I

"CHEMICAL AND BIOCHEMICAL STUDIES ON SOME TRADITIONAL VIRILISING PREPARATIONS IN USE IN NIGERIA".

BY

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A B S T R A C T

The decline in the sexual activity of the male with increase in age used to be a major concern of clinicians and scientists from the beginning of knowledge. Modern medicine has, however, provided a means, in terms of testosterone propionate injections, of abrogating such symptoms.

In Africa, the age-long tradition of cure by herbs and mixtures of plants and animals parts, commonly referred to as "agbo" in the Yoruba speaking parts of West Africa still persists; and many are known for restoring partly this decline in sexual activities of the older male.

We have analysed four of the known Yoruba virilising medicinal preparations for their biological activity and potency. Using the famous biological assay methods of the growth of the capon's comb and increase in the weights of the rat's prostate glands and seminal vesicles, as well as the rat's urine when these local drugs were injected into the castrated animals , we found that all the local drugs contain, to some extent, androgenic hormones, which are capable of stimulating the growth of these organs.

The various local drugs were hydrolysed with acid and a modified method is presented by which they were extracted

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with ether, followed by chemical and physical tests which indicated the presence of these androgenic steroids in the local drugs. Other tests on the "drugs" showed the presence of such substances as proteins, and fatty acids in the various local drugs, outside the sex hormones.

Thin-layer chromatography was used to separate and purify the various steroids extracted from each of the local drugs and infrared spectrophotometry was used to show the presence of the sex steroids in the local medicines. The rat experiments gave comparable results with those of the whole crude drug when the steroidal extracts from the local drugs were re-injected, while the response of capons to the steroidal extracts were lower compared with the whole crude drug.

Of major importance is the presence of androgenic steroids in one of the local drugs which is made strictly from plant roots. This is very significant because androgenic steroids are usually associated with animal parts.

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First, I should like to thank the Professor and Head of Department of Biochemistry, University of Ibadan, for the large range of facilities he put at my disposal and also the University for a grant with which the project was carried out.

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Finally, I thank Professor R. T. Williams of Saint Mary's Hospital, London, whose regular visits to our department during the period of this study went a long way in strengthening my belief in good research work.

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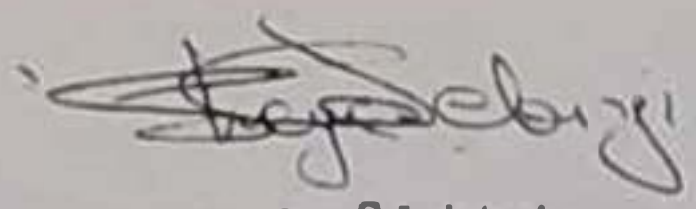
M E M O R A N D U M

Except where acknowledgement is made, the experimental work reported in this thesis is my own unaided work, performed in the Department of Biochemistry, University of Ibadan, under the supervision of Professor O. Bassir, between October 1969 and June, 1972.



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CHAPTER ONE

INTRODUCTION

Most men begin to exhibit decreased sexual functions in the late 40's or 50's similar to the decreased in the sexual function at menopause in women. This has been related to a decrease in Testosterone secretion as depicted in Fig. 1 (Hooker 1937; Guyton 1961; Kinsey et al 1948; Woolhouse 1967).

The decrease in male sexual function is called the "male climateric", occasionally, the male climateric is associated with symptoms of hot flushes, suffocation, decreased energy in sexual performance and psychic disorders similar to the menopausal symptoms of the female. These symptoms, by the modern curative methods can be corrected by the administration of testosterone. However, some esters of testosterone have been found to be more potent androgens than testosterone itself.

This may be due to a decrease in the rate of absorption and consequent improvement in the utilisation. This is perhaps why testosterone propionate is often administered by injection instead of testosterone in such clinical disturbances.

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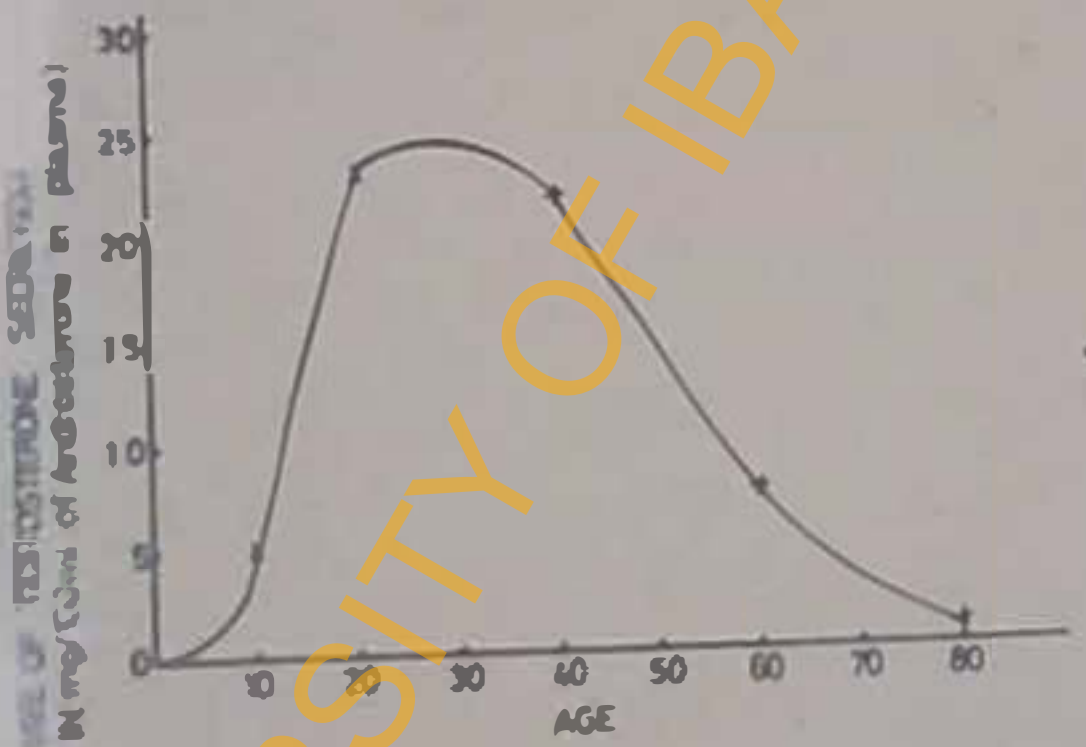
FIGURE 1

RATE OF TESTOSTERONE SECRETION AT DIFFERENT AGES AS ALDGED BY THE CONCENTRATION OF ANDROSTERONE IN PLASMA.
(AFTER A.C. GUYTON 1961).



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The decline in sexual activity of the older male is partly or perhaps primarily the result of a general decline in physical and physiological capacity; (Pincus et al 1955), which is also affected by psychological fatigue, a loss of interest in repetition of the same sort of experience, an exhaustion of the possibilities for exploring new techniques, new types of contacts and new situations.

Evidence of this is to be found in many cases of older males whose performances had dropped materially until they met new partners, adopted new sexual techniques or embraced totally new sources of outlet. Under new situations, their rates materially rise to drop again, however, within a few months, or in a year or two to the old level.

In Africa, the herbalists and traditional healers are "expert botanists knowledgeable in the properties of local plants" (Gelfand 1961). There are various methods of healing in Africa ranging from the use of extracts of plants and animals to suggestive cure. In relation to loss of sexual activity, a large variety of herbal mixtures are often used. Among the Nsehonas of Southern Rhodesia, Gelfand (1956) stated that "treatment of impotency in the male is based on sympathetic magic".

In other parts of Africa, including Western Nigeria, herbal mixtures for impotency or decrease in sexual

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activity often contain sex organs of male animals alone or sometimes mixed with plant parts.

We have selected four of these medicinal preparations (commonly called agbo mokole) which are claimed to have a virilising effect on the male. Since it has been established beyond reasonable doubts that the mating activities of many species are heavily dependent on testicular secretions, we decided to analyse for sex steroids in the locally purchased drugs which are claimed to have stimulatory effects on sexual activity.

