		EXCELLENT
B.G.	26	2	6.6		3.9		MODERATE
S.B.	27	3	-		0.4		EXCELLENT
E.R.	28	3	2.4		1.9		EXCELLENT
R.McI.	29	4	0.7		0.4		GOOD
S.McD.	30	4	5.0		4.0		SEVERE
E.R.	28	4	-		<0.4		EXCELLENT
N.W.	31	4	-		0.1		MILD
J.L.	32	4	0.3		0.3		GOOD
W.F.	33	5	-	29	<0.4		GOOD
C.N.	34	5	0.7		<0.3		SEVERE
A.G.	35	5	1.4		<0.3		MODERATE
J.L.	32	5	18.5		9.3	6.3	GOOD
M.D.	36	5	7.3		5.3		MODERATE
C.C.	37	6	11.8		7.8		SEVERE
A.K.	38	7	1.0		<0.5		MODERATE

A	B	C	D	E	F	G	H
L.C.	39	8	3.6		2.9		MODERATE
C.H.	40	8	7.8		7.8		MILD
A.J.	41	9	-	22	<1.4	58.3	SEVERE
L.C.	42	9	7.3		7.0		GOOD
H.H.	43	11	53.3		8.7		MILD
		12	14.1		9.0		
A.McM.	44	12	228.2	172	102		SEVERE
V.C.	45	13	95.2		90.6		MILD
L.McK.	46	14	59.6		34.7	273	MODERATE
M.McK.	47	14	92.6		75.8		MILD
S.C.	48	15	4.7	273	0.2		MODERATE

* Berger et al. (1970)

\+ Loras et al. (1966)

TABLE XIX. PLASMA DEHYDROEPIANDROSTERONE SULPHATE IN PATIENTS WITH CYSTIC FIBROSIS

MALE PATIENTS				FEMALE PATIENTS			
Name	Patient Number	Age	µg DHAS per 100 ml	Name	Patient Number	Age	µg DHAS per 100 ml
E.B.	1	4	4.8	S.B.	27	3	6.3
C.B.	2	4	16.4	E.R.	28	3	** 3.0
S.C.	3	5	2.1	R.McI.	29	4	<1.7
D.C.	5	7	5.4	S.McD.	30	4	2.5
D.S.	6	7	3.7	J.L.	32	4	1.9
R.S.	7	7	1.0				** 8.9
T.R.	8	8	6.5	W.F.	33	5	4.3
A.McG.	9	8	6.0	A.G.	35	6	0.9
J.S.	10	9	1.2	C.C.	37	6	22.9
J.R.	11	9	2.4	L.C.	39	8	1.0
R.R.	12	9	6.2	C.H.	40	8	1.7
R.G.	13	10	2.1	A.J.	41	9	0.3
F.B.	14	10	* 325.6	L.C.	42	9	0.4
S.C.	15	11	1.2	H.H.	43	11	12.0
J.H.	16	12	9.1	A.McM.	44	12	* 9.3
G.F.McM.	17	14	2.2	V.C.	45	13	31.4
D.McG.	20	15	11.8	M.McK.	47	14	5.0
M.C.	21	15	<2.8	S.C.	48	15	5.6
P.S.	22	16	** 1.5				

Under treatment with:- * Ethyl Estrenol

** Oxymetholone

of this finding in relationship to the thesis, which concerns, in part, the role of DHA in the masculinising processes, will be discussed fully in the next chapter. The three patients classified as severe (numbers 7, 8 and 14) all had a low excretion of total DHA. Of the older male patients 8 out of 11 had low urinary levels of DHA (Table XVII). Only three boys (numbers 19, 22 and 24) approximated the normal levels quoted by Berger et al. (1970). Patients 19 and 24 have high levels of total DHA which is excreted mainly as the sulphate. These boys have satisfactorily entered into puberty and the possible significance of these high levels will be considered in the next chapter. Female patients with cystic fibrosis demonstrate a more strikingly low level of total DHA (Table XVIII). The apparent anomalous finding of a single high level of DHA in a severely affected child of 12 years (patient 44) is due to her having had anabolic steroid therapy at the time of urine collection (see next chapter for the effects of anabolic hormones).

PLASMA LEVELS OF DEHYDROEPIANDROSTERONE IN CHILDREN WITH CYSTIC FIBROSIS

Dehydroepiandrosterone was assayed in plasma samples from 36 patients with cystic fibrosis - 19 boys (4 to 16 years) and 17 girls (3 to 15 years). The results are shown in Table XIX. Patients on anabolic hormone therapy are indicated. Circadian rhythms in the /

TABLE XX. PLASMA LEVELS OF DEHYDROEPIANDROSTERONE IN NORMAL CHILDREN (μg per 100 ml)

(A REVIEW OF THE LITERATURE)

MALE CHILDREN			FEMALE CHILDREN		
Age Range	μg DHA per 100 ml	Author(s); Method of Quantitation	Age Range	μg DHA per 100 ml	Author(s); Method of Quantitation
3- 9	0.06 ± 0.05	Gandy and Peterson, 1968 DHA (DOUBLE ISOTOPE DILUTION)	3- 8	.07 ± .03	Gandy and Peterson, 1968 DHA (DOUBLE ISOTOPE DILUTION)
10-15	0.24 ± 0.20				
3-10	15 ± 15	Yamaji and Ibayashi, 1969 DHAS (G.L.C.)	1-11	0.14	Forest and Migeon, 1970 DHA (DOUBLE ISOTOPE DILUTION)
9-15	81 ± 42				
16-20	180 ± 37.7				
1- 5	2.7	Rosenfield and Eberlein, 1969 (G.L.C.)	2-10	14 ± 19	Yamaji and Ibayashi, 1969 DHAS (G.L.C.)
6- 9	13.5		11-15	95 ± 37.6	
12	27.6		16-20	148 ± 42.7	
13	36.2				
14	15.6		9	35.2	Rosenfield and Eberlein, 1969 DHAS (G.L.C.)
15	106		10	1.2	
17	114		11	15.4	
			13	43.9	
2- 5	12.05	Saez and Bertrand, 1968 DHA + DHAS (DOUBLE ISOTOPE DILUTION)	14	136	
6- 9	17.4		15	65	
11-13	68.9				
3- 6	6.3	Rosenfield et al. 1971 DHA + DHAS (COMPETITIVE BINDING)			

the plasma 17-oxosteroids in normal adult subjects have been investigated by Migeon et al. (1957) who found peak levels of DHA at 8 a.m. Conversely Laatikainen and Vihko (1968) reported highest levels of DHA to occur in the late afternoon. Vaitukaitis et al. (1969) withdrew blood from 7 subjects at 8 a.m. and at 9 p.m. and were unable to demonstrate any diurnal variation in DHAS. The majority of blood samples taken for this investigation were withdrawn in the afternoon between 2 and 5 p.m.

Migeon et al. (1957), employing a micro-Zimmermann reaction, were unable to detect plasma 17-oxosteroids until the age of 5 to 7 years. Rosenfield and Eberlein (1969), employing g.l.c. analyses, demonstrated DHAS to be present in plasma pools from normal male children from 1 to 5 years. Normal values for plasma dehydroepiandrosterone in children, as reported in the literature, are given in Table XX. The results of Yamaji and Ibayashi (1969) obtained from g.l.c. analyses of plasma extracts from normal children will be used for comparison with the results obtained from children with cystic fibrosis.

Gas liquid chromatographic analyses of plasma samples are demonstrated in the following traces. Patient 14, during treatment with ethyl estrenol, had a high level of plasma DHA (Fig. 37).

On /

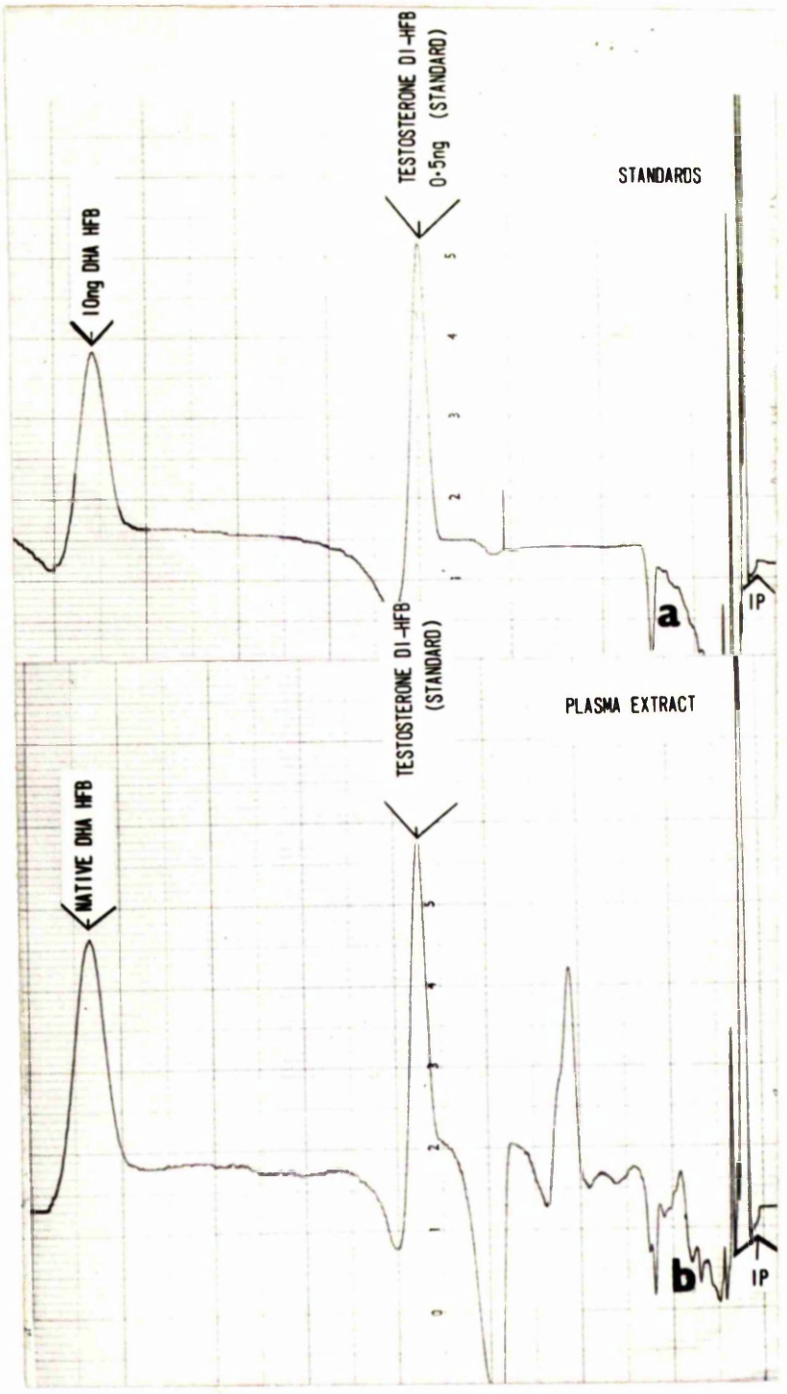


Fig. 37. Tracing obtained on g.l.c. analysis of plasma from patient 14 (male; 10 years) after treatment with ethyl estrenol. 4 ml of plasma processed; 3 µl of 250 µl injected.

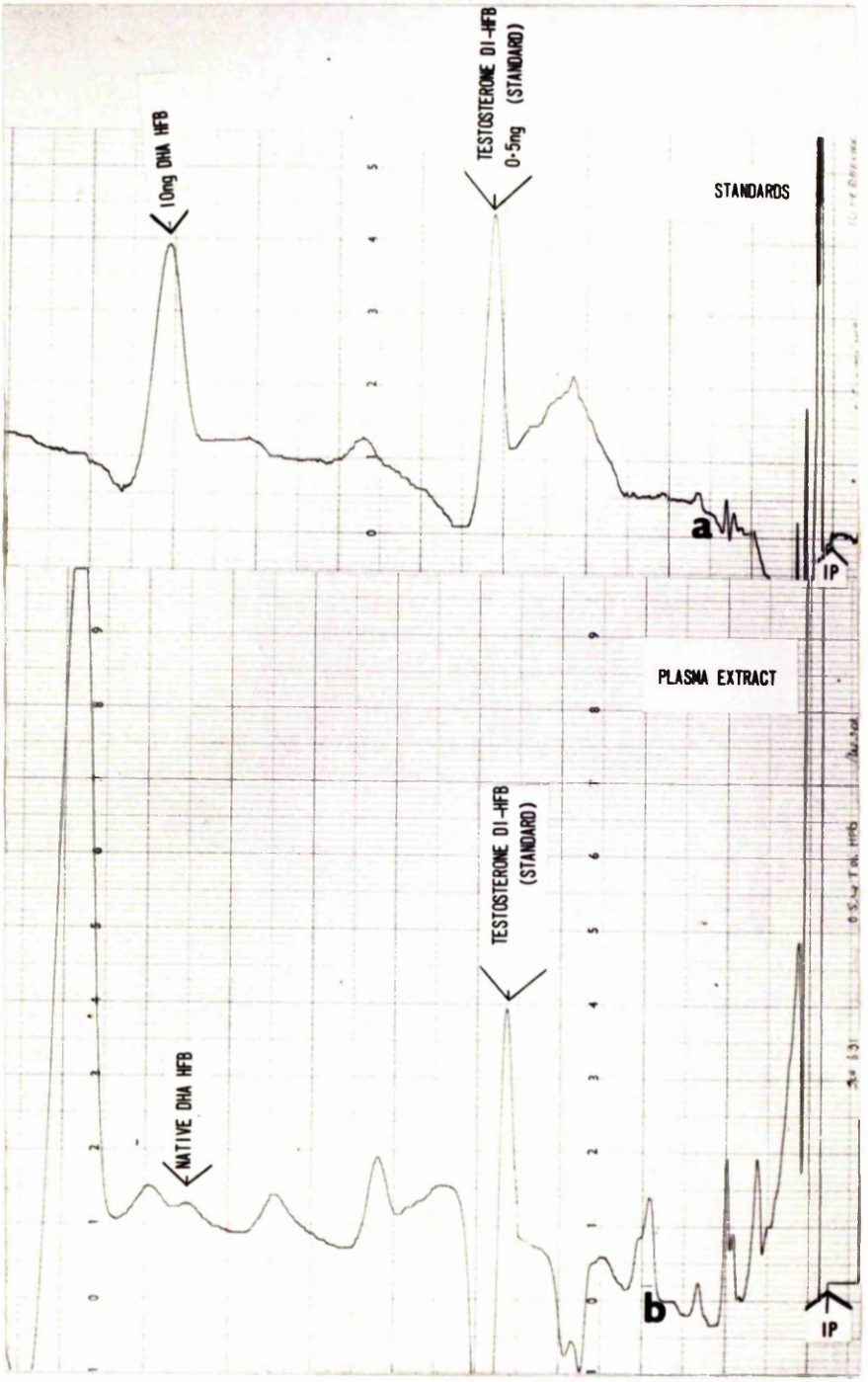


Fig. 38. Tracing obtained on g.l.c. analysis of plasma from patient 44 (female; 12 years) during a period of treatment with ethyl estrenol.