(1) Historical background

In the pre-endocrine era, Aristotle (300 B.C.), observed the relation between the testes and virility the high-pitched voice and failure of sexual hair growth in the young castrate. He was also the first to compare the effects of castration in the birds and men. Agur-Veda (1400 B.C.) also prescribed testes for impotence.

Much of our knowledge concerning the role of the testes in the growth and function of male accessory organs is an offshoot of the early experiments on castration and implantation, which date from John Hunters' (1792) famous observations on the post-castrate regression of the prostate and seminal vesicle. In 1849, Berthold, proved that the

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influence of the testis is carried by something that travels in the oiroulating blood.

He castrated six cookerels of 2 to 3 months old. Their combs, wattles and spurs were not yet developed. He developed into typical capons, fat, docile, without cock's comb and unable to crow. In the next two, he removed the testes but put one of them back, dropping it among the intestines. He examined the cocks months later and found that the testes had bocoms attached to the intestines and had acquired a good blood supply. Both these cookerels became typical cocks.

In the last two, each had one testis removed, then Berthold exchanged the remaining testes, giving each bird the other's testis, which he implanted on the intestines. These also became typical cocks. This experiment showed that the testis was not dependent on any specific nerves to maintain its control of the secondary sex characters but works through the blood. Berthold's little contribution belongs to the fundamental classics of endocrinology.

After this discovery, a lot of efforts were made to follow up. For instance, there was C. E. Brown-Sequard, a French-Irish-American (1817 - 1894) who made two impacts

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on the field of internal secretions. In 1856, he was the first to remove the adrenal glands from animals and to observe the fatal disorder thus produced. In 1889, when he was 72 years old, he began to dose himself with extracts of dog's testicles. He was feeling the effects of aging and hoped to rejuvenate himself. He claimed that after the injections, he felt much "stronger and more alert". But many people including the medical profession did not believe him then. We now know that when a man grows old, he ages, not only in his testicles but all over (Pincus et al 1955) and that Brown-Sequard could have had little of the genuine testes hormone in his extract.

Berthold's (1849) experiment of transplantation of the testis had also been used in the rejuvenation of senile men. The most widely publicised of these was that of the Franco-Russian surgeon, Serge Voronoff, who, between 1912 - 1925, was implanting monkeys' testes into human patients. Some of the patients reported hopeful results. But according to Corner (1947), more often, no doubt, the benefit was entirely "psychic". It is now known that tissues from one species of mammals can grow with difficulty in another species.

Pezard (1911) following the steps of Berthold castrated some cocks and injected them with saline extracts of

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testor. He observed that they developed like typical cocks. Between this time to 1927, various investigators reported that they had extracts of the testes which induced growth of the head furnishings of the cocks. None of these experiments however was fully convincing then.

The road to chemical identification of the sex hormones was opened in 1927, when Hochhaim and Zondek, then in Berlin, discovered that the urine of pregnant women contained considerable amounts of a hormone which produced sexual heat when injected into mice or rats. Adolf Windaus, the father of sterol chemistry was asked by a German chemical firm to explore this substance. He elected to turn over the new problem to a young student of his, named Adolf Butenandt.

Working independently, both Butenandt and Doisy in 1929, succeded in isolating the first known sex hormone - Estrone. In 1927, a post-graduate student in biochemistry under Professor F. C. Koch at the University of Chicago, L. C. McGee, applied the new methods of extraction to the tissues of the bull's testes and promptly secured a relatively pure extract that was capable of producing rapid growth of the capon's comb. This lead was followed up by a group of workers in biochemistry and zoology in the

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University of Chicago, including McGee, Koch, Moore, Dunn and Mary John and by various workers.

In 1929, Loewe and Voss, and Funk and Herrow independently extracted androgenic substances from male human urines. Gallagher and Koch (1929) also extracted these hormones from testes tissues (Gallagher 1928).

The untiring Butenandt in 1931, together with Tscherning isolated from 15,000 litres of male urine, 15mg of a hormonal substance which they named androsterone. Butenandt (1932) later deduced the nature of the molecule and its structure, which was independently proved correct by Leopold Ruzicka, who produced androsterone by splitting off the 8-carbon side chain from a derivative of cholesterol through oxidation. Ruzicka and his colleagues made androsterone synthetically in 1934.

In 1935, David and others succeeded in the difficult task of purifying the potent hormone from extracts of the steer testes which they called Testosterone. Butenandt and Ruzicka (1935) were able to synthesise a related substance which could be produced in more plentiful yield. This substance named dehydro-epi-androsterone, was made from cholesterol by burning off the side chain while the essential hydroxy group attached to the first ring, and

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the double bond at the 5, 6 position in the second ring, were protected by stable chemical combinations at these positions.

Butenandt and Ruzicka (1935) shortly afterwards synthesised testosterone from dehydro-epi-androsterone. It became clear that testosterone was the true hormone; androsterone and dehydro-epi-androsterone were metabolites of the hormone in the urine.

II. Chemical properties of androgens.

An androgen is a substance capable of stimulating male secondary sex characteristics (Dorfman and Shipley 1956). The word androgen is from the Greek words Andros - man; gennao-produce. The androgens belong to a widely occuring group of colourless compounds called steroids. The generic term 'steroids' was suggested by Callow and Young (1936) for the group of biologically important compounds related to the familiar plant and animal sterols.

The steroids include the bile acids, cardiac aglycones, sapogenins, adrenal cortical hormones and the female and male sex hormones - oestrogens and androgens. For the most part all steroids are saturated natural products possessing the 4-ring nucleus, consisting of a cyclopentane

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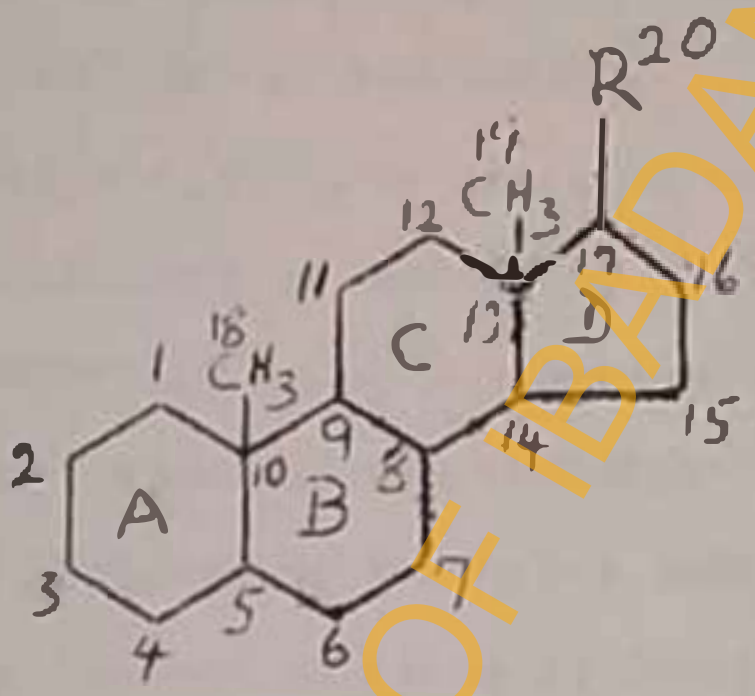
ring, attached to a hydrogenated phenanthrene ring system named 'cyclopentanoperhydrophenanthrene'. This is illustrated in formula (I), in which the group R is a saturated or unsaturated hydrocarbon radical. The group R is referred to as the side chain, the remainder



(I)

of the molecule being termed the nucleus. In addition most steroids have methyl groups placed as shown in formula (I). The positions of these methyl groups ordinarily are indicated by a short line. The formula also shows the system used for numbering the carbon atoms of the nucleus.