4 ml of plasma processed; 1 µl of 150 µl injected.

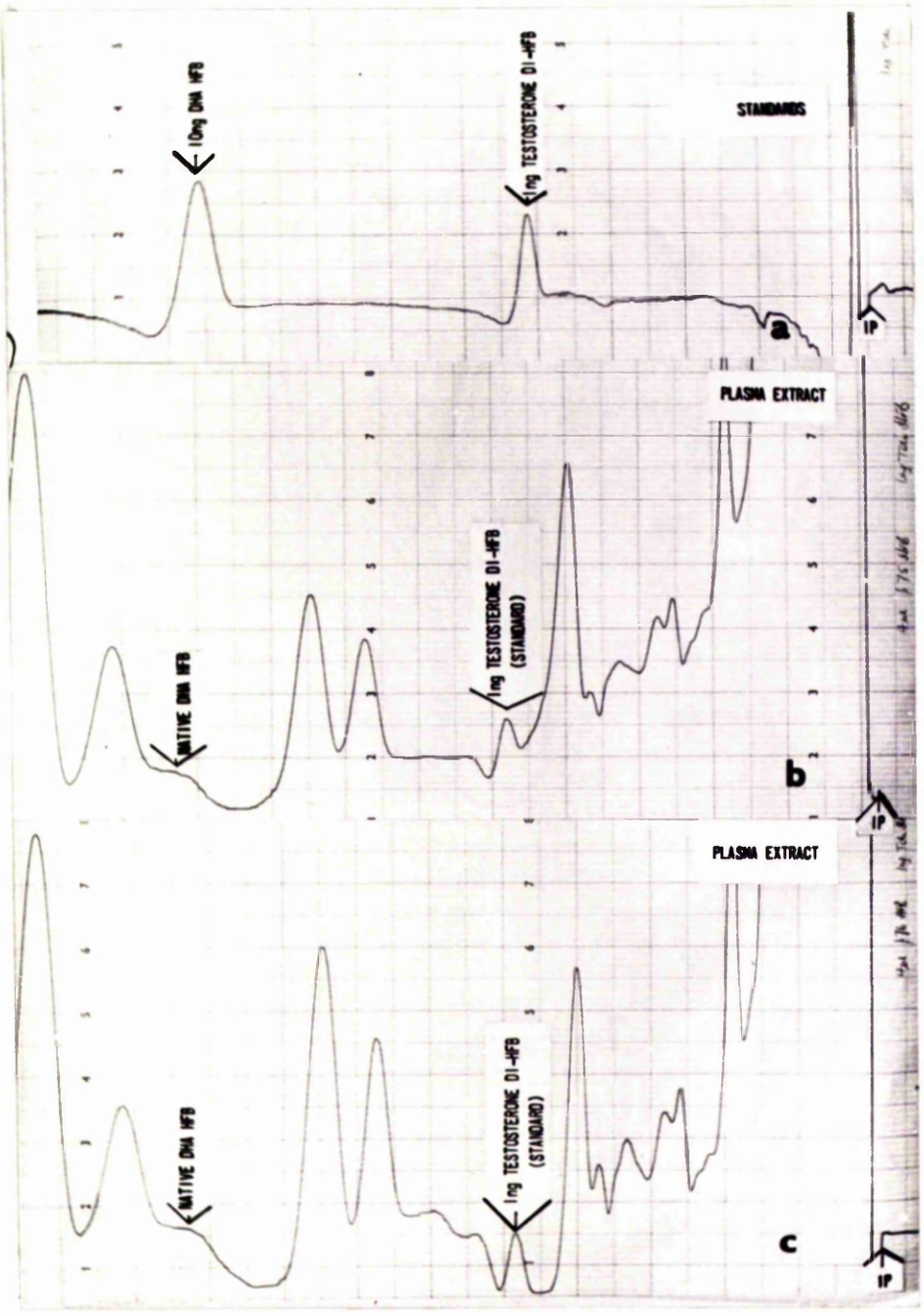


Fig. 39. Tracing obtained on g.l.c. analysis of plasma from a 6 year old normal girl (b) and from patient 30 (c) (female; 4 years).

Fig. 39 b: 11.2 ml of plasma processed; 2 µl of 50 µl injected.

Fig. 39 c: 12 ml of plasma processed; 2 µl of 50 µl injected.

On the other hand, a much smaller level was recorded from patient 44 who was receiving the same treatment (Fig. 38). Small peaks corresponding to DHA were observed in plasma extracts from a 6 year old normal girl and from a 4 year old child with cystic fibrosis (patient number 30) (Fig. 39).

The final recovery of $[7\alpha\text{-}^{3}H]$ DHA, added initially to plasma samples as $[7\alpha\text{-}^{3}H]$ DHAS, was 33.65 ± 7.6 per cent from 15 random samples (Table XXI). The percentage recovery of labelled-steroid at each stage of the analytical procedure is given after correction for aliquots taken. Prior to solvolysis recovery is estimated by liquid scintillation counting of $[7\alpha\text{-}^{3}H]$ DHAS and to facilitate solubilisation of this water-soluble conjugate in the organic scintillator, a diluent of 20 per cent methanol was incorporated into the scintillator.

A comparison of plasma levels of DHA in patients with cystic fibrosis with normal values (Yamaji and Ibayashi, 1969) revealed a significant difference in the older age range (11 to 15 years) of both sexes. The levels in affected girls of 3 to 10 years old did not differ significantly from the normal values whereas boys of the same age group had levels just significantly lower than normal (Table XXII).

SUMMARY OF RESULTS

The excretion of total 17-hydroxycorticosteroids in children with /

TABLE XXI. PERCENTAGE RECOVERY OF [7α - ^{3}H] DHA in 15 RANDOM PLASMA SAMPLES AT VARIOUS STAGES IN THE ANALYSIS

	PERCENTAGE RECOVERY OF ^{3}H-DHAS and ^{3}H-DHA				
	STAGE IN ANALYTICAL PROCEDURE				
Sample Number	* 9	* 11	13	16	21
1	95.66	81.14	60.57	48.88	36.71
2	92.19	84.15	66.68	34.63	35.82
3	90.62	83.42	69.33	50.89	39.67
4	86.31	81.11	63.73	45.02	37.85
5	95.30	68.60	54.30	34.10	29.30
6	89.30	52.80	77.65	54.80	38.99
7	89.00	57.00	55.50	43.50	30.10
8	86.10	54.90	62.60	38.50	26.30
9	81.10	60.20	43.80	29.40	22.00
10	62.00	56.70	44.10	42.00	41.10
11	66.40	58.50	50.60	50.50	50.30
12	74.70	61.80	55.50	42.60	36.09
13	63.20	51.70	46.70	39.20	24.47
14	78.16	64.80	68.10	42.60	26.50
15	81.21	72.40	62.57	45.60	29.59
Mean ± 1 Standard Deviation	82.08±11.1	65.95±11.7	58.78±9.9	42.81±7.03	33.65±7.6

Stage 9 - Following Extraction with Diethyl Ether:Ethanol (3:1, v/v)
Stage 11 - After First t.l.c. separation (t-Butanol:Ethyl Acetate: 5N NH_4OH, 82:100:40, v/v)
Stage 13 - After Solvolysis
Stage 16 - After Second t.l.c. separation (Benzene:Ethyl Acetate, 6:4, v/v)
Stage 21 - Prior to g.l.c..

* Counted as ^{3}H-DHAS using a diluent of 20 per cent methanol in the toluene-based scintillator.

TABLE XXII. PLASMA DEHYDROEPIANDROSTERONE SULPHATE LEVELS IN PATIENTS+ WITH CYSTIC FIBROSIS COMPARED WITH LEVELS FROM NORMAL CHILDREN*.

MALE PATIENTS Age Range (Years)	CYSTIC FIBROSIS PATIENTS µg per 100 ml (Mean ± 1 Standard Deviation)	NORMAL VALUES* µg per 100 ml (Mean ± 1 Standard Deviation)	
3-10	4.82 ± 4.15 (n = 12)	15 ± 15 (n = 10)	JUST SIFNIFICANTLY DIFFERENT
11-15	6.07 ± 5.2 (n = 4)	81 ± 42 (n = 49)	SIGNIFICANT DIFFERENCE
FEMALE PATIENTS Age Range (Years)	CYSTIC FIBROSIS PATIENTS µg per 100 ml (Mean ± 1 Standard Deviation)	NORMAL VALUES* µg per 100 ml (Mean ± 1 Standard Deviation)	
3-10	4.22 ± 6.83 (n = 10)	14 ± 19 (n = 8)	NO SIGNIFICANT DIFFERENCE
11-15	13.49 ± 12.35 (n = 4)	95 ± 37.6 (n = 53)	SIGNIFICANT DIFFERENCE

\+ Omitting Patients on Steroid Therapy.

*Yamaji and Ibayashi (1969).

with cystic fibrosis does not differ significantly from normal. Likewise the excretion of 17-oxosteroids in the age range 1 to 10 years is not abnormally low in these children. However, urinary 17-oxosteroids in affected children of 11 to 17 years is significantly lower than accepted normals. Normally the urinary excretion of 17-oxosteroids rises gradually from the age of 7 years to puberty and normal adult levels are generally reached between 17 and 20 years. Prior to puberty the adrenal cortex is the main site of production of the precursors of the urinary 17-oxosteroids and this is reflected by the similarity of 17-oxosteroid levels in male and female children of this age range. However, Rosenfield and Eberlein (1969) considered that plasma DHAS levels, attributed in large part to adrenal secretion, could reflect the maturation of the adrenal cortex. They reported that increased secretion of DHAS may start as early as 9 years in the female child and may reach adult levels at 13 or 14 years of age. In this study plasma DHAS was significantly lower than normal in the 11 to 15 years age range (Table XXII). Thus adrenal function in the cases of cystic fibrosis here investigated is normal in the whole paediatric age range. However, in the pubertal and post-pubertal age ranges, the ability to synthesise DHAS, as reflected by low plasma levels, is impaired. This /

This impaired function is a feature mainly of the adrenal and in the male may also be partly testicular. It might be postulated that the phenomenon called "adrenarche" is inadequate but, whatever the aetiology, there is defective production of dehydroepiandrosterone sulphate in both sexes during puberty and the period which follows. Further discussion of these findings takes place in the next chapter.

CHAPTER FIVE

DISCUSSION

The recently described defect in the male genital tract, consideration of which has already occupied part of the introductory sections of this work, has been attributed both to a failure in differentiation of the mesonephric duct (Kaplan et al. 1968; Gracey et al. 1969; Landing et al. 1969) and also to secondary obstruction of that duct (di Sant' Agnese, 1968; Oppenheimer and Esterly, 1969). The male subjects studied in Landing's survey (1969) ranged from 3 days to 25 years and in 31 of 32 an absent vas deferens and reduced epididymis were demonstrable. Wang et al. (1970) observed that these defects were neither age dependent nor related to the clinical severity of the disease. It therefore seems unlikely that a progressive obstruction of mesonephric duct structures occurs. Olson and Weaver (1969) lent further evidence for this view when they reported investigations of the genital tracts of 15 male infants who died within the first year of life. All demonstrated characteristic abnormalities without any evidence of inflammation or obstructive changes. These findings strongly suggest a developmental failure.

Generally, the absence of the vas deferens is considered to be a relatively rare abnormality of the male genital tract, bilateral absence /

absence occurring in only 1 to 10 per cent of men with aspermia. The gonads of such patients usually exhibit normal hormonal function and gamete formation. Further, fertility may be restored in men who have undergone vasectomy by anastomosing the vas deferens even up to 18 years post-operatively (Waller and Turner, 1962). Therefore the findings of impaired spermatogenesis, with arrest at the spermatocyte and spermatid stages (Kaplan et al. 1968; Denning et al. 1968), as observed from histological examination of testicular tissue from post-pubertal males with cystic fibrosis, can not be related to an obstruction phenomenon.

Valman and France (1969) suggested that differentiation of the mesonephric duct was normal until 10 weeks after conception since recognisable fragments of epididymis and vas deferens were present in some subjects with cystic fibrosis. However, the reduction of the mesonephric duct to a fibrous cord suggests a failure in development soon after the appearance of the metanephric duct, probably between the 10th and 12th foetal week (Valman and France, 1969).

These observations suggest a possible androgen deficit in foetuses with cystic fibrosis reflected by a failure in the differentiation and maintenance of the mesonephric duct structures and in the arrest in spermatogenesis, operative at spermatocyte and spermatid stages. Together /

Together with the malabsorption and chronic disease experienced by these patients, an androgen deficiency may also contribute to the retarded bone age, general failure to thrive, delayed puberty and the delayed and limited pubertal growth spurt so characteristic of the disease.

Using foetal testes in incubation systems with radioactive steroid substrates, Hamilton et al. (1970) demonstrated a greater conversion of Δ^5-androstenediol and DHA to testosterone than of androstenedione to testosterone in testes of less than 12 weeks. After 12 weeks the adult pattern of greater conversion to testosterone from androstenedione rather than from DHA and Δ^5-androstenediol is evident (Lucis et al. 1967). Thus DHA is an important precursor of the most androgenic steroid, testosterone, during the period of differentiation of the male genital tract. It will be remembered that higher levels of androgen (Jost, 1968) are required to differentiate the male internal duct structures than are required at a later stage to masculinise the male external genitalia. The role of foetal androgens on both somatic and psychological development is now well recognised and is diagramatically shown in Fig. 40. The fact that androgens are required for male psychosexual identity is well demonstrated in reverse in the feminising testicular syndrome wherein the /

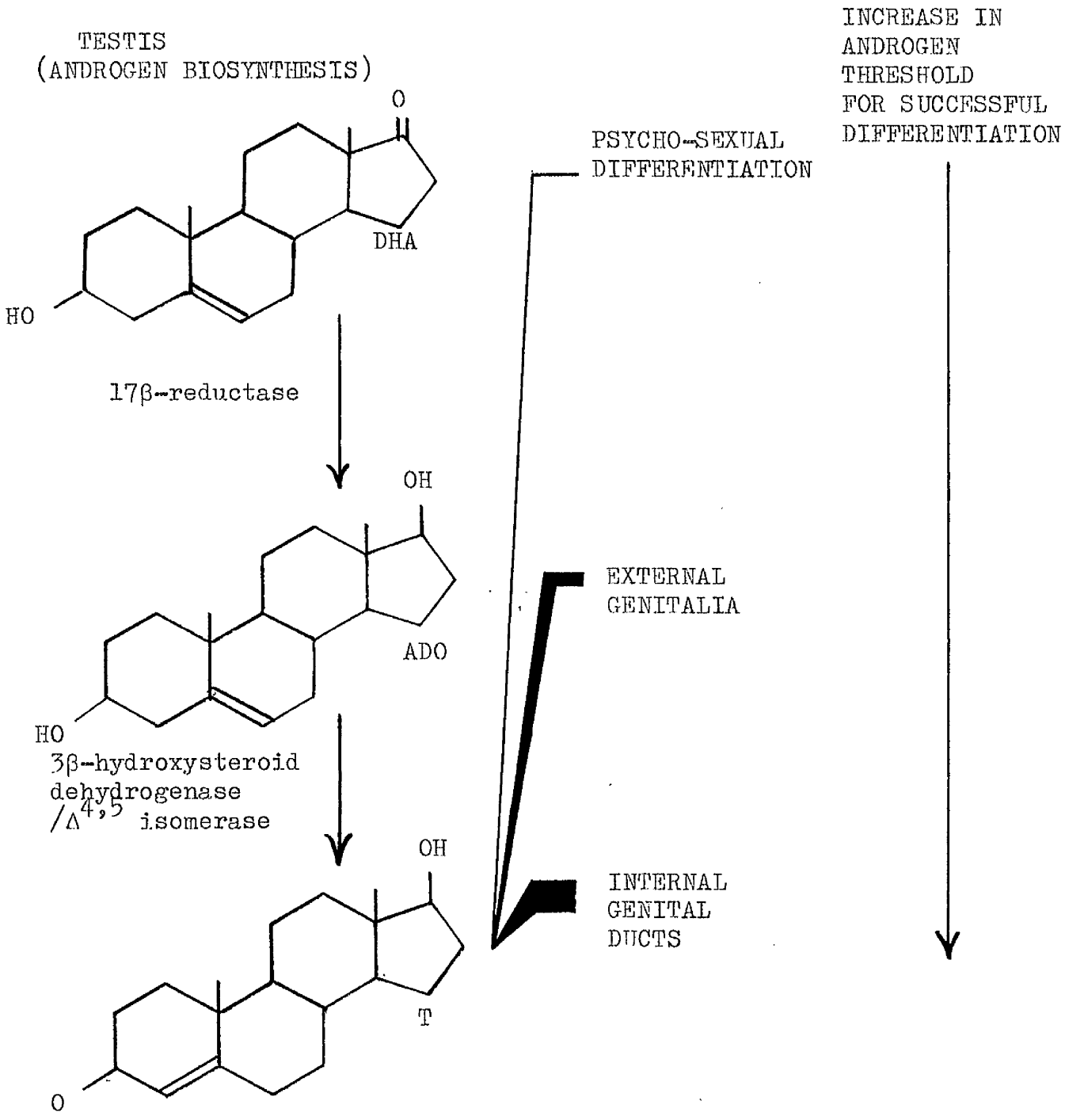


Fig. 40. Testicular androgen synthesis and action during sexual differentiation of the male foetus.

DHA: Dehydroepiandrosterone

ADO: Δ^5 - androstenediol

T : Testosterone

the phenotypic female (XY chromosomal constitution) thinks and acts completely in a female fashion because "she" lacks the necessary peripheral ability to respond to normal and even elevated levels of circulating testosterone.

Gupta (1965) has calculated that on its own merit DHA has an activity of 2 per cent of testosterone on the development of the male reproductive tract. The androgenicity of DHA as bio-assayed by the comb-growth test is $^{1}/_{13}$th that of testosterone (Fieser and Fieser, 1959). As an important intermediate of testosterone synthesis during the differentiation period, its contribution to the imposition of masculinity on the foetus must be considerable.

These arguments and the extent of the physiological and metabolic importance of DHA such as are now recognised and which have been considered in Chapter 2 have led me to a consideration of the possible significance of this hormone in cystic fibrosis.

If DHAS circulated in suboptimal concentrations then an endocrine aetiology for the maldevelopment of the mesonephric duct in the male foetus with cystic fibrosis could be proposed. Such a diminished availability of steroid might result from reduced secretion of DHAS by the adrenal, or from reduced sulphatase for DHAS so decreasing the availability of the free active DHA at a cellular level, or from reduced sulphokinase for DHA which would then /

then result in rapid elimination of DHA from the body. The investigations presented here have demonstrated normal DHA levels in urine from pre-adolescent children and low levels in the pubertal and post-pubertal age ranges. It is therefore unlikely that a sulphokinase defect is operative in cystic fibrosis.

Two periods of intense androgen activity can be recognised - one during foetal development associated with the differentiation of androgen dependent tissues, a process which is almost complete by the 18th week of gestation (Table I, p. 47), and the other at puberty associated with the development of the secondary sexual characteristics. The capacity of the foetal testis to synthesise androgens and the temporal relationship of steroidogenic activity for differentiation of the mesonephric duct have been discussed fully in Chapter 2. Large quantities of DHA and DHAS are secreted by the human foetal adrenal cortex and are attributed to the relative deficiency of 3β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$ isomerase activity in this tissue. On the basis of this enzyme deficiency it appeared unlikely that the foetal adrenal cortex secretes androstenedione or testosterone in significant amounts. However, while the conversion of pregnenolone to progesterone has proved to be insignificant in human foetal adrenal tissue (Taylor, 1971), in vitro /

in vitro conversion of DHA to Δ^4-3-oxosteroids has been demonstrated by foetal adrenal (Bloch et al. 1962; Shirley and Cooke, 1969; Taylor, 1971). Taylor (1971) conceived that substrate specific dehydrogenase/Δ^4 3β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$ isomerase activity in the foetal adrenal might operate for DHA. He also suggested that the capacity of Δ^4-3-oxosteroid formation might be restricted to the male foetus and that the resulting androstenedione (the major Δ^4-3-oxosteroid extracted from the incubation mixture) might also be involved in the maintenance of an androgenic environment for the male foetus.

Thus since androgen deficiency during foetal differentiation could account for the anomalies of the male genital tract in the patient with cystic fibrosis, it is therefore possible that subnormal levels of DHA are implicated in the maldevelopment. This follows from the fact that DHA is a pre-hormone of testosterone, at least at testicular level, during this active period of differentiation.

At birth the adrenal gland is proportionately 10 to 20 times greater than that of the adult, this being due to the hyperplasia of the foetal zone thought to be responsible for the secretion of large quantities of DHAS (Cooke and Taylor, 1971). With the involution of the foetal zone of the adrenal cortex during the neonatal period, the plasma levels and urinary excretion of Δ^5-3β-hydroxysteroids fall to very low levels, which state persists until the onset of adolescence. It /

It is not until after puberty that the adrenal reattains its birth weight. Plasma DHAS levels are age dependent (Rosenfield and Eberlein, 1969) and begin to rise at about 8 years of age. Rosenfield and Eberlein (1969) related an increase in plasma DHA and DHAS to an increased secretion by a maturing adrenal cortex. The similarity in 17-oxosteroid excretion by male and female children in the pre-pubertal age range reflects the common adrenal origin of precursors of this steroid group.

Unfortunately, the unique opportunity to study adrenal and testicular tissue from a foetus which might have been affected by cystic fibrosis did not present itself. However, study of affected adolescent children has shown that plasma DHAS does not have the usual relationship to age (Rosenfield and Eberlein, 1969). In my series, 5 boys and 4 girls of 11 to 15 years have significantly low circulating levels of DHAS (Table XIX, patients 15, 16, 17, 20, 21, 43, 45, 47 and 48.). Also, plasma DHAS was within the normal range in female patients of 3 to 10 years and just significantly lower than normal in male patients of the equivalent age range (Table XXII, p.170a). It seems, therefore, that a failure of the maturation process in the adrenal cortex for DHAS occurs in patients with cystic fibrosis and is reflected by low plasma DHAS levels. Because of the slow clearance rate of this conjugate from the blood, /

blood, plasma DHAS levels approximate the secretion of DHAS from the adrenal.

A paucity of information is available regarding adrenocortical function in children with cystic fibrosis. Di Sant' Agnese et al. (1953) reported an increase in 17-oxosteroids in the urine of a 14 year old pre-pubertal boy following ACTH administration. He also noted the anomalous, though characteristic, normal response in urine electrolyte excretion following salt restriction as opposed to the lack of response of the sweat electrolytes to subcutaneous deoxycorticosterone administration.

Chodos et al. (1965) investigated a series of 19 patients with cystic fibrosis. They estimated total 17-hydroxycorticosteroid excretion, 17-oxosteroid excretion, plasma concentrations of 17-hydroxycorticosteroids, cortisol and corticosterone before and after ACTH administration and the disappearance rates of hydrocortisone and corticosterone administered intravenously.
They found that these parameters of adrenocortical function were not strikingly different from those of normal children. The results presented in the preceding chapter show too that the patients investigated had normal adrenocortical function i.e. total 17-hydroxycorticosteroid levels in urine from 32 patients with cystic fibrosis (2 to 17 years) did not differ significantly from levels excreted /

excreted by normal children (Table XII).

Note too that in my extensive group of patients the 17-oxosteroid excretion, in patients aged 1 to 10 years, did not differ significantly from normal. However, children from 11 to 17 years did have a significantly lower excretion of 17-oxosteroids. Excretion of DHA in the older age ranges (11 to 20 years) was subnormal in 8 of 11 boys and in 6 of 7 girls (the one exceptional case (patient 44, Table XVIII, p. 162) having had anabolic hormone therapy during the period of urine collection). Circulating levels of DHAS were significantly lower than normal in both males and females in the 11 to 15 age range. Plasma levels did not differ significantly from normal values in the 5 to 10 years age range in female patients and were just significantly lower than normal for the corresponding males.

From these results it is apparent that the increase in 17-oxosteroid excretion and increase in plasma DHAS associated with puberty and reflecting a complete maturation of the adrenal cortex and also of the testes in the male, is not well defined in the adolescent with cystic fibrosis.

Compare these results with those of Chodos et al. (1965) and Flensborg and Johnsen (1972). Their patient age ranged from

5 /

5 months to 13 years and from 2 months to 15 years respectively. The levels of 17-oxosteroids did not differ from those of normal control children though Flensborg and Johnsen reported subnormal values for 11-keto-aetiocholanolone and for 11-hydroxy-aetiocholanolone. The significance of these findings is not known and they suggest none. In a series of 18 children with cystic fibrosis (aged 10 months to 23 years) Flensborg found normal urinary levels of DHA (personal communication).