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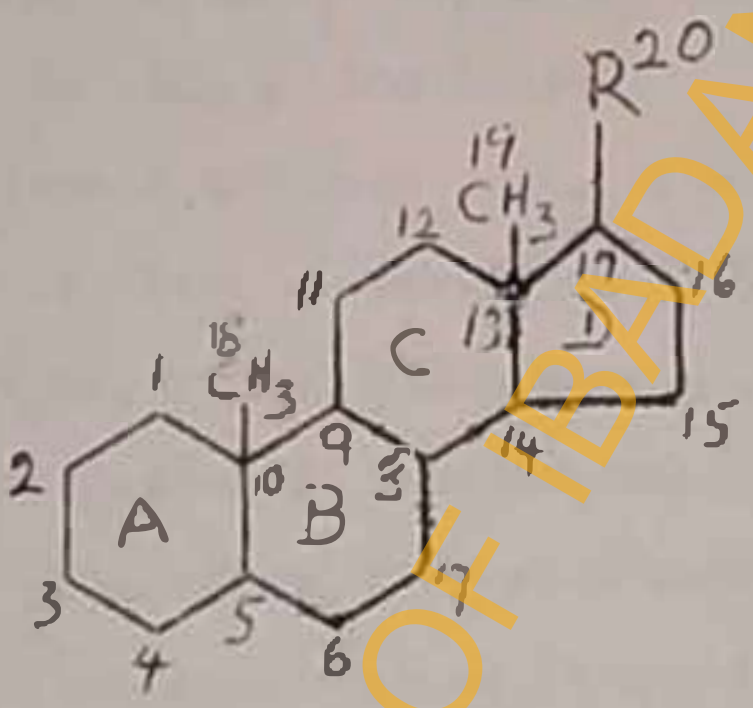
ring, attached to a hydrogenated phenanthrene ring system named 'cyclopentonoperhydrophenanthrene'. This is illustrated in formula (I), in which the group R is a saturated or unsaturated hydrocarbon radical. The group R is referred to as the side chain, the remainder



(I)

of the molecule being termed the nucleus. In addition most steroids have methyl groups placed as shown in formula (I). The positions of these methyl groups ordinarily are indicated by a short line. The formula also shows the system used for numbering the carbon atoms of the nucleus.

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The four ring systems which are usually, though not always fully reduced are designated A, B, C and D. The system of numbering and alphabetic designation of the four rings was that originally proposed by Fieser (1937), extended by Reichstein and Shoppee (1943) and later modified by Mason (1948) and Fieser and Fieser (1935; 1949). It was finally adopted at the International CIBA conference in 1957 by the IUPAC.

The steroid molecule contains eight asymmetric carbon atoms, <u>namely</u> those at positions 3, 5, 8, 9, 10, 13, 14 and 17. Hence, it has 256 stereoisomeric forms. However, the variations in stereochemical configuration encountered in natural products of the types under consideration are confined to the carbon atoms at positions 3 and 5 and the relative configurations of rings A and B.

The conventions usually adopted for the description and representation of substituted steroids have been expressed clearly by Shoppee (1946). The configuration of substituents at the nuclear carbon atoms is represented by the suffix (α) or (β) according to whether the substituent lies below the plane of the molecule or projects forward from the plane of the molecule. Furthermore, whenever it is necessary to indicate a specific configuration;

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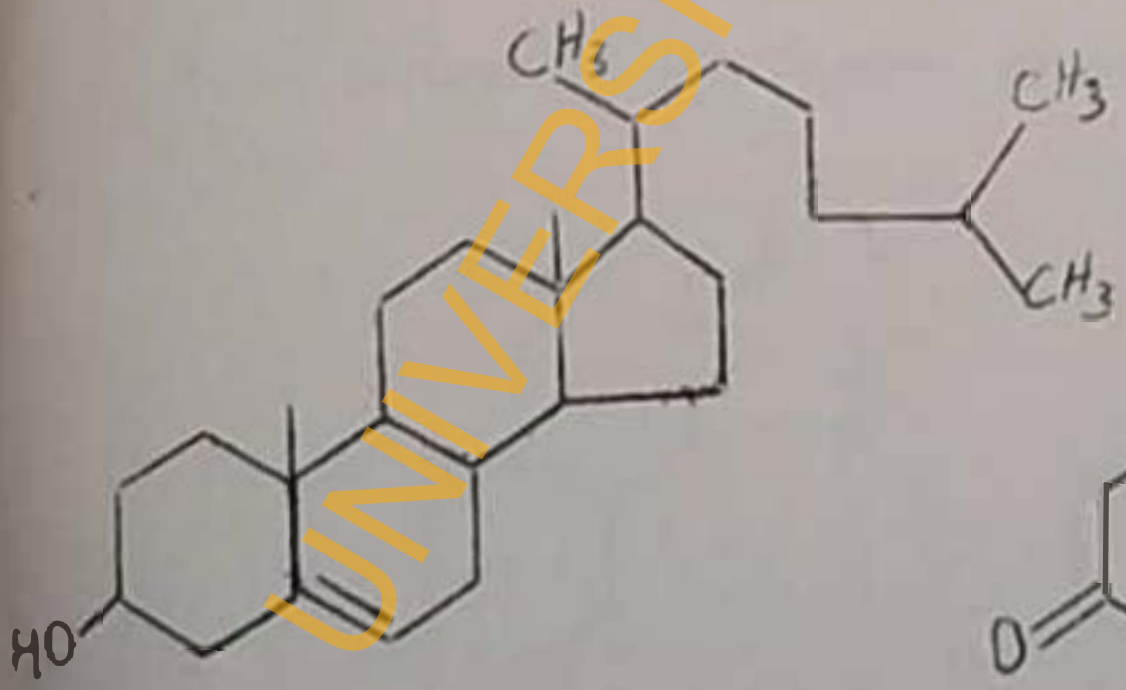
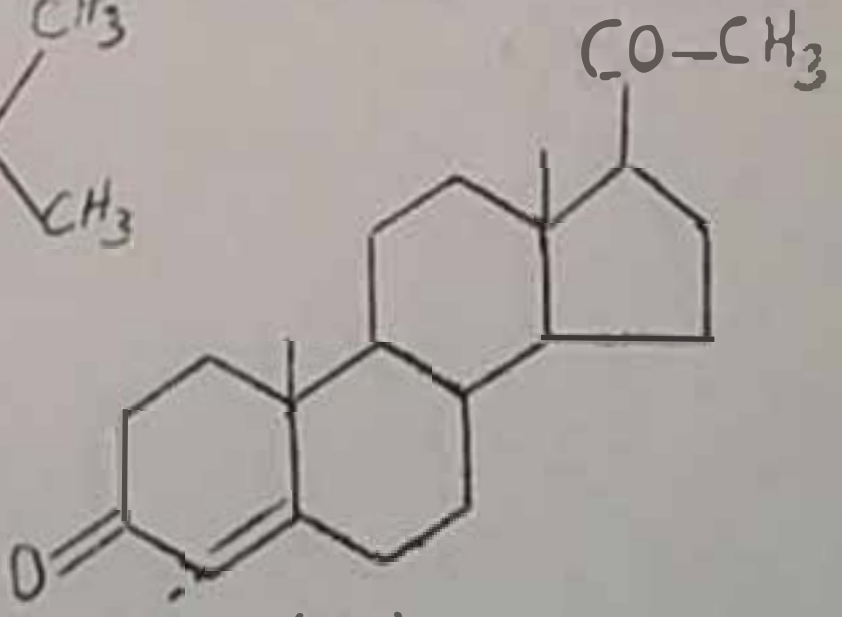
(β) - orientation is represented in the formula by a full-line bond, whereas (α) - orientation is represented by a broken-line bond. An excellent report of our knowledge of the stereochemical configuration of the steroid nucleus and of nuclear substituents was been published by Shoppee (1946).

Androgens are often C_{19}-steroids and are easily produced by complete oxidative removal of the side-chain in cholesterol (II) and progesterone (III). Not all C_{19}-steroids have androgenic activity (Heftmann and Hoesttig 1960). The systematic names of androgens are derived.