Thus in summary, it seems probable that during the two peak periods (the 6th to 18th intra-uterine week and at puberty) of adrenocortical function for DHAS production a failure can be postulated, which accounts in theory for the "endocrine" features of cystic fibrosis.

Since the defects of the mesonephric duct, characteristic of cystic fibrosis, are now here believed to be developmental in origin and since this failure in differentiation may be the result of the failure to attain the prerequisite threshold of androgen (p. 175) it might be expected that further anomalies of the male genital tract could occur in patients with this disease.

Wang et al. (1970) were able to investigate 141 male patients with cystic fibrosis and of these 9 boys were found to have an inguinal hernia /

hernia and 3 had a hydrocele. This combined incidence of both inguinal hernia and hydrocele was 8.5 per cent. The incidence of these anomalies in the general male population is 1 per cent for inguinal hernia and less than 1 per cent for hydrocele (Swenson, 1969; Campbell, 1964). Holsclaw and Shwachman (1971) investigated 325 male patients with cystic fibrosis and reported an increased incidence of herniae, hydrocele and undescended testes (unilateral and bilateral).

Of the 24 male patients in this investigation one (patient 5) had hypospadias and one (patient 22) had a left-sided hydrocele. These abnormalities associated with the urogenital tract were thus present in 8.3 per cent of the present series. It is interesting to postulate that these abnormalities are also related to the factors which have been incriminated as causing the failure in development of the mesonephric duct. There is some evidence that this could well be so, the evidence being based on recent knowledge of the functioning of the various components of testicular tissue.

The steroidogenic capacity of the interstitial (Leydig) cells of the testis is well established. Studies of testicular function in incubation systems have largely employed slices or homogenates of testicular tissue and any steroidogenic activity has been attributed to Leydig cell function.

However, /

However, recent developments in the techniques of micro-dissection have enabled the isolation of individual seminiferous tubules. Tubular cells isolated in this way from rats were cultured and then incubated with radioactive-substrates (Dufau et al. 1971). While Leydig cells have been confirmed to be the major source of testicular androgen (Christensen and Mason, 1965), the tubular components have been shown to have a steroid converting capacity with the ability to form testosterone from androstenedione, Δ^5-androstenediol and DHA. Much smaller quantities of testosterone were formed from more remote substrates such as pregnenolone, and 17α-hydroxyprogesterone (Dufau et al. 1971). Since high levels of testosterone are known to be necessary for the maintenance of spermatogenesis, Dufau and colleagues suggest that the tubular cells produce high local concentrations of testosterone necessary for the maintenance of spermatogenesis from such precursors as DHA. Bell (1972) has confirmed these findings of differential androgen production in the human testis. The Leydig cells, then, produce testosterone which is released into the peripheral circulation for systemic use and intermediate steroid metabolites which diffuse into the tubules there to be converted to testosterone so maintaining a high local concentration presumably to stimulate spermatogenesis. If in cystic fibrosis circulating levels of DHA/DHAS /

DHA/DHAS are subnormal, as the results of this thesis would indicate, then suboptimal concentrations of such testosterone pre-hormone would be present at the tubular level. Defective spermatogenesis could then be related to a failure in local testosterone production and hence a failure to maintain an androgenic environment.

There is now evidence in the literature that clinical syndromes arise as a result of defective testosterone biosynthesis. Also in certain diseases testosterone production is progressively diminished. Thus Zachman et al. (1972) were able to show absent 17,20 desmolase activity in a child with male pseudohermaphroditism (a phenotypic female with an XY chromosomal constitution). Also, testicular incubation studies using testicular material from patients with Klinefelter's syndrome have demonstrated a progressive deficiency of testicular enzymes (Hamilton, 1971). Patients with myotonic dystrophy have also a deficient testosterone production which could be related to the characteristic degeneration of testicular tissue (Hamilton, 1973). Thus not only in cystic fibrosis but in other subjects such defects as hypospadias, undescended testes and herniae may result from yet unrecognised defects in testosterone synthesis.

While the investigation of testicular function in male adolescent children with cystic fibrosis was outwith the scope of this investigation, /

investigation, the results presented in Tables XVII and XXII (pp. 161 and 170a) demonstrate low plasma DHAS levels and low urinary DHA levels in this age range. As in any study, particularly those concerned with cystic fibrosis where the extent and degree of involvement of the various tissues is so variable, exceptions to the thesis must occur. Patients 9, 19 and 24 have increased levels of urinary DHA as compared with accepted normal values. The DHA in these cases was excreted predominantly as the sulphate conjugate. None of the patients were undergoing treatment with anabolic hormones (see later discussion, p. 188). Two, patients 9 and 24, were given "good" clinical ratings and patient 19 had a "moderate" degree of involvement. Despite the finding that the abnormality of the male genital tract may be one of the most invariable features of the disease, it is recognised that exceptions do occur and claims to paternity, though few, have been made. It may be that these three patients have, and have always had, adequate androgen biosynthesis for normal differentiation and testicular function. Two (patients 19 and 24) no longer reside in the Glasgow area. Analysis of plasma obtained from the third (patient 9) showed DHAS to be present at a concentration of 6 μg per 100 ml (Table XIX, p. 163a). This result was incorporated in the overall results for the 3 to 10 year old males which /

which on the whole were just significantly lower than normal (Table XXII, p.170a). However, on its own merit, a level of 6 μg DHAS per 100 ml plasma was probably well within the normal range.

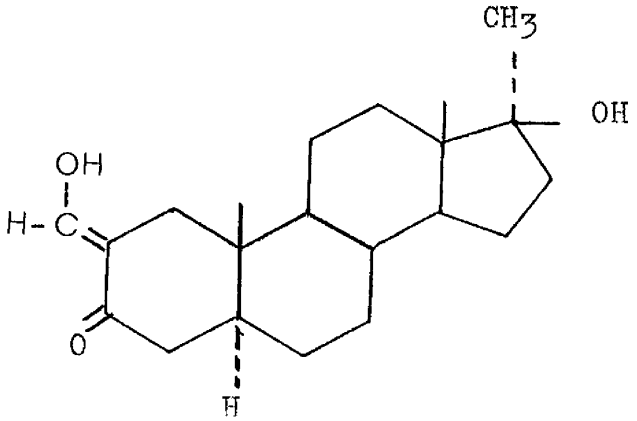
Patient 5 also excreted high levels of DHA although the sulphate:glucuronoside ratio was reversed, the DHA being mainly conjugated with glucuronic acid. One can but speculate on the significance of such a finding, particularly on its possible relationship to the hypospadias noted in this patient. The hypospadias suggests a deficient androgen concentration during the period of external genital differentiation but clearly his pre-pubertal plasma level of DHAS (5.4 μg DHAS per 100 ml plasma) was normal (Table XIX, p.163a).

One could postulate an enzyme deficiency at testicular level wherein either 3β-hydroxysteroid dehydrogenase and/or 17β-reductase were deficient, so resulting in high levels of DHA (i.e. failure to convert DHA to androstenedione, Δ^5-androstenediol and thence to testosterone). The accumulated DHA might then be preferentially conjugated with glucuronic acid for rapid clearance from the body (it will be recalled that normally the gut and liver resulphate DHA and in cystic fibrosis the gut and liver are known to be involved). It is known that the clearance rate for DHAG approximates to the glomerular filtration rate.

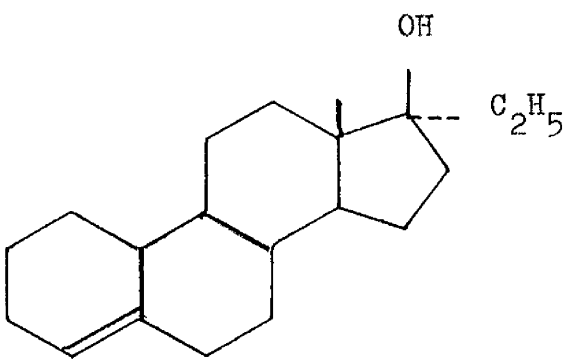
There /

There is a parallel for this thinking in the now well-recognised variety of congenital adrenal hyperplasia due to defective 3β-hydroxysteroid dehydrogenase which is associated with hypospadias in the male.

A number of patients undergoing treatment with anabolic hormones had near normal or elevated levels of total urinary DHA and normal or high plasma DHAS levels (patients 14, 16, 28, 32 and 44; Tables X, XI and XIX.). The type of anabolic hormone given to each of these patients is indicated in the aforementioned tables.
Two preparations with a favourable anabolic/androgenic ratio were given to a selected number of patients with cystic fibrosis.
The rationale for this step taken by the physician (W.H.) was as follows.
Incubation studies on foetal testicular tissue, using as substrate DHA and androstenedione, showed that, oxymetholone, a C2-substituted C19 steroid, and ethyl estrenol were inhibitors of 3β-hydroxysteroid dehydrogenase so blocking the conversion of DHA to androstenedione.



OXYMETHOLONE
(17α-methyl-2-hydroxymethylene dihydrotestosterone)



ETHYL ESTRENOL
(17α-ethyl-3-desoxo-19-nortestosterone)

Oxymetholone is believed to block enzyme action by competitive inhibition through the formation of the enol at C2 and C3. It is thought that the enol binds irreversibly at the site of enzyme activity. This compound has been further shown to inhibit 17β-reductase for DHA and androstenedione. The combined action then is to raise the level of circulating DHA (Hamilton, 1972; Hamilton, unpublished observations). It thus seemed logical that these drugs could be used in an attempt to raise circulating levels of DHA in patients with cystic fibrosis. Oxymetholone has been used largely to treat hypoplastic anaemia, the dosage levels being in the region of 100 to 300 mg daily. At this dosage level some workers have suggested that it may have carcinogenic effects (Johnson et al. 1972) but, in children with cystic fibrosis, a much smaller dose of 2.5 to 5 mg daily was used.

The use of anabolic hormones in cystic fibrosis is not unprecedented (Dennis and Panos, 1965; Good and Bessman, 1966; Dooley et al. 1969; Harris and Waring, 1970) although previous administration has been purely for anabolic effect. However, it is of interest that Dooley et al. (1969) noted that pre-adolescent female patients with cystic fibrosis derived greatest benefit from anabolic steroid therapy. The purpose of administering these agents /

TABLE XXIII. THE EFFECTS OF ANABOLIC HORMONE ON URINARY AND PLASMA DEHYDROEPIANDROSTERONE.

Patient	Patient Number	Age	Urinary DHA µg per 24 hrs.		Plasma DHAS µg per 100 ml
			Total DHA	DHAS	
F.B.	14	10	* 21.35	15.8	325.6
			18.4	12.5	
			** 33.4	27.0	
			33.6	7.8	
		11	7.9	6.7	
P.S.	22	16	* 490.6	453.0	
			** 280.7	13.7	1.5
			** 242.8	179.4	
J.L.	32	4	0.3	0.3	1.9
			** 18.5	9.3	8.9
A.McM.	44	12	* 228.2	102	9.3

Under treatment with:- * Ethyl Estrenol

** Oxymetholone

agents to a selected number of patients with cystic fibrosis in this hospital was as an attempt to exploit their enzyme-blocking properties to raise levels of circulating DHA (Hamilton, 1970) which, in this investigation, were found to be subnormal, particularly in the adolescent age range. It must be emphasised that the use of anabolic hormone therapy in cystic fibrosis is still very much in the experimental stage. Patients chosen for this form of treatment were either severely affected children (patients 14 and 44) or children with a good clinical status who were likely to benefit from an anabolic stimulus (patients 22 and 32) (Table XXIII). Ethyl estrenol was the first anabolic hormone preparation to be administered but unwanted oestrogen-like side effects (e.g. gynaecomastia) were observed. The administration of this drug, however, seemed to result in near-normal to high levels of total urinary DHA. In patients 14 and 22 this was predominantly excreted as the sulphate ester. In patient 44, however, the sulphate/glucuronoside ratio for urinary DHA was less than unity. Patient 14 responded to ethyl estrenol therapy with a very high plasma level of DHAS (Fig. 37, p. 165). However, patient 44, after the same treatment, still had low plasma levels of DHAS compared with normal values (Fig. 38, p. 166). Patient 22 also had low plasma DHAS levels despite treatment with oxymetholone (Table XXIII). Blood and urine had been collected one /

one month after replacing ethyl estrenol therapy with oxymetholone. Near-normal values of total urinary DHA were then maintained though, for a short time after the change of therapy, DHA was excreted mainly as the glucuronoside. A urine sample collected 3 months after the start of oxymetholone therapy indicated urinary DHAS levels to be again in excess of DHAG levels. In this patient ethyl estrenol therapy resulted in higher urinary levels of total DHA than did oxymetholone administration. This is in contrast with the findings from analyses of urine samples from patient 14. One month after cessation of oxymetholone therapy in patient 14 near-normal levels of urinary total DHA were maintained although the DHA was excreted mainly as the glucuronoside. Six months afterwards, low levels of urinary total DHA were detected. Patient 32, after 6 months therapy with oxymetholone, had increased urinary levels of DHA and also of plasma DHAS. It can be seen that the results of anabolic steroid therapy on this group of patients with cystic fibrosis are variable. However, treatment did result in near-normal levels of DHA in urine and this coincided with a period of increased linear growth (Hamilton, 1972). Frequent plasma analyses would be advantageous to relate dosage of anabolic hormone to response of DHAS levels in an attempt to maintain physiological plasma concentrations of DHAS. It might then be possible

over /

over several years to speak more authoritatively to the effects of normal plasma DHAS levels in patients with cystic fibrosis.

Thus, from the results of the investigations done on children with cystic fibrosis presented in this thesis it would appear that there is a real failure in adrenal production of DHAS at puberty which persists even after the appearance of clinical puberty. This is consistent with the concept that adrenal androgen activity is responsible for the early signs of puberty before the gonads take over. To be more explicit there is a failure to increase secretion of DHAS in the pubertal and post-pubertal age ranges. Adrenocortical function as determined by the excretion of total 17-hydroxycorticosteroids is within the normal range. The thesis is therefore offered that were more known regarding the total cellular actions of DHA, this demonstrated failure might account for more of the clinical features of cystic fibrosis.

Attempts to raise circulating levels of DHA by using anabolic steroids, in particular oxymetholone, resulted in impressive height and weight gains which were associated with increased urinary DHA excretion. In the future were this therapy or another achieving similar effects to be practised as routine, frequent assay of plasma DHAS levels would be necessary to monitor the raising of plasma DHAS to /

to physiological concentrations thus ensuring the desired effects with the accompanying effects on osseous maturation.

Other forms of treatment for children with cystic fibrosis have tended to follow popular opinion. It will be remembered that cloxacillin was occasionally found to interfere with steroid colorimetric estimations (in 9 of 59 urine samples) and that the "affected" urine samples were either collected during hospitalisation or from children whose parents rigorously applied therapeutic measures at home. This in itself speaks to the capricious value of any treatment which cannot readily be monitored and it seems that even the fundamental, though essential, administration of antibiotics is often neglected by the parents.

Though this thesis does not resolve the quest for the basic aetiology of cystic fibrosis, it points to a defect at cellular and metabolic levels. It must be remembered that cystic fibrosis affects many cell types. If a single transmittable genetic defect is to account for the range of abnormalities associated with the disease then the common defect in the many cell types must surely rest at such a basic metabolic or cellular level.

CHAPTER SIX

REFERENCES

Acevedo, H.F., Axelrod, L.R., Ishikawa, E., and Takaki, F. (1963). Studies in fetal metabolism. II Metabolism of progesterone-4-^{14}C and pregnenolone-7α-^{3}H in human fetal testes. J. clin. Endocr., 23^{2}, 885.

Allen, W.M. (1950). A simple method for analysing complicated absorption curves of use in the colorimetric determination of urinary steroids. J. clin. Endocr., 10, 71.

Andersen, D.H. (1938). Cystic fibrosis of the pancreas and its relation to coeliac disease. Am. J. Dis. Child., 56, 344.

Andersen, D.H. (1939). Cystic fibrosis of the pancreas, vitamin A deficiency and bronchiectasis. J. Pediat., 15, 763.

Andersen, D.H. (1942). Pancreatic enzymes in duodenal juice in coeliac syndrome. Am. J. Dis. Child., 63, 643.

Andersen, D.H., and Hodges, R.G. (1946). Coeliac syndrome. V Genetics of cystic fibrosis of the pancreas with consideration of aetiology. Am. J. Dis. Child., 72, 62.

Appleby, J.I., Gibson, G., Norymberski, J.K., and Stubbs, R.D. (1955). Indirect analysis of corticosteroids. I The determination of 17-hydroxycorticosteroids. Biochem. J., 60, 453.

Balfe, /

Balfe, J.W., Cole, C., and Welt, L.G. (1968). Red cell transport defect in patients with cystic fibrosis and in their parents. Science, 162, 689.

Barach, A.L., Silberstein, F.H., Oppenheimer, E.T., Hunter, T., and Soroka, M. (1945). Inhalation of penicillin aerosol in patients with bronchial asthma, chronic bronchitis, bronchiectasis and lung abscess. Ann. Int. Med., 22, 485.

Barbero, G.J., and Chernick, W. (1958). Function of the salivary glands in cystic fibrosis of the pancreas. Pediatrics, 22, 945.

Baulieu, E.E. (1962). Studies of conjugated 17-ketosteroids in a case of adrenal tumor. J. clin. Endocr., 22, 501.

Baulieu, E.E. (1965). A new development in steroid endocrinology: the metabolism and the biological importance of the conjugated forms of hormones and their metabolites. In "Proceedings of the IInd Int. Congress of Endocrinology, London, 1964" p. 2. Ed. S. Taylor (Excerpta Medica International Congress Series No. 83). Amsterdam, New York, London, Milan, Tokyo, Buenos Aires: Excerpta Medica Foundation.

Beas, F., Zürbrügg, R.P., Cara, J., and Gardner, L.I. (1962). Urinary C19 steroids in normal children and adults. J. clin. Endocr., 22, 1090.

Bell, J.G.B. (1972). Interconversion of testosterone and androstenedione in the human testis: a comparison between the activities displayed by the interstitium and the seminiferous tubules. Experientia, 28, 212.

Berger, /

Berger, H., Fink, M., Fritz, H.J., Gleispach, H., Heidemann, P., and Wolf, J. (1970). Normal values for the various 17-oxosteroids, pregnanes and testosterone excreted in the urine of healthy boys and girls. Z. klin. Chem. u. klin. Biochem., 8, 354.

Besley, G.T.N., and Patrick, A.D. (1969). The effect of cystic fibrosis plasma and saliva on enzyme systems of cell membranes. In "Proceedings of the Fifth International Cystic Fibrosis Conference, Churchill College, Cambridge, September, 1969". p. 14. Ed. D. Lawson. Cystic Fibrosis Research Trust, London.

Blackfan, K.D., and May, C.D. (1938). Inspissation of secretion, dilatation of ducts and acini, atrophy and fibrosis of pancreas in infants: clinical note. J. Pediat., 13, 627.

Blackfan, K.D., and Wolbach, B.S. (1933). Vitamin A deficiency in infants. J. Pediat., 3, 679.

Bloch, E. (1964). Metabolism of 4-^{14}C-progesterone by human fetal testis and ovaries. Endocrinology, 74, 833.

Bloch, E. (1967). *In vitro* steroid synthesis by gonads and adrenals during mammalian fetal development. In "Proceedings of the Second International Congress on Hormonal Steroids, Milan, 1966". p. 675. Eds. L. Martini, F. Fraschini and M. Motta. (Excerpta Medica International Congress Series No. 132). Amsterdam, New York, London, Milan, Tokyo, Buenos Aires: Excerpta Medica Foundation.

Bloch, /

Bloch, E., Tissenbaum, B., Rubin, B.L. and Deane, H.W. (1962). Δ^5-3β-hydroxysteroid dehydrogenase activity in human fetal adrenals. Endocrinology, 71, 629.

Blunck, W. (1968). Die α-ketolischen Cortisol-und Corticosteron-metaboliten sowie die 11-Oxy-und 11-Desoxy-17-ketosteroide im Urin von Kindern. Acta endocr., Copnh., Suppl., 134.

Boat, T.F. (1970). Pathogenesis of turbid submaxillary saliva in patients with cystic fibrosis. Cystic Fibrosis Club Abstracts, Eleventh Annual Meeting, Atlantic City, p. 30.

Brooks, C.J.W., and Norymberski, J.K. (1952). Characterisation of the corticosteroid side-chain. Chem. and Ind., p. 804.

Brooks, C.J.W., and Norymberski, J.K. (1953). Oxidation of corticosteroids with sodium bismuthate. Biochem. J., 55, 371.

Brown, G.A., Black, S.C., and Anderson, C.M. (1970). Sulphate uptake kinetics in leucocytes from fibrocystic children and their parents. First Annual Meeting, European Working Group for Cystic Fibrosis, Stockholm, Sweden.

Brownsey, B., Cameron, E.H.D., Griffiths, K., Gleave, E.N., Forrest, A.P.M., and Campbell, H. (1972). Plasma dehydroepiandrosterone levels in patients with benign and malignant breast disease. Europ. J. Cancer, 8, 131.

Bryson, V., Sansome, E., and Laskin, S. (1944). Aerosolization of penicillin solutions. Science, 100, 33.

Burstein, /

Burstein, S., and Dorfman, R.I. (1963). Determination of mammalian steroid sulphatase with 7α-^{3}H-3β-hydroxyandrost-5-en-17one sulphate. J. biol. Chem., 238, 1656.

Burstein, S., and Lieberman, S. (1958). Hydrolysis of ketosteroid sulphates by solvolysis procedures. J. biol. Chem., 233, 331.

Butenandt, A., and Tscherning, K. (1934). Über Androsterone, ein krystallisiertes männliches Sexual-hormon. I Isolierung und Reindarstellung aus Männerharn. Hoppe-Seyler's Z. physiol.Chem., 229,167

Callow, R.K. (1938). The significance of the excretion of sex hormones in the urine. Proc. Roy. Soc. Med., 31, 841.

Calvin, H.I., and Lieberman, S. (1964). In vitro conversion of pregnenolone-^{3}H-sulphate-^{35}S to 17α-hydroxypregnenolone-^{3}H-^{35}S sulphate. Biochemistry, 3, 259.

Campbell, M.F. (ed.), (1964). Urology, 2nd Ed. p. 1998. W.B. Saunders Co., Philadelphia.

Cavallero, C., and Magrini, U. (1967). Histochemical studies on 3β-hydroxysteroid dehydrogenase and other enzymes in the steroid secreting structures of human foetus. In "Proceedings of the Second International Congress on Hormonal Steroids, Milan, 1966". p. 667. Eds. L. Martini, F. Fraschini, and M. Motta. (Excerpta Medica International Congress Series No. 132). Amsterdam, New York, London, Milan, Tokyo, Buenos Aires: Excerpta Medica Foundation.

Chernick, /

Chernick, W.S., Barbero, G.J., and Parkins, F.H. (1961). Studies on submaxillary saliva in cystic fibrosis.
J. Pediat., 59, 890.

Chernick, W.S., and Barbero, G.J. (1963). Studies on human tracheobronchial and submaxillary secretions in normal and pathophysiological conditions.
Ann. New York Acad. Sc., 106, 698.

Chodos, D.D.J., Ely, R.S., Limbeck, G.A., and Kelley, V.C. (1965). Corticosteroid metabolism in cystic fibrosis.
Am. J. Dis. Child., 110, 76.

Christensen, A.K., and Mason, N.R. (1965). Comparative ability of seminiferous tubules and interstitial tissue of rat testes to synthesize androgens from progesterone-4-^{14}C *in vitro*.
Endocrinology, 76, 646.

Clark, S.J., and Wotiz, H.H. (1963). Separation and detection of nanogram amounts of steroids.
Steroids, 2, 535.

Clarke, C., and Hadfield, G. (1924). Congenital pancreatic disease with infantilism.
Quart. J. Med., 17, 358.

Cooke, B.A., and Taylor, P.D. (1971). Site of dehydroepiandrosterone sulphate biosynthesis in the adrenal gland of the previable foetus. J. Endocr., 51, 547.

Crawfurd, /

Crawfurd, M. d'A. (1969). Experience of the cilia test for a factor in the serum of patients and carriers of cystic fibrosis. In "Proceedings of the Fifth International Cystic Fibrosis Conference, Churchill College, Cambridge, September, 1969". p. 42. Ed. D. Lawson. Cystic Fibrosis Research Trust, London.

Crawfurd, M. d'A. (1970). Preliminary report on the isolation and characterisation of the C.F. serum factor. In "Cystic Fibrosis Research Trust Research Conference Abstracts. Abstract 1." Owens Park, Manchester, December, 1970.

Danes, B.S. (1969). Cell culture studies in cystic fibrosis. In "Proceedings of the Fifth International Cystic Fibrosis Conference. Churchill College, Cambridge, September, 1969". p. 67. Ed. D. Lawson. Cystic Fibrosis Research Trust, London.

Danes, B.S., and Bearn, A.G. (1968). A genetic cell marker in cystic fibrosis of the pancreas.
Lancet, 1, 1061.

Danes, B.S., and Bearn, A.G. (1969). Cystic fibrosis of the pancreas: A study in cell culture.
J. exp. Med., 129, 775.

Dantchakoff, V. (1937). Sur la faculté des tissus induits par l'hormone male, d'édifier de nouvelles structures chez l'embryon de cobaye femelle.
Compte rendu Soc. de biol., 124, 516.

Denning, /

Denning, C.R., Sommers, S.C., and Quigley, H.J. (1968). Infertility in male patients with cystic fibrosis. Pediatrics, 41, 7.

Dennis, J.L., and Panos, T.C. (1965). Growth and bone-age retardation in cystic fibrosis. Response to an anabolic steroid. J.A.M.A., 194, 113.

Di Sant' Agnese, P.A. (1953). Bronchial obstruction with lobar atelectasis and emphysema in cystic fibrosis of the pancreas. Pediatrics, 12, 178.

Di Sant' Agnese, P.A. (1968). Fertility and the young adult with cystic fibrosis. New Engl. J. Med., 279, 103.

Di Sant' Agnese, P.E.A., and Andersen, D.H. (1946). Coeliac syndrome. IV Chemotherapy in infections of the respiratory tract associated with cystic fibrosis of the pancreas; observations with penicillin and drugs of sulphonomide groups with special reference to penicillin aerosol. Am. J. Dis. Child., 72, 17.