(II)



(III)

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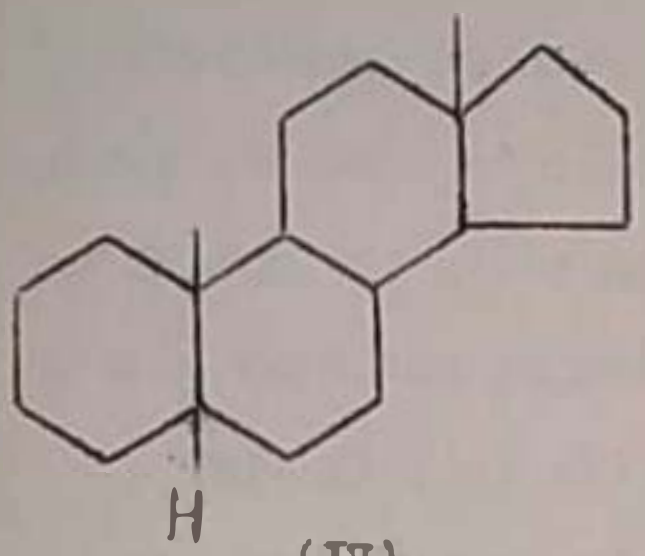
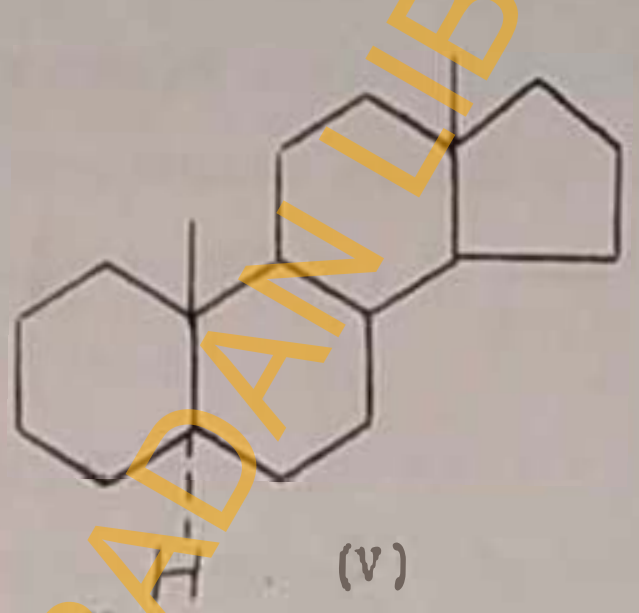
from those of the hydrocarbons Etiocholane (Greek, aitio-fundamental) (IV) in which the A/B ring junction is cis; and adrostane (V) in which the A/B ring junction is trans.

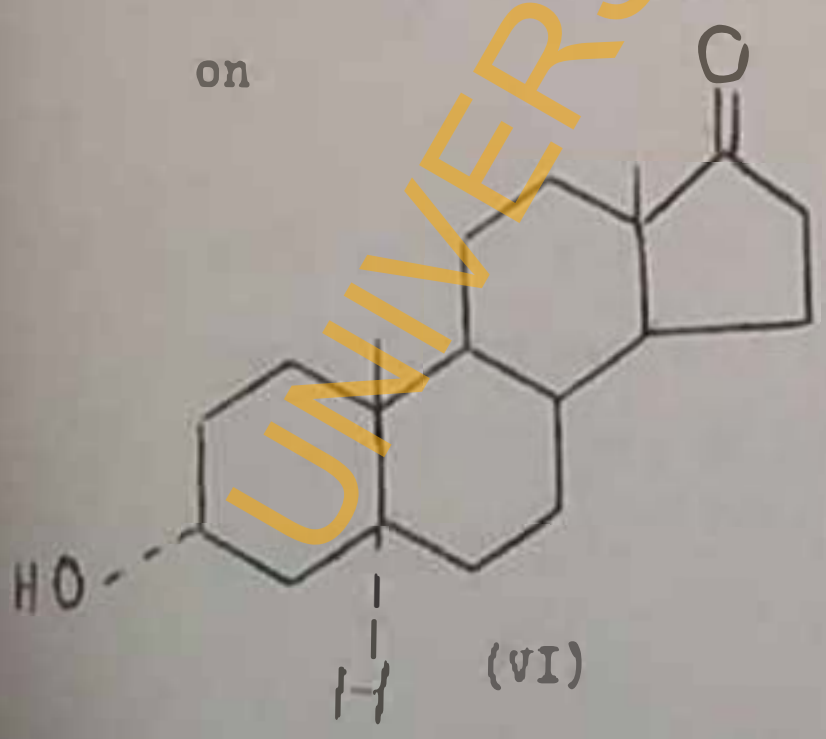


(IV)

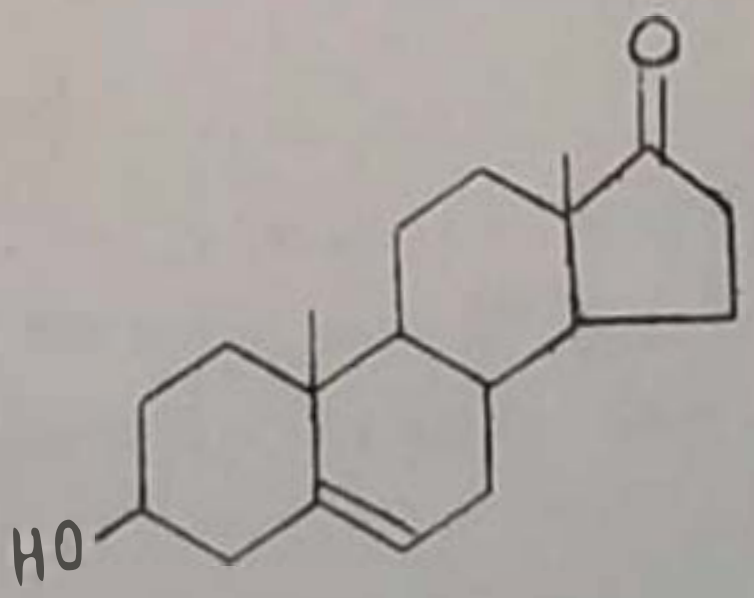


(V)

Androsterone (VI) (3α-hydroxy, 17-ketoandrostane), first isolated by Butenandt and Tscherning (1931) from human male urine is a saturated compound possessing a secondary alcohol group as well as a ketone group. It gives typical derivatives such as the acetate (m.p. 164°), the oxime (m.p. 209°) and the semicarbazone (m.p. 276°). On oxidation, androsterone gives androstandione, which, on



(VI)



(VII)

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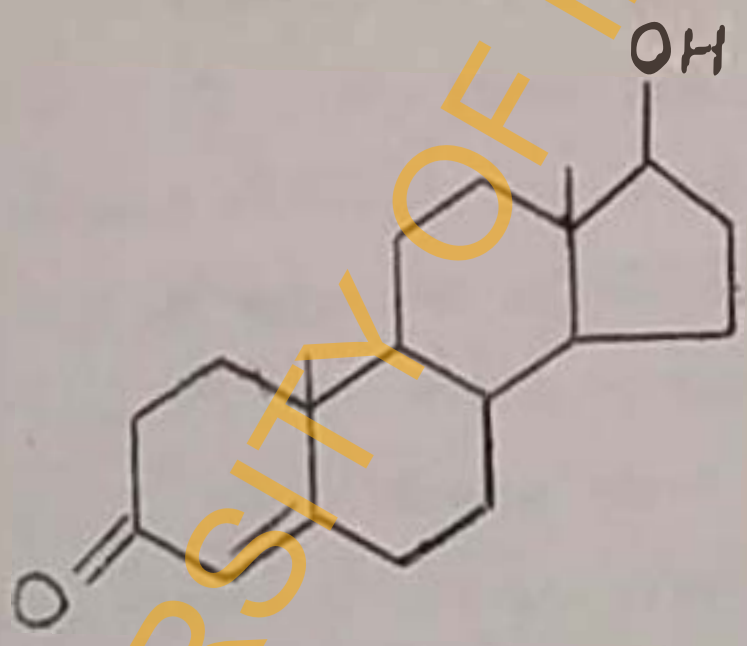
reduction by Clemmonsen's method with zinc amalgam and hydrochloric acid, gives the parent saturated hydrocarbon androstane.

Dehydroisoandrosterone (VII) like androsterone, is a hydroxyketone but with a 3 -hydroxy group and a double bond between the carbon atoms at position 5 and 6. It gives the same reaction products as androsterone. On oxidation, it gives an androstendione, in which tho doubls bond has migrated into ring A.