Di Sant' Agnese, P.A., and Blanc, W.A. (1956). A distinctive type of biliary cirrhosis of the liver associated with cystic fibrosis of the pancreas. Pediatrics, 18, 189.

Di Sant' Agnese, P.A., Darling, R.C., Perera, G.A., and Shea, E. (1953). Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas: clinical significance and relationship to disease. Pediatrics, 12, 549.

Di Sant' Agnese, /

Di Sant' Agnese, P.A., Dische, Z., and Danilezenko, A. (1957). Physico-chemical differences of mucoproteins in duodenal fluid of patients with cystic fibrosis of pancreas and controls: clinical aspects. Pediatrics, 19, 252.

Di Sant' Agnese, P.A., Grossman, H., Darling, R.C., and Denning, C.R. (1958). Saliva, tears and duodenal contents in cystic fibrosis of the pancreas. Pediatrics, 22, 507.

Di Sant' Agnese, P.A., and Powell, G.F. (1962). The eccrine sweat defect in cystic fibrosis of the pancreas (mucoviscidosis). Ann. New York Acad. Sc., 93, 555.

Diczfalusy, E. (1967). Steroid metabolism in pregnancy and in the foeto-placental unit. In "Proceedings of the Second International Congress on Hormonal Steroids, Milan, 1966". p. 82. Eds. L. Martini, F. Fraschini and M. Motta. (Excerpta Medica International Congress Series No. 132). Amsterdam, New York, London, Milan, Tokyo, Buenos Aires: Excerpta Medica Foundation.

Dische, Z. (1970). In "Cystic Fibrosis and related human and animal diseases". p. 164. Ed. S. Jakowska. Gordon and Breach, New York, London, Paris.

Dische, Z., di Sant' Agnese, P.A., Pallavicini, C., and Youlos, J. (1959). Composition of mucoprotein fractions from duodenal fluid of patients with cystic fibrosis and from controls. Pediatrics, 24, 74.

Dische, Z., Pallavicini, J.C., Smirnow, N., and di Sant' Agnese, P.A. (1961). Abnormalities in composition of urinary nondialyzable glycoproteins in cystic fibrosis of pancreas. Am. J. Dis. Child., 102, 733.

Dorgett, /

Doggett, R.G., and Harrison, G.M. (1969). Significance of the bacterial flora associated with chronic pulmonary disease in cystic fibrosis. In "Proceedings of the Fifth International Cystic Fibrosis Conference. Churchill College, Cambridge, September, 1969". p. 175. Ed. D. Lawson. Cystic Fibrosis Research Trust, London.

Dooley, R.R., Moss, A.J., Wright, P.M., and Hassakis, P.C. (1969). Norethandrolone in cystic fibrosis of the pancreas. J. Pediat., 74, 95.

Dufau, M.L., de Kretser, D.M., and Hudson, B. (1971). Steroid metabolism by isolated rat seminiferous tubules in tissue culture. Endocrinology, 88, 825.

Edwards, R.W.H., Kellie, A.E., and Wade, A.P. (1953). The extraction and oxidation of urinary steroid conjugates. In "The determination of adrenocortical steroids and their metabolites". Memoirs of the Society for Endocrinology No. 2. p. 53. Eds., P. Eckstein and S. Zuckerman.

Elger, W. (1966). Die Rolle der fetalen Androgene in der Sexualdifferenzierung des Kaninchens und ihre Abgrenzung gegen andere Hormonale und somatische Faktoren durch Anwendung eines starken Antiandrogens. Arch. Anat. micr. Morphol. exper., 55, 657.

Ericsson, R.J., Cornette, J.C., and Buthala, D.A. (1967). Binding of sex steroids to rabbit sperm. Acta endocr., Copnh., 56, 424.

Exley, /

Exley, D. (1966). The ultramicro-determination of testosterone using gas liquid chromatography with electron capture detection. In "Androgens in Normal and Pathological conditions". p. 11. Ed. A. Vermeulen. Proceedings of the Second Symposium on Steroid Hormones, Ghent, 1965. (Excerpta Medica International Congress Series No. 101) Amsterdam: Excerpta Medica Foundation.

Exley, D., and Chamberlain, J. (1967). Properties of steroidal 3 enol heptafluorobutyrates.
Steroids, 10, 509.

Fanconi, G., Uehlinger, E., and Knauer, C. (1936). Das Coeliakiesyndrom bei angeborener zystischer Pankreasfibromatose und Bronchiektasien.
Wien. med. Wchnschr., 86, 753.

Farber, S. (1944). Pancreatic function and disease in early life. V Pathologic changes associated with pancreatic insufficiency in early life. Arch. Path., 37, 238.

Farber, S., Shwachman, H., and Maddock, C.L. (1943). Pancreatic function and disease in early life. I Pancreatic enzyme activity and coeliac syndrome.
J. clin. Invest., 22, 827.

Feather, E.A., and Russell, G. (1970). Sputum viscosity in cystic fibrosis of the pancreas and other pulmonary diseases.
Brit. J. Dis. Chest, 64, 192.

Few, /

Few, J.D. (1961). A method for analysis of urinary 17-hydroxy-corticosteroids. J. Endocrin., 22, 31.

Fieser, L.F., and Fieser, M. (1959). Steroids. p. 519. Reinhold Publishing Corporation, New York.

Flensborg, E.W., and Johnsen, S.G. (1972). Significant changes in two 17-ketosteroid fractions in cystic fibrosis. Acta paediat. scand., 61, 382.

Forest, M.G., and Migeon, C.J. (1970). Percentage of testosterone, androstenedione, and dehydroepiandrosterone bound to plasma protein in preadolescent children. J. Pediat., 76, 732.

Fotherby, K. (1959). A method for the estimation of dehydroepiandro-sterone in urine. Biochem., J., 73, 339.

Frankel, A.I., and Eik-Nes, K.B. (1970). Testosterone and dehydroepiandrosterone in the epididymis of the rabbit. J. Reprod., Fert., 23, 441.

Friedmann, T., and Johnson, P. (1967). Structure of Tamm-Horsfall mucoproteins in fibrocystic disease. J. lab. & clin. Med., 70, 404.

Gandy, H.M., and Peterson, R.E. (1968). Measurement of testosterone and 17-ketosteroids in plasma by the double isotope dilution derivative technique. J. clin. Endocr., 28, 949.

Gardner, /

Gardner, J.D. (1969). In "GAP" Conference Reports. Conference on Cell Biochemistry. Salk Institute for Biological Studies, La Jolla, California. Second Session: Membranes.

Garrod, A.E., and Hurtley, W.H. (1912). Congenital family steatorrhoea. Quart. J. Med., 6, 242.

Gibian, H., and Bratfish, G. (1956). Eine Steroidsulfatase aus Säugetierleber. Zeitsch. f. physiol. Chem., 305, 265.

Gibson, L.E., and Cooke, R.E. (1959). A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine by iontophoresis. Pediatrics, 23, 545.

Goldberg, A., Moore, M.R., Beattie, A.D., Hall, P.E., McCallum, J., and Grant, J.K. (1969). Excessive urinary excretion of certain porphyrinogenic steroids in human acute intermittent porphyria. Lancet, 1, 115.

Good, T.A., and Bessman, S.P. (1966). Anabolic steroids in cystic fibrosis of the pancreas. Am. J. Dis. Child., 111, 272.

Gordon, R.S., and Cage, G.W. (1966). Mechanism of water and electrolyte secretion by eccrine sweat gland. Lancet, 1, 1246.

Gracey, M., Campbell, P., and Noblett, H.R. (1969). Atretic vas deferens in cystic fibrosis. New Engl. J. Med., 280, 276.

Grand, /

Grand, R.J., di Sant' Agnese, P.A., Talamo, R.C., and Pallavicini, J.C. (1967). Effects of exogenous aldosterone on sweat electrolytes. II Patients with cystic fibrosis of pancreas. J. Pediat., 70, 357.

Grand, R.J., Talamo, R.C., di Sant' Agnese, P.A., and Schwartz, R.H. (1966). Pregnancy in cystic fibrosis of pancreas. J.A.M.A., 195, 993.

Granick, S., and Kappas, A. (1967). Steroid induction of porphyrin synthesis in liver cell culture. J. biol. Chem., 242, 4587.

Greenberg, J. (1933). An attempt to reproduce coeliac disease experimentally in young animals by excluding the external pancreatic secretion from the intestine. Yale J. Biol. & Med., 6, 121.

Greene, R.R., Burrill, M.W., and Ivy, A.C. (1939). Experimental intersexuality. The effect of antenatal androgens on sexual development of female rats. Am. J. Anat., 65, 415.

Gupta, D. (1965). The excretion of steroids by pre-pubertal children during growth. Ann. paediat., 204, 414.

Gurpide, E., MacDonald, P.C., VandeWiele, R.L. and Lieberman, S. (1963). Measurement of the rates of secretion and of peripheral metabolism of two interconvertible compounds: dehydroisoandrosterone - dehydroisoandrosterone sulphate. J. clin. Endocr., 23, 346.

Haahti, /

Haahti, E.O., Vandenheuvel, W.J., Horning, E.C. (1961). Separation of urinary 17-ketosteroids by gas chromatography. Anal. Biochem., 2, 182.

Hadorn, B., Johansen, P.G., and Anderson, C.M. (1968). Pancreozymin-secretin test of exocrine pancreatic function in cystic fibrosis and the significance of the result for the pathogenesis of the disease. Aust. paediat. J., 4, 8.

Hamilton, W. (1970). Ethyl estrenol and oxymetholone in the treatment of cystic fibrosis. First Annual Meeting of the European Working Group for Cystic Fibrosis. Abstracts p. 32. Stockholm, Sweden, 1970.

Hamilton, W. (1971). Testosterone synthesis in the Klinefelter testis. Proc. XIIIth International Congress of Pediatrics, Wien. Vol. VIII. Endocrinology, p. 17.

Hamilton, W. (1972). Drugs inhibiting corticosteroid synthesis. In "The Therapeutic Choice in Paediatrics". p. 113. Eds. W.L. Burland and B.M. Laurance. Churchill Livingstone, Edinburgh and London.

Hamilton, W. (1973). Testicular function in myotonic dystrophy of childhood. In press.

Hamilton, W., Grant, J.C., and Wilson, R.R. (1970). Enzyme activity of human undescended testes. In "Errors of Phenylalanine, Thyroxine and Testosterone Metabolism". p.105. Ed. W. Hamilton and F.P. Hudson. Livingstone, Edinburgh.

Handwerger, /

Handwerger, S., Roth, J., Gorden, P., di Sant' Agnese, P., Carpenter, D.F., and Peter, G. (1969). Glucose intolerance in cystic fibrosis. New Engl. J. Med., 281, 451.

Hansson, V., and Tveter, K.J. (1971). Uptake and binding in vivo of ^{3}H labelled androgen in the rat epididymis and ductus deferens. Acta endocr., Copnh., 66, 745.

Harper, M.H. (1938). Congenital steatorrhoea due to pancreatic defect. Arch. Dis. Childh., 13, 45.

Harris, J.A., and Waring W.W. (1970). Studies of dianabol in patients with cystic fibrosis. Cystic Fibrosis Club Abstracts. p. 27. Eleventh Meeting, Atlantic City, New Jersey, April, 1970.

Holsclaw, D.S. (1969). Cystic fibrosis and fertility. Brit. Med. J., iii, 356.

Holsclaw, D.S., and Shwachman, H. (1971). Increased incidence of inguinal hernia, hydrocele, and undescended testicle in males with cystic fibrosis. Pediatrics, 48, 442.

Howard, E. (1962). Steroids and bone maturation in infant mice: relative activities of dehydroepiandrosterone and testosterone. Endocrinology, 70, 131.

Howard, E. (1963). Effects of steroids on epiphysiodiaphyseal union in prepuberal mice. Endocrinology, 72, 11.

Ivy, /

Ivy, A.C. (1936). Certain aspects of the applied physiology of external pancreatic secretion.
Am. J. dig. Dis. & Nutr., 3, 377.

James, V.U.T., and de Jong, M. (1961). The use of tetramethylammonium hydroxide in the Zimmermann reaction.
J. clin. Path., 14, 425.

Johansen, P.G. (1963). Some observations on mucous secretions in cystic fibrosis of pancreas. Ann. New York Acad. Sc., 106, 755.

Johansen, P.G. (1970). In "Cystic Fibrosis and related human and animal diseases". p. 172. Ed. S. Jakowska. Gordon and Breach, New York, London, Paris. 1970.

Johansen, P.G., (1970). An in vitro system for studying mucus secretion and other physiological activities in human intestinal mucosa. Experientia, 26, 130.

Johnson, F.L., Feagler, J.R., Lerner, K.G., Majerus, P.W., Siegel, M., Hartmann, J.R., and Thomas, E.D. (1972). Association of androgenic-anabolic steroid therapy with development of hepatocellular carcinoma. Lancet, 2, 1273.

Johnston, W.H. (1956). Salivary electrolytes in fibrocystic disease of the pancreas.
Arch. Dis. Childh., 31, 477.

Jost, /

Jost, A. (1947). Reserches sur la différenciation sexuelle de l'embryon de lapin. III Rôle des gonades foetales dans la différenciation somatique.
Arch. Anat. micr. Morphol. expér., 36, 27

Jost, A. (1953). Problems of fetal endocrinology: The gonadal and hypophyseal hormones.
Recent Progr. Horm. Res., 8, 379.

Jost, A. (1955). Modalities in the action of gonadal and gonadostimulating hormones in the foetus.
Mem. Soc. Endocr., 4, 237.

Jost, A. (1967). Steroids and sex differentiation of the mammalian foetus. In "Proceedings of the Second International Congress on Hormonal Steroids, Milan, May, 1966". p. 74. Eds. L. Martini, F. Fraschini and M. Motta. (Excerpta Medica International Congress Series No. 132). Amsterdam, New York, London, Milan, Tokyo, Buenos Aires: Excerpta Medica Foundation.

Jost, A. (1968). Modalities in the action of androgens on the foetus. In "Research on Steroids III. Transactions of the IIIrd Meeting of the International Study Group for Steroid Hormones, Rome". p. 207. Eds. C. Cassano, M. Finkelstein, A. Klopper and C. Conti. Il Pensiero Scientifico , Rome.

Jost, A. (1970). Hormonal factors in the development of the male genital system. In "The Human Testis." p. 11. Eds. E. Rosemberg and C.A. Paulsen. Plenum Press, New York.

Kaplan, /

Kaplan, E., Shwachman, H., Perlmutter, A.D., Rule, A., Khaw, K-T., and Holsclaw, D.S. (1968). Reproductive failure in males with cystic fibrosis.
New Engl. J. Med., 279, 65.

Kellie, A.E. (1961). The metabolism of steroid conjugates: androstenolone sulphate and glucuronoside.
J. Endocr., 22, i.

Kellie, A.E., and Wade, A.P. (1957). The analysis of urinary 17-oxo-steroids by gradient elution.
Biochem. J., 66, 196.

Kessler, W.R., and Andersen, D.H. (1951). Heat prostration in fibrocystic disease of the pancreas and other conditions.
Pediatrics, 8, 648.

Kim, M.H., and Herrmann, W.L. (1968). In vitro metabolism of dehydroepiandrosterone sulphate in foreskin, abdominal skin and vaginal mucosa.
J. clin. Endocr., 28, 187.

Kochakian, C.D. (1965). Mechanism of anabolic action of androgens. In "Mechanisms of Hormone Action". p. 192. Ed. P. Karlson. Academic Press, New York and London.

Kollberg, H. (1971). Infant screening by neutron activation analysis. Proceedings of the Second Annual Meeting of the European Working Group for Cystic Fibrosis. Institute of Child Health, London, p. 29.

Kopito, /

Kopito, L., Mahmoodian, A., Townley, R.R.W., Khaw, H.T., and Shwachman, H. (1965). Studies in cystic fibrosis: analysis of nail clippings for sodium and potassium. New Engl. J. Med., 272, 504.

Kowalewski, K. (1958). Uptake of radiosulphate in growing bones of cockerels treated with cortisone and certain anabolic-androgenic steroids. Endocrinology, 63, 759.

Laatikainen, T. and Vihko, R. (1968). Diurnal variation in the concentrations of solvolyzable steroids in human plasma. J. clin. Endocr., 28, 1356.

Landing, B.H., Wells, T.R., and Wang, C-I. (1969). Abnormality of the epididymis and vas deferens in cystic fibrosis. Arch. Path., 88, 569.

Landing, B.H., Wells, T.R., and Williamson, M.L. (1970). Anatomy of eccrine sweat glands in children with chronic renal insufficiency and other fatal chronic diseases. Am. J. clin. Path., 54, 15.

LeBeau, M.C., Alberga, A., and Baulieu, E.E. (1964). Adrenal biosynthesis of dehydroisoandrosterone sulphate. Biochem. biophys. Res. Commun., 17, 570.

Lipsett, M.B., and Tullner, W.W. (1965). Testosterone synthesis by fetal rabbit gonad. Endocrinology, 77, 273.

Lobeck, /

Lobeck, C.C. (1966). Cystic Fibrosis of the Pancreas. In "The Metabolic Basis of Inherited Disease". p. 1305. Eds. J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson. Second Edition. McGraw Hill. New York, London.

Lobeck, C.C., and Huebner, D. (1962). Effect of age, sex, and cystic fibrosis on the sodium and potassium content of human sweat. Pediatrics, 30, 172.

Lobeck, C.C., and McSherry, N.R. (1963). Response of sweat electrolyte concentrations to 9-alpha-fluorohydrocortisone in patients with cystic fibrosis and their families. J. Pediat., 62, 393.

Loraine, J.A., and Bell, E.T. (1971). In "Hormone Assays and their Clinical Application". p. 513. Third Edition. E. & S. Livingstone, Edinburgh and London.

Loras, B., Roux, H., Cautenet, B., Ollagnon, C., Forest, M., de Peretti, E., and Bertrand, J. (1966). Determination of the 17-ketosteroids in children of various ages by paper chromatography. In "Androgens in Normal and Pathological Conditions". p. 93. Ed. A. Vermeulen. Proceedings of the Second Symposium on Steroid Hormones, Ghent, 1965. (Excerpta Medica Congress Series, No. 101). Amsterdam: Excerpta Medica Foundation.

Lucis, O.J., Reheja, M.C., Millard, O., and Morse, W.I. (1967). Utilization of circulating precursors for the synthesis of testosterone by human testis *in vivo*. Canad. J. Biochem., 45, 1213.

Mandel, /

Mandel, I.D., Kutscher, A., Denning, C.R., Thompson, R.H., and Zegarelli, E.V. (1967). Salivary studies in cystic fibrosis. Am. J. Dis. Childh., 113, 431.

Mangos, J.A., and Braun-Schubert, G. (1967). Micropuncture study of the rat parotid gland. Biblthca. paediat., 86, 107.

Mangos, J.A., and McSherry, N.R. (1967). Sodium transport. Inhibitory factor in sweat of patients with cystic fibrosis. Science, 158, 135.

Mangos, J.A., McSherry, N.R., and Benke, P.J. (1967). A sodium transport inhibitory factor in the saliva of patients with cystic fibrosis of the pancreas. Pediat. Res., 1, 436.

Marks, B.L., and Anderson, C.M. (1960). Fibrocystic disease of the pancreas in a man aged 46. Lancet, 1, 365.

Marsden, J.C. (1969). A reappraisal of cystic fibrosis. Nature, 223, 214.

Matalon, R., and Dorfman, A. (1968). Acid mucopolysaccharides in cultured fibroblasts of cystic fibrosis of the pancreas. Biochem. biophys. Res. Commun., 33, 954.

Maxfield, M., and Wolins, W.A. (1962). Molecular abnormality of urinary mucoprotein in cystic fibrosis. J. clin. Invest., 41, 455.

May, /

May, J.R., and Roberts, D.E. (1969). Bronchial infection in cystic fibrosis. Lancet, 1, 602.

Medical Research Council. (1963). A standard method of estimating 17-oxosteroids and total 17-oxogenic steroids. Lancet, 1, 1415.

Medical Research Council. (1969). A critical appraisal of a method of estimating urinary 17-oxosteroids and total 17-oxogenic steroids. Lancet, 1, 124.

Metcalf, M.G. (1967). Interference with urine 17-oxogenic steroid analysis. Clin. Biochem., 1, 172.

Migeon, C.J., Keller, A.R., Lawrence, B., and Shepard, T.H. (1957). Dehydroepiandrosterone and androsterone levels in human plasma. Effect of age and sex: day-to-day and diurnal variations. J. clin. Endocr., 17, 1051.

Migeon, C.J., and Plager, J.E. (1954). Identification and isolation of dehydroepiandrosterone from peripheral human plasma. J. biol. Chem., 209, 767.

Mikulaskova, J., and Linet, O. (1964). Weitere histochemische Studien über den Einfluss von Dehydroepiandrosteron auf die experimentelle Atheromatose bei Kaninchen. Acta biol. med. germ., 12, 602.

Miller, R., and Perkins, H. (1920). Congenital steatorrhoea. Quart. J. Med., 14, 1.

Milner, /

Milner, A.D. (1969). Blood glucose and serum insulin levels in children with cystic fibrosis.
Arch. Dis. Childh., 44, 351.

Mounib, M.S. (1964). Effects of certain hormones on the metabolism of bull spermatozoa.
Acta endocr., Copnh., 45, 631.

Munger, B.L., Brusilow, S.W., and Cooke, R.E. (1961). Electron microscopic study of eccrine sweat glands in patients with cystic fibrosis of pancreas.
J. Pediat., 59, 497.

Munson, P.L., Gallagher, T.F., and Koch, F.C. (1944). Isolation of dehydroepiandrosterone sulphate from normal male urine.
J. biol. Chem., 152, 67.

Neuberger, A. (1970). Tamm and Horsfall glycoprotein in cystic fibrosis. Cystic Fibrosis Research Trust Research Conference Abstracts. Owens Park, Manchester, December, 1970.

Neumann, F., von Bersworth-Wallrabe, R., Elger, W., Steinbeck, H., and Hahn, J.D. (1969). Effects of antiandrogens. In "Progess in Endocrinology. Proceedings of the IIIrd International Congress of Endocrinology, Mexico City, 1968". p. 823. Eds. C. Gual and F.J.G. Ebling (Excerpta Medica International Congress Series No. 184). Amsterdam: Excerpta Medica Foundation.

Oertel, /

Oertel, G.W., (1971). Dehydroepiandrosteron in biochemischer und klinischer Sicht. Arzneim. Forsch., 21, 543.

Olson, J.R., and Weaver, D.K. (1969). Congenital mesonephric defects in male infants with mucoviscidosis. J. clin. Path., 22, 725.

Oppenheimer, E.A., Case, A.L., Esterly, J.R., and Rothberg, R.M. (1970). Cervical mucus in cystic fibrosis: A possible cause of infertility. Am. J. Obstetrics and Gynecology, 108, 67

Oppenheimer, E.H., and Esterly, J.R. (1969). Observations on cystic fibrosis of the pancreas. V. Developmental changes in the male genital system. J. Pediat., 75, 806.

Oppenheimer, E.H., and Esterly, J.R. (1970). Observations on cystic fibrosis of the pancreas. VI. The uterine cervix. J. Pediat., 77, 991.

Pal, S.B., and Teller, W.M. (1972). Urinary excretion of dehydroepiandrosterone in normal children and in patients with adrenocortical disorders. Clin. Chim. Acta, 38, 371.

Pallavicini, J.C. (1970). In "Cystic Fibrosis and Related Human and Animal Diseases". p. 165. Ed. S. Jakowska. Gordon and Breach, New York, London, Paris.

Pallavicini, J.C., Gabriel, O., di Sant' Agnese, P.A., and Buskirk, E.R. (1963). Isolation and characterisation of carbohydrate-protein complexes from human sweat. Ann. New York Acad. Sc., 106, 330.

Parmelee, /

Parmelee, A.H. (1935). The pathology of steatorrhea. Am. J. Dis. Child., 50, 1418.

Paulsen, E.P., Sobel, E.H., and Shafran, M.S. (1966). Urinary steroid metabolites in children. I. Individual 17-ketosteroids in children with normal sexual development. J. clin. Endocr., 26, 329.

Percival, E., and McDowell, R.H. (1967). In "Chemistry and Enzymology of marine algal polysaccharides". p. 122. Academic Press Inc., London.

Picon, R. (1971). Effect of the rat fetal testis on the regression of the Müllerian ducts in vitro. In "Hormones in Development" p. 645, Eds. M. Hamburgh and E.J.W. Barrington. Meredith Corporation, New York.

Pierrepoint, C.G., Griffiths, K., Grant, J.K., and Stewart, J.G.S. (1965). Formation of neutral steroid sulphates by a feminizing Leydig cell tumor from a human subject. Biochem. J., 95, 55P.

Potter, J.L., Matthews, L.W., Lemm, J., and Spector, S. (1963). Human pulmonary secretions in health and disease. Ann. New York Acad. Sc., 106, 692.

Price, D., and Ortiz, E. (1965). The role of fetal androgen in sex differentiation in mammals. In "Organogenesis". p. 629. Eds. R.L. de Haan and H. Ursprung. Holt, Rinehart and Winston, Inc., New York.

Principe, /

Principe, U., and Bellucci, G. (1952). Azione del propionato di testosterone iniettato in loco sulla guarigione delle fratture (ricerche sperimentali).
Sperimentale, 102, 266.

Prout, M. and Snaith, A.H. (1958). Urinary excretion of 17-ketosteroids in children.
Arch. Dis. Childh., 33, 301.

Puche, R.C., and Romano, M.C. (1970). The effect of androgens on the carbohydrate metabolism of bone.
Calc. Tiss. Res., 6, 133.

Raynaud, A. (1937). Intersexualité provoquée chez la souris femelle par injection d'hormone male a la mère en gestation.
Compte rendu Soc. de biol., 126, 866.

Raynaud, A., and Frilley, M. (1947). Destruction des glandes génitales de l'embryon de souris, par une irradiation au moyen des rayons X, à l'âge de treize jours.
Ann. d'endocrinol., 8, 400.

Reed, G.B., Bain, A.D., McCrae, W.M., and Scott, F.M. (1970). Cellular metachromasia in cystic fibrosis.
J. Path., 101, 251.