Testosterone (VIII), (17-hydroxy-keto- -androstene), is the most potent of the androgens. It is an unsaturated hydroxy-ketone. It gives rise to typical derivatives.



(VIII)

such as the acetate (m.p. 140°), propionate (m.p. 121°), benzoate (m.p. 198°) and the oxime (m.p. 222°). Its activity is lost by treatment with hot alkali (Gallagher and Koch 1934).

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Androgens and various derivatives of these substances have high melting points. This has been very useful in their identification and characterisation. They are highly soluble in fat solvent up to a certain concentration, after which, it would be necessary to heat before they would dissolve .

(a) Extraction

As stated earlier, modern work on the androgens began with the efforts of Berthold in 1849, who related the shrinkage of the cocks comb after castration to the loss of some material in the testis. This was confirmed by Pezard in 1911, who showed that extracts of testis could actually stimulate the cock's comb.

Androgens have been extracted from the testes (David et al 1935), adrenal cortex (Short 1960), ovaries Horton et al 1966), various other tissues and the urine of a large number of animals (Womack and Koch (1932 a, b); Kritzer and Cunningham 1937). Extraction of androgens from tissues dates back to 1889 when Brown-Sequad began to dose himself with extracts of dogs testicles. He extracted tissues with saline solution. Pezard (1911) also injected capons with saline extracts of testes.

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The work of early investigators had shown that androgens in urine occur in water-soluble biologically inactive form. On treatment with acid and heat, the water-soluble complex was split yielding the fat soluble, water insoluble androgen, which, now, is biologically active. (Allan et al 1928); Funk et al 1929a).

Hydrolysis of the conjugates of androgens present a problem which has not been satisfactorily solved. Present methods of hydrolysis with inorganic acids may destroy the androgens and it is known that in some cases they do cause alteration of the molecule. (Pincus and Pearlman 1943).

Some authors, (Baulann and Metzger (1940); Talbot et al (1940), have recommended simultaneous hydrolysis and extraction to avoid production of dehydration products through loss of the 3-hydroxyl group of some of the androgens. It is now known, however, that such procedures do not entirely prevent the loss of the 3-hydroxyl group and that with solvents such as carbon tetrachloride or benzene the temperature of the mixture may not allow complete hydrolysis. (Friedgood et al 1943).

There is no general agreement on the conditions of hydrolysis which lead to optimal results. Ideally,

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hydrolysis should quantitatively free the neutral steroids from their conjugated state and should do so without alteration or destruction of their molecular structure. Most often the urine is boiled for 10 minutes after addition of 0.1 of its volume of concentrated hydrochloric acid. It is then cooled quickly and extracted. Some workers prefer to use an equivalent or even greater amount of sulphuric acid (Doorinar et al (1948); Patterson et al (1942). Others, (Holtorff and Koch 1940) increase the amount of hydrochloric acid to 0.15 of the volume of urine. The boiling time may be reduced to 7 minutes (Pincus (1945); Salter et al 1948); or extended to 15 minutes (Strickler et al 1941). Autoclaving of acidified urine at 15 pounds pressure for 15 minutes, as described by Cuyler and Baptist (1941), or prolonged boiling (Peterson et al 1937), tends to destroy some of the androgens (Callow et al 1939).

In 1928, Koch et al introduced a standard method of extracting androgens from the tissues. They used benzene to fractionate the 95% ethanolic extract of the bull's testes. Other workers, including Moore and McGee (1928), Gallagher (1928), Dodds et al 1930) and Funk et al (1930) confirmed and extended the effectiveness

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of this method. Dodds et al (1930) prepared a water-soluble biologically active androgen by a method similar to that of McGee et al (1928). They started with the benzene extract of the tissues, but this was later re-extracted with butyl alcohol and ether, after saponification with barium hydroxide.

Butenandt and Tscherning (1931) extracted 15mg of androsterone with chloroform from acidified concentrates of man's urine and demonstrated its androgenic activity. Adler (1934), confirmed the work of earlier investigators when he showed that benzene extracts of male urine which were inactive by the capon's comb test could be converted to biologically active material by heating with trichloracetic acid. Other workers, like Gallagher and Koch (1934), Peterson et al 1937), also confirmed and extended these findings. Dingemanse et al (1937), Callow et al (1939) extracted compounds related to steroid hormones from human urine with benzene.

The suggestion that androgens are initially present in the urine as water-soluble biologically inactive compound was confirmed by Venning et al (1942), using water as solvent, they extracted androsterone sulphate from the urine of a patient with an interstitial cell tumour of the testis

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Munson et al (1944), also extracted dehydroisoandrosterone in the form of its sulphate ether from man's urine with butyl alcohol at pH. 7.0.

In 1943, Robbie and Gibson introduced the use of carbon tetrachloride for the extraction of androgens in urine. The method was widely accepted and was adopted by the Medical Research Council Committee on Clinical Endrocrinology in 1951. Between 1951 to 1960, a lot of methods were introduced for the extraction of androgens. Drekter et al (1952) produced a pigment free extract from urine with ether. Dobrinor and Lieberman (1950) had earlier extracted androgens from urine by continuous extraction with ether.

Jensen and Tottorman (1952a, b), suggested a new fractional hydrolytic method believed to aid better extraction of androgen with ether from urine. (Talbot et al 1943). This was confirmed and modified by Schneider and Lambert (1959). However, a lot of workers found that the solvents for extraction and the preliminary hydrolytic procedure were not fully adequate hence a new series of solvents were again applied. Smith (1960) applied a mixture of ethanol-diethyl ether to extract androgens from urine and found that 50 to 63 per cent of the androgens

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present in the urine were extracted.

In 1963, the Medical Research Council's Committee on Clinical Endocrinology met again and recommended the use of ethylene dichloride for extracting androgens from urine. This was intended to provide a standard extraction solvent for all endocrinologists. However, in 1964, both Zarrow et al, and King (1964) demonstrated their confidence in the continuous extraction with ether when they reported the extraction of androgens from urine. They claim that diethyl ether extracts more of the urinary pigments than does carbon tetrachloride or benzene.

Hence, it is obvious that there is no consistent method of hydrolysing and extracting conjugates of androgens, although it is very clear that organic solvents, more often, ether, are used after the hydrolysis, for extracting androgens.

(b) Isolation.

Androgens have been isolated from various tissues and urines of normal individuals as well as those with pathological conditions. Several quantitative methods have been described (Talbot et al (1940); Pincus and Pearlman (1943); Pincus (1945) for the separation of the crude neutral extract of urine into non-ketonic and the biologically active ketonic fractions. Some of the

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non-ketonic fractions are also active while some of the ketonic fractions are also inactive.

The use of the Girard T(trimethyl-acet-hydrazide ammonium chloride) for the isolation of the ketonic androgen by their conversion to their hydrazones was first proposed by Girard and Sandulesco (1936). This method was extensively used but soon gave way, because of its obvious limitations when adsorption techniques were introduced. Column chromatography was the first partition method used for the isolation of androgens. Callow and Callow (1940) reported the separation of androsterone, dehydroepiandrosterone and etiocholane-3&-ol-17one by a chromatographic technique in which aluminium oxide was the adsorbent and carbon tetrachloride-ethanol system was used for elution. Bauden and Motzger (1940) absorbed the steroids from pentane solution and eluted them with other.

In order to separate alcoholic from non alcoholic androgens, Talbot et al (1941) allowed a solution of the urinary extract in carbon tetrachloride to percolate through a column of activated alumina. The alumina retained the alcoholic steroids and permitted non-alcoholic ketones to pass through. Dobriner et al (1948); Lieberman et al (1948) extended the use of aluminium oxide for steroid chromatography and also used magnesium silicate,

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obtaining separation of 42 different urinary steroids by means of repeated chromatographing of extracts from large amount of urine. The isolated compounds were characterized by melting points and infrared analysis.

Dingemanse et al (1946) published a method for the determination of individual 17-ketosteroids in 24 hour urine by a single chromatographic process. using alumina as adsorbent and benzene and benzene-ethanol mixtures for elution. A ratio of 2,500 parts of adsorbent to 1 part of 17-ketosteroid was used, and 48 fractions of 50ml were collected. The amount of 17-ketosteroids in each fraction was determined by the Zimmermann reaction. Three of the fractions were identified as dehydroiso-androsterone, androsterone and etiocholanolone. Other fractions have since been identified. (Dingemanse et al (1948 a, b) (Kellie and Wade 1957).