Reid, L. (1967). Secretion of bronchial mucous glands: in "Cystic Fibrosis". p. 45. Eds. Porter, R., and O'Conner, M.. Ciba Foundation Study Group No. 32. J. & A. Churchill Ltd., London.

Reid, /

Reid, L., and de Haller, R. (1967). The bronchial mucous glands - their hypertrophy and change in intracellular mucus. Biblthca paediat., 86, 195.

Robel, P., Emiliozzi, R., and Baulieu, E.E. (1967). Metabolism of dehydroisoandrosterone glucuronide. J. clin. Endocr., 27, 1290.

Roberts, K.D., VandeWiele, R.L., and Lieberman, S. (1961). The conversion in vivo of dehydroisoandrosterone sulphate to androsterone and etiocholanolone glucuronidates. J. biol. Chem., 236, 2213.

Roelfs, R.E., Gibbs, G.E., and Griffin, G.D. (1967). Composition of rectal mucus in cystic fibrosis. Am. J. Dis. Child., 113, 419.

Rosan, R.C., Shwachman, H., and Kulczycki, L.L. (1962). Diabetes mellitus and cystic fibrosis of the pancreas. Am. J. Dis. Child., 104, 625.

Rosenfield, R.L., and Eberlein, W.R. (1969). Plasma 17-ketosteroid levels during adolescence. J. Pediat. 74, 932.

Rosenfield, R.L., Grossman, B.J., and Ozoa, N. (1971). Plasma 17-ketosteroids and testosterone in pre-pubertal children before and after ACTH administration. J. clin. Endocr., 33, 249.

Roy, A.B. (1957). The sulphatase of ox liver. 6. Steroid sulphatase. Biochem. J., 66, 700.

Rule, /

Rule, A.H., Kopito, L., and Shwachman, H. (1970). Chemical analysis of ejaculates from patients with cystic fibrosis. Fertility and Sterility, 21, 515.

Saez, J.M., and Bertrand, J. (1968). Studies on testicular function in children: plasma concentrations of testosterone, dehydroepiandrosterone and its sulphate before and after stimulation with human chorionic gonadotrophin. Steroids, 12, 749.

Saggers, B.A. (1970). Mucoid Pseudomonas aeruginosa and its relationship to cystic fibrosis. Cystic Fibrosis Research Trust Research Conference Abstracts. Abstract 25. Owens Park, Manchester, December, 1970.

Salmon, W.D., Bower, P.H., and Thompson, E.Y. (1963). Effect of protein anabolic steroids on sulphate incorporation by cartilage of male rats. J. lab. & clin. Med., 61, 120.

Schneider, J.J., and Lewbart, M.L. (1956). Enzymatic synthesis of steroid sulphates. J. biol. Chem., 222, 787.

Schulz, I.J., and Peter, G. (1967). Micropuncture studies of abnormality in sweat formation in cystic fibrosis (CF). Fed. Proc., 26, 287.

Shirley, I.M.S., and Cooke, B.A. (1969). Metabolism of dehydroepiandrosterone by the separated zones of the human foetal and newborn adrenal cortex. J. Endocr., 44, 411.

Shwachman, /

Shwachman, H., and Antoniwicz, I. (1962). The sweat test in cystic fibrosis. Ann. New York Acad. Sc., 93, 600.

Shwachman, H., and Kulczcki, L.L. (1958). Long-term study of one hundred five patients with cystic fibrosis. Am. J. Dis. Child., 96, 6.

Solomon, S. (1967). The formation and metabolism of neutral steroids in the human fetus and placenta. In "Proceedings of the Second International Congress on Hormonal Steroids, Milan, May, 1966". p. 653. Eds. L. Martini, F. Fraschini, and M. Motta. (Excerpta Medica International Congress Series No. 132) Amsterdam, New York, London, Milan, Tokyo, Buenos Aires: Excerpta Medica Foundation.

Spicer, S. (1970). In "Cystic Fibrosis and related human and animal diseases". p. 170. Ed. Jakowska, S. Gordon and Breach, New York, London, Paris.

Spock, A., Heick, H.M.C., Cress, H., Logan, W.S. (1967). Abnormal serum factor in patients with cystic fibrosis. Pediat. Res., 1, 173.

Spock, A., and Renuart, A.W. (1970). Elevated sweat chloride levels in mucopolysaccharidosis. In "Cystic Fibrosis Club Abstracts. Eleventh Annual Meeting". p. 51. Atlantic City, New Jersey, 1970.

Steinberger, /

Steinberger, E., and Steinberger, A. (1969). The spermatogenic function of the testes. In "The Gonads". p. 715. Ed. K.W. McKerns. North-Holland Publishing Company, Amsterdam.

Sturgess, J.M. and Reid, L., (1969). A new pattern of sputum viscosity. In "Proceedings of the Fifth International Cystic Fibrosis Conference. Churchill College, Cambridge". p. 368. Ed. D. Lawson. Cystic Fibrosis Research Trust, London.

Swenson, O. (Ed) (1969). Pediatric Surgery. Third Edition. p.561. Appleton-Century-Crofts, New York.

Talalay, P., Fishman, W.H., and Higgins, W. (1946). Chromogenic Substrates. II Phenolphthalein glucuronic acid as substrate for assay of glucuronidase activity. J. biol. Chem., 166, 757.

Taylor, T. (1971). Ph.D. Thesis, University of Glasgow. p. 216.

Tveter, K.J., and Attramadal, A. (1968). Selective uptake of radioactivity in rat ventral prostate following administration of testosterone-1, 2-^{3}H.
Acta endocr., Copnh., 59, 218.

Tveter, K.J., and Unhjem, O. (1969). Selective uptake of androgen by rat seminal vesicle.
Endocrinology, 84, 963.

Uozumi, /

Uozumi, T., Manabe, H., Tanaka, H., Hamanaka, Y., Kotoh, K., Suzuki, K., and Matsumoto, K. (1969). Urinary steroids of healthy subjects from childhood to old age. Acta endocr., Copnh., 61, 17.

Vaitukaitis, J.L., Dale, S.L., and Melby, J.C. (1969). Role of ACTH in the secretion of free dehydroepiandrosterone and its sulphate ester in man. J. clin. Endocr., 29, 1443.

Valman, H.B., and France, N.E. (1969). The vas deferens in cystic fibrosis. Lancet, 2, 566.

VandeWiele, R.L., and Lieberman, S. (1960). The metabolism of dehydroisoandrosterone. In "Biological Activities of Steroids in Relation to Cancer". p. 93. Eds. G. Pincus and E.P. Vollmer. Academic Press, New York.

VandeWiele, R.L., MacDonald, P.C., Bolté, E. and Lieberman, S. (1962). Precursors of the urinary 11-desoxy-17-ketosteroids: Estimation of the secretory rate of dehydroisoandrosterone. J. clin. Endocr., 22, 1207.

VandeWiele, R.L., MacDonald, P.C., Gurpide, E., and Lieberman, S. (1963) Studies on the secretion and interconversion of the androgens. Recent Progr. Horm. Res., 19, 275.

VanWagenen, G., and Hurme, V.O. (1950). Effect of testosterone propionate on permanent canine tooth eruption in the monkey (Macaca mulatta). Proc. Soc. exp. biol. Med., 73, 296.

Vestergaard, /

Vestergaard, P. (1965). Urinary excretion of individual 17-ketosteroids in children.
Acta endocr., Copnh., 49, 436.

Villee, C.A., and Fujii, T. (1968). Growth of seminal vesicle and prostate: Role of RNA following testosterone stimulation. In "Hormones in Development". p. 619. Eds. M. Hamburgh and E.J.W. Barrington. Meredith Corporation, New York.

Wallace, E., and Lieberman, S. (1963). Biosynthesis of dehydroisoandrosterone sulphate by human adrenocortical tissue. J. clin. Endocr., 23, 90.

Wallace, E., and Silberman, N. (1964). Biosynthesis of steroid sulphates by human ovarian tissue.
J. biol. Chem., 239, 2809.

Waller, J.I., and Turner, T.A. (1962). Anastomosis of the vas after vasectomy. J. Urol., 88, 409,

Wang, C.I., Kwok, S., and Edelbrock, H. (1970). Inguinal hernia, hydrocele, and other genitourinary abnormalities.
Am. J. Dis. Child., 119, 236.

Warren, J.C., and French, A.P. (1965). Distribution of steroid sulphatase in human tissues.
J. clin. Endocr., 25, 278.

Wiesmann, U., and Neufeld, E.F. (1970). Metabolism of sulphated mucopolysaccharide in cultured fibroblasts from cystic fibrosis patients. J. Pediat., 77, 685.

Witschi, /

Witschi, E. (1967). In "The Biochemistry of Animal Development". Vol. II, p. 193. Ed. R. Weber, Academic Press, New York.

Yamaji, T., and Ibayashi, H. (1969). Plasma dehydroepiandrosterone sulphate in normal and pathological conditions. J. clin. Endocr., 29, 273.

Zaaijer, J.J.P., and Price, D. (1968). Early secretion of androgenic hormones by human fetal gonads and adrenal glands in organ culture and possible implications for sex differentiation. In "Hormones in Development". p. 537. Eds. M. Hamburgh and E.J.W. Barrington. Meredith Corporation, New York.

Zachmann, M., Vollmin, J.A., Hamilton, W., and Prader, A. (1973). Steroid 17,20-desmolase deficiency. A new cause for male pseudo-hermaphroditism. Clin. Endocr., 1, 369.

Zoppi, G., Shmerling, D.H., Gaburro, D., and Prader, A. (1970). The electrolyte and protein contents and outputs in duodenal juice after pancreozymin and secretin stimulation in normal children and in patients with cystic fibrosis. Acta paediat. scand., 59, 692.

The possible significance of dehydroepiandrosterone in cystic fibrosis.

By

Christina E. Gray, B.Sc.

A SUMMARY

of the thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy.

April, 1973.

The most consistent physical manifestations of cystic fibrosis in the male are abnormalities of the vasa deferentia, epididymes and seminal vesicles. These abnormalities of the male genital tract have been attributed by others to a failure in differentiation of the mesonephric duct at about the 10th to 12th foetal week.

From the literature, incubation studies employing human foetal testicular material have demonstrated dehydroepiandrosterone (DHA) to be an important pre-hormone of testosterone at this time. Thus DHA must be regarded as important in the maintenance of an androgenic environment necessary for the stabilisation of the mesonephric duct and its derivatives in the male foetus. It is accepted that DHA and testosterone advance bone maturation which in children with cystic fibrosis is frequently retarded. Also, androgens are required for the maintenance of normal spermatogenesis. This process is known to be arrested at spermatocyte and spermatid stages in children with cystic fibrosis. DHA, then, is an important precursor of androgens (the Δ^5 pathway being important during foetal development) and on its own account has widespread metabolic activity.

Since /

Since tissues from a foetus known to have cystic fibrosis were not obtained, a study of dehydroepiandrosterone production pre-natally was not possible. However, a study of DHA in urine and blood samples collected from 48 children (from 1 to 20 years old) with cystic fibrosis was undertaken.

Following addition of $[7\alpha\text{-}^{3}H]$ DHAS for recovery purposes, aliquots of urine from 24-hour collections were submitted to 6 hours hot hydrolysis at neutral pH (Fotherby, 1959). This hydrolytic method is specific for Δ^{5}-3β-hydroxysteroid sulphate: DHA conjugated with glucuronic acid in urine was hydrolysed by a 24 hour incubation with β-glucuronidase (1,000 F.u. per ml urine) at 37°C employing a sulphatase inhibitor (potassium dihydrogen orthophosphate). Following extraction, purificati by column chromatography and t.l.c. was effected. The dried residues were derivatised with benzene:heptafluorobutyric anhydride (1:1, v/v) for 30 minutes at 70°C and quantitation of the DHA ester was made by electron capture detection on a Pye 104 g.l.c. system. A correction was made according to the recovery of $[7\alpha\text{-}^{3}H]$ DHA added initially to urine samples.

Group estimations of 17-oxosteroids, 17-oxogenic steroids and total 17-hydroxycorticosteroids were also made on the urine samples /

samples when sufficient urine was available.

Plasma DHAS was estimated in 36 children (3 to 16 years of age) with cystic fibrosis according to the method of Brownsey et al. (1972).

Levels of total 17-hydroxycorticosteroids excreted by children with cystic fibrosis, as presented in this thesis, are not significantly different from those of normal children and are thus in agreement with the findings of Chodos et al. (1965). The 17-oxosteroid excretion in affected patients from 1 to 10 years did not differ significantly from that of corresponding normal children. However, patients with cystic fibrosis in the 11 to 17 years age group had a significantly lower excretion of 17-oxosteroids than normal.

DHA was detected in the urine of some children as young as 4 years of age and was found to be within the normal range in children up to 11 years of age. Excretion of DHA in the older age group (11 to 20 years) was subnormal in the majority of children with cystic fibrosis (in 8 of 11 boys and 6 of 7 girls). Circulating levels of DHAS were also significantly lower than normal in both males and females in the 11 to 15 years age range.

From /

From these results it is apparent that the increase in 17-oxosteroid excretion and the increase in plasma DHAS associated with normal puberty does not take place in the adolescent with cystic fibrosis even although clinical puberty may be well established, albeit at a later than average time. These findings reflect a failure in the maturation of the adrenal cortex and possibly also of the teste in the male.