The method of Dingemanse et al (1946) has been used by other workers, including Robinson and Goulden (1949); Barton and Klyne (1948); Lieberman et al (1948). Micro scale adaptations have been devised by Zygmuntowicz et al (1951), and by Pond (1951); 1952). Rubin et al (1953) used the alpha-ketonic fraction of the urine for chromatography. This fraction was obtained by reaction with Girard reagent P, then digitonin precipitation

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according to the method of Butt et al (1948). The digitonin non-precipitable fraction is called the alpha-ketonic fraction. They used silica gel instead of alumina on the column.

The technique of paper partition chromatography originally devised by Consden et al (1944) for the separation of amino acid mixtures was adopted for the identification of steroids by Zaffaroni et al (1949). This method has also been successfully applied to the separation of corticoids (Burton et al (1951) cardiac glycosides and aglycones (Hoffmann and Levant 1952) and for other steroid hormones. (Axelrod (1952); Savard (1952).

Generally strips of filter paper (Whatman No.1) are used. The chromatogram could be run by descending or ascending technique. Originally, Zaffaroni et al (1949) used water as the stationary phase which was bound to the cellulose fibre of the filter paper. They used an organic solvent immiscible with water as the mobile phase. However, the idea of using water as the stationary phase was soon found to be unsatisfactory, when applied to such compounds as steroids which have limited water solubility. Among the various suggestions to circumvent this difficulty was the preparation of derivatives having greater solubility

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(Zaffaroni et al (1949), modification of the partition coefficient in favour of the polar stationary phase, by incorporating a polar phase other than water in the paper support (Zaffaroni et al 1950) or reversed phase chromatography on paper (Kritchevsky and Tiselius 1951).

Separation and identification of steroids by paper chromatography has been accomplished in relatively few cases. Attempts at separation have been carried out using untreated filter paper (Zaffaroni et al (1949) Heftmann (1950); Nyc et al (1950), with varying degrees of success. In two cases of oestrogens (Heftmann 1950), and androgens (Zaffaroni et al 1949), derivatives were used rather than the free steroids. The use of derivatives (Burton et al 1948) was in part necessitated by the difficulty in detecting the free steroids. In a case where free androgens were used, (Nyc et al 1950), the Rf values were rather close together. More successful separations have been achieved using paper treated with formamide or propylene glycol (Zaffaroni et al 1950) or impregnated with alumina (Bush 1950), Silicon (Kritchevsky and Tiselius 1951).

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During the past few years thin-layer chromatography (TLC) has gained an ever increasing importance in the investigation of organic compounds. TLC, first applied to steroids in 1959 by Barbier et al, is by now one of the most important analytical methods in the steroid field. It has proved complementary to paper chromatography and, in many cases, has replaced the paper chromatographic method (Bush (1961); Nahor 1963). The entire subject of steroid chromatography was reviewed by Heftmann (1961) A more recent review was by Nahor (1963).

Generally, chromatography on sheets has the advantage that a series of samples can be compared simultaneously and it is therefore more rapid, accurate and convenient for qualitative analysis than column chromatography. Gas chromatography is superior in all these respects for quantitative analysis, but owing to the lack of specificity of detectors, it requires purified samples only. In this instance, TLC has been used for preliminary separation (Morlick and Avison (1963).

Silica gel G. containing calcium sulphate (E. Merck. Darmstadt) has been found to be the best choice of adsorbent for steroids on TLC. Impregnation of silica gel with silver nitrate aids in the separation of unsaturated compounds, which form silver complexes

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(Avigan et al (1963); Morris (1963). Other adsorbents that have been tried include, Alumina (Lederer and Lederer 1957) and Kieselguhr G.

Thin-layer plates are normally prepared by use of commercially available equipment. Micro-chromatoplates are conveniently prepared by dipping microscope slides into a suspension of 35 gm of silica gel G in 100ml-chloroform - methanol (2:1) and, after the solvent has evaporated, exposing them to steam.

The choice of developing solvents in partition chromatography is not too difficult. In most cases, organic solvents and binary mixtures usually do very well. The most generally applicable solvent mixtures are prepared from benzene or chloroform and acetone or methanol. Ethanol or ethyl acetate mixed with a hydrocarbon like cyclohexane is also widely used. There is no standard developing solvent system, the choice depends on the type of steroid and the investigator. However, the solvents should be of highest purity, especially for preparation work and free of peroxides, which tend to concentrate on the adsorbents.

Location of steroids on chromatograms have been a source of concern to all scientist. This is because

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they are not very specific to one type of steroid. Detecting reagents may be divided into two categories. The non-selective reagents, which produce colours or fluorescence with the majority of steroids, while the response to the selective reagents is due more or less to specific structural feature. Non-selective reagents such as sulphuric acid (Jones et al (1964); Anthony and Beher (1964), for sterols, steroids, bile acids and gibberellins; phosphoric acids, (Starka and Malikova (1961); Nehor and Wettstein 1952), for sterols and steroids; antimony trichloride, (Takeda et al 1963), for carotenoids, steroid sapogenins, steroidglycosides, and flavonoids; and various aromatic alcohydes give characteristic colours in visible or ultraviolet light with some steroids.

Non-destructive detection on TLC can be achieved by using known fluorescent absorbents, the fluorescent is quenched by ultra-violet absorbing substances. Convenient fluorescent dyes such as Rhodamine 6G, 2 7'-dibromo or 2, 7' dichlorofluorescein have been used (Morris (1963).

(c) Synthesis

The natural androgens and their transformation products are derivatives of 5α-androstane (IX) and 5β-androstane (X).

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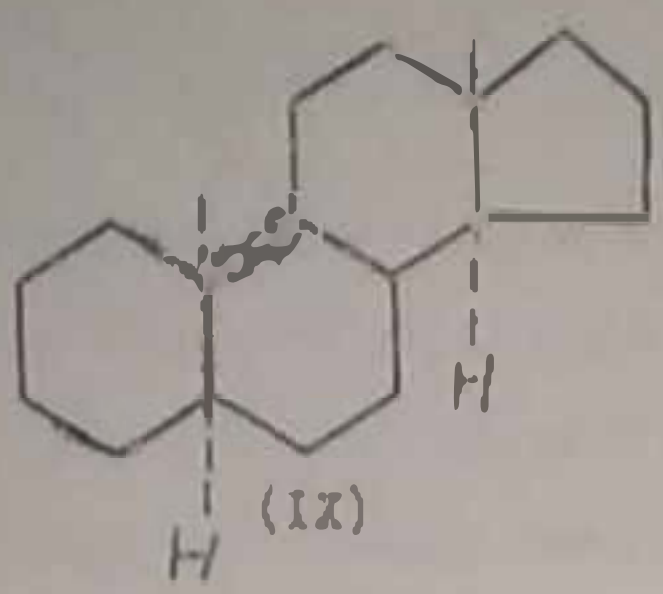

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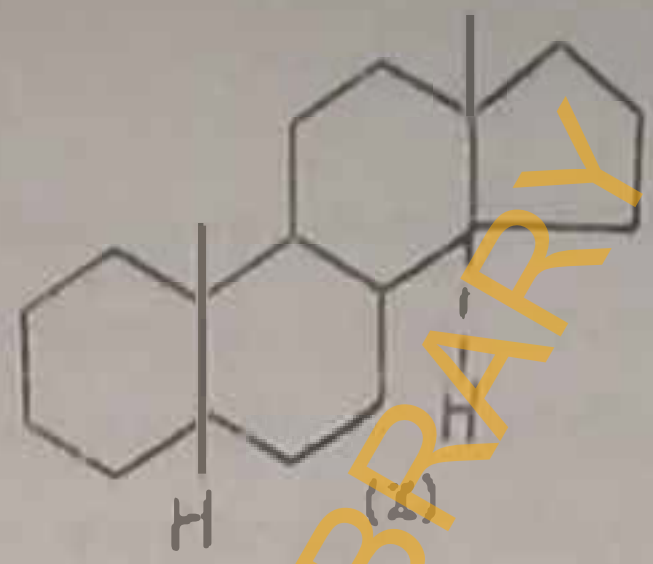

(X)

Androsterone, ($C_{19}H_{30}O_2$) melting point 183°, optical rotation (α) D + 94°, was isolated in 1931 by Butenandt. 15,000 litres of normal male urine gave 15mg. of crystalline androsterone. The hormone was characterised as a hydroxy ketone and on the basis of the molecular formula which indicates the presence of four reduced rings, Butenandt suggested the correct formula (VI).

At this point, Ruzicka et al (1934) started to explore the possibility of degrading cholesterol (C_{27}) to androsterone (C_{19}). Using the chromic acid oxidation method earlier suggested by Windaus (1927), they converted 5 -cholestanyl acetate by oxidative removal of the side chain with chromic acid into a ketone of the structure suggested by Butenandt. The product was Epi-androsterone (formerly called iso-androsterone) melting point 174°, [α] D + 86°, one-seventh as active as androsterone on the capon's comb (Deanesly and Parkes 1936). Androsterone was

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similarly obtained from epi-5α-cholestanyl acetate (Ruzicka et al 1934), 1935), while 5B-androsterone and epi-5B-androsterone were obtained from 5B-cholestanyl and epi-5B-cholestanyl acetate respectively (Ruzicka et al 1934).

In the brief period when androsterone was the only known androgen of significant potency, Ruzicka's original procedure and related routes were actively investigated, but no process affording a good yield was found. Callow and Deanesly (1935) made some improvements, including separation of androsterone with Girard reagent, but under the best conditions obtained the hormone in 0.2% overall yield from cholesterol. Marker (1935) also oxidised B-cholestanyl chloride to 3B-chloroandrostanone and hydrolysed the latter to androsterone. Despite poor yields, these degradations, established the structures of androsterone, epi-androsterone and their isomerides, (Fig. 2)

The total synthesis of a non-aromatic steroid, epi-androsterone was first achieved by Cornforth and Robinson in 1949. The synthesis starts with reduction of 1, 6-diethoxy-naphthalene with sodium and alcohol to 5-methoxytetralone-2. Methylation, acetylation, isomerisation, hydrogenation, alkylation and finally hydrolysis of this compound produce epi-androsterone.

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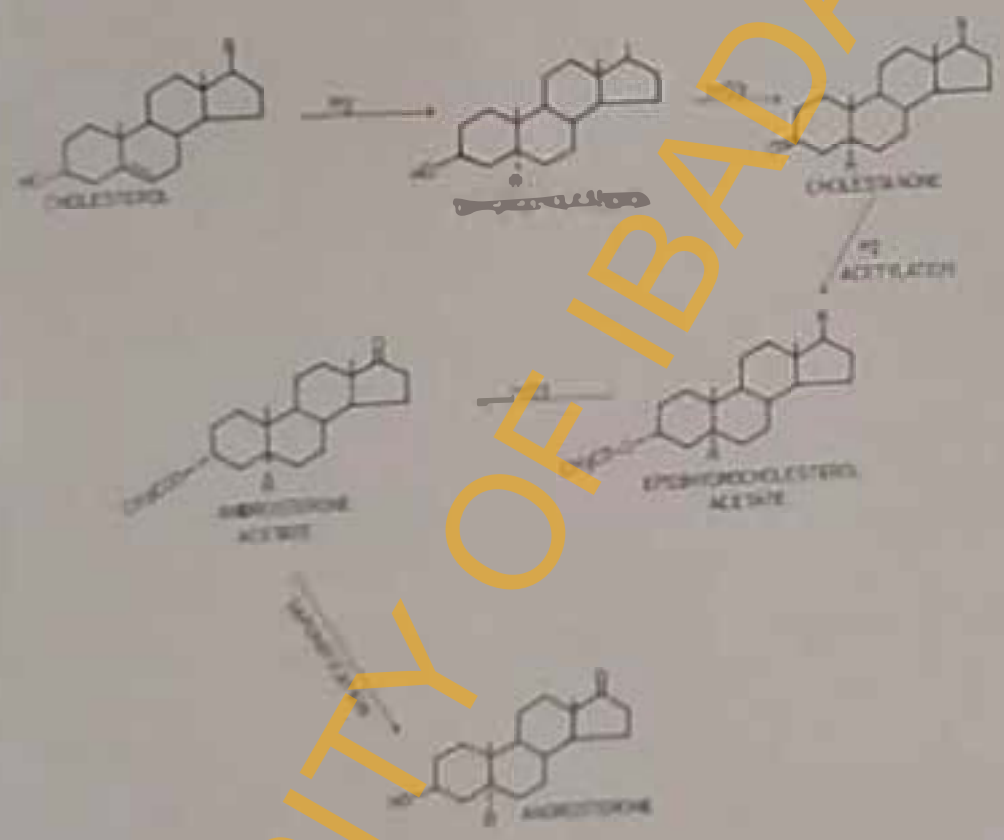
FIGURE 2.



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The best method of preparation of androsterone from dehydro-epi-androsterone was suggested by Wallis and Fernholz (1937); Shoppee (1946). Total synthesis of androsterone has been described in detail by Woodward et al (1952).

Dehydro-epi-androsterone or Androstenolone is an intermediate in the commercial production of testosterone. With androstenolone now available, a simple route to androsterone consists in hydrogenation of the double-bond and epimer-isation at C_3 via the tosylate (Johnson et al 1953). Another method suggested by Djerassi et al (1935) involve the reduction of androstane-3, 7-dione with aged (non-pyrophoric) Raney nickel in ethanol to a mixture of 17-ketones, separated with digitonin to afford androsterone (50%) and epi-androsterone (34%).

Dehydroepi-androsterone (androstenolone) ($C_{19}H_{28}O_2$), melting point 153°, (&) D + 11°, an unsaturated weakly androgenic hydroxyketone, and the related 3B-chloroandrostenone were isolated in 1934 from male urine by Butenandt. The chloroketone was formed by the action of hydrochloric acid on androstenolone during acid hydrolysis of the urine. Androstenolone has a 3B-hydroxy group, and is the 5.6-dehydro derivative of epi-androsterone. The chloride derivative

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The best method of preparation of androsterone from dehydro-epi-androsterone was suggested by Wallis and Fernholz (1937); Shoppee (1946). Total synthesis of androstorone has been described in detail by Woodward et al (1952).

Dehydro-epi-androsterone or Androstenolone is an intermediate in the commercial production of testosterone. With androstenolone now available, a simple route to androsterone consists in hydrogenation of the double-bond and epimer-isation at C_3 via the tosylate (Johnson et al 1953). Another method suggested by Djerassi et al (1935) involve the reduction of androstane-3, 7-dione with aged (non-pyrophoric) Ranay nickel in ethanol to a mixture of 17-ketones, separated with digitonin to afford androsterone (50%) and epi-androsterone (34%).

Dehydroepi-androsterone (androstenolone) ($C_{19}H_{28}O_2$), melting point 153°, (&) D + 11°, an unsaturated weakly androgenic hydroxyketone, and the related 3B-chloroandrostenone were isolated in 1934 from male urine by Butenandt. The chloroketone was formed by the action of hydrochloric acid on androstenolone during acid hydrolysis of the urine. Androstenolone has a 3B-hydroxy group, and is the 5.6-dehydro derivative of epi-androsterone. The chloride derivative

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could also be obtained by use of phosphorus pentachloride with retention of configuration at C_3 (Shoppee 1946), and regenerating androstenolone by treatment with benzoate ions in benzoic acid and subsequent alkaline hydrolysis (Fig. 3). Hydrogenation of the chloroketone with palladium in ethanol or acetic acid yield 3 -chloroandrostanone, which by acetolysis undergoes substitution with inversion to give, after hydrolysis androsterone, (Fig. 4).

The structure of androstenolone was confirmed by partial synthesis of its acetate from cholesteryl acetate dibromides (Fig. 5) (Ruzicka and Wettstein (1935); Wallis and Fernholz (1935), and from sitosteryl acetate dibromide (Oppenauer 1935), and by hydrogenation to epi-androstorone accompanied by 10% of epi-5 -androsterone (Reichstein et al 1941; 1944). In general synthesis of androstenolone is by oxidative degradation of a suitably protected cholesterol derivative with chromic acid in acetic acid. Various modifications have been introduced to improve the yield. Androstenolone formate reduction with sodium borohydride gives androst-5-ene-3 , 17 diol formate. This as the 17-acetate or propionate has been converted into testosterone acetate or propionate by Oppenauer oxidation (Ringold et al 1956).

By 1935, a large number of compounds derived

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FIGURE 3.

CONVERSION OF 3β-CHLOROANDROSTENONE TO DEHYDRO-EPI-ANDROSTE-RONE (ANDROSTENOLONE) (SHOPPEE 1966)

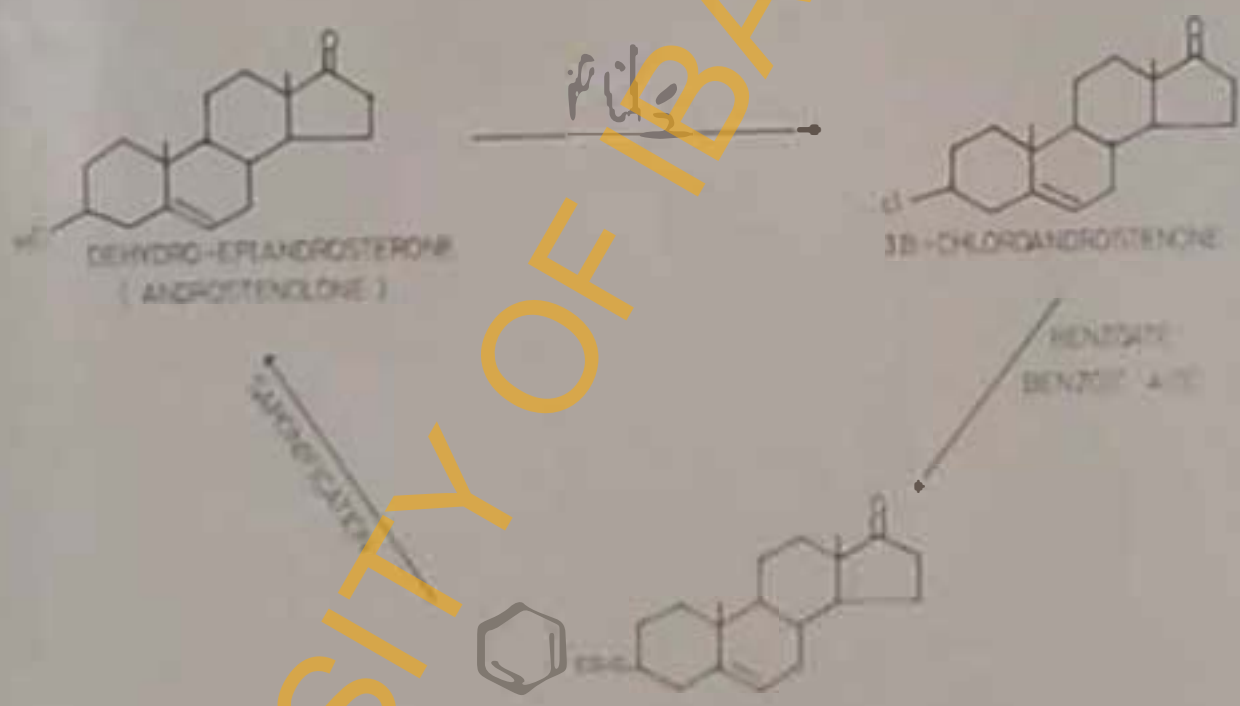

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FIGURE 4.

SYNTHESIS OF ANDROSTERONE FROM ANDROSTENOLONE.

(STOPPEE 1946)

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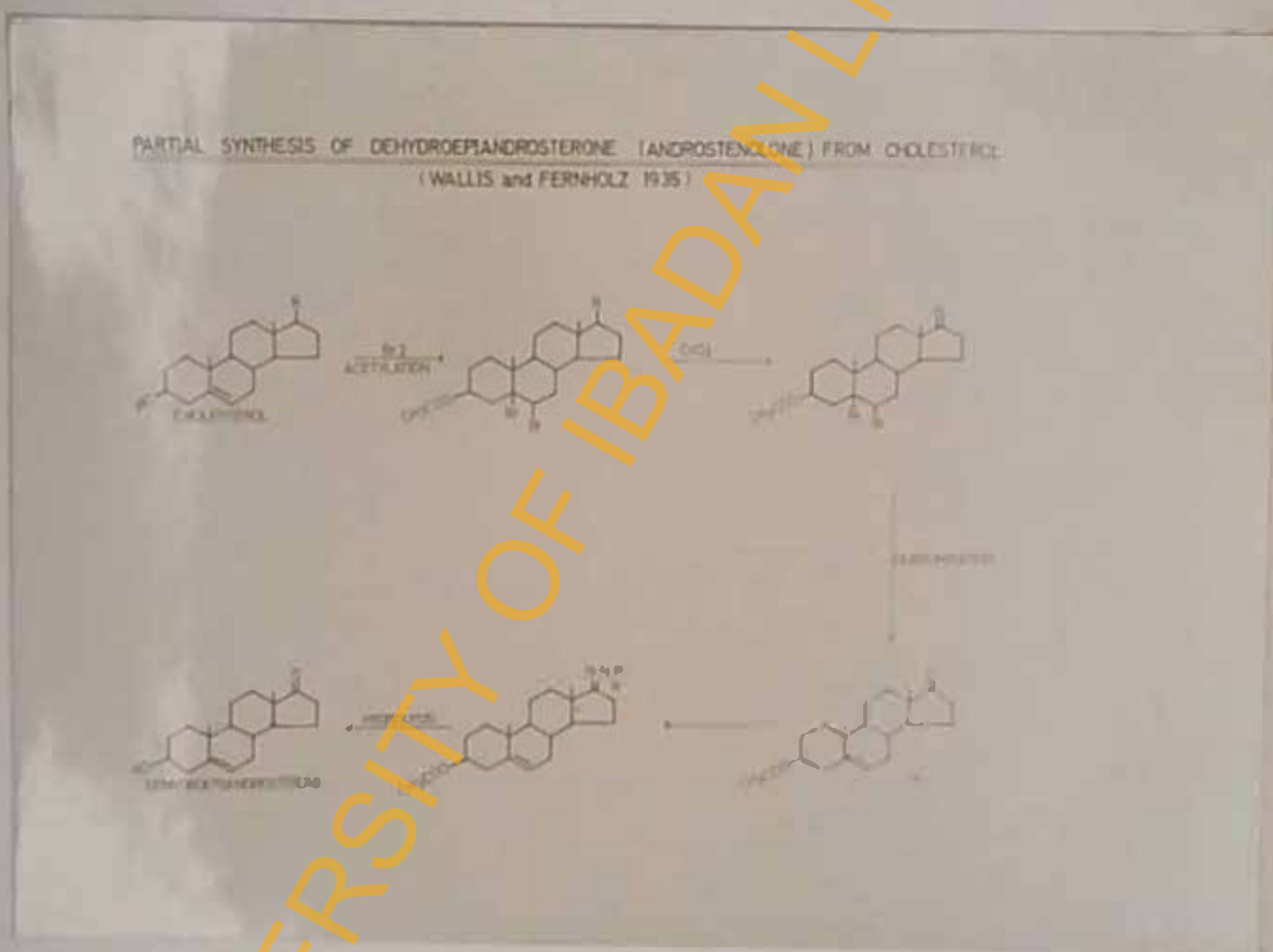
FIGURE 5.



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from androsterone and androstenolone had been prepared and assayed for androgenic activity in capons, (Gallagher and Koch (1930); Koschevsky (1932); Callow and Parkes (1935); Greenwood et al 1935), and in the castrated rats, (Callow and Deanesly (1935); Moore and Price (1937); Kochakian (1938).

In 1935, David and his co-workers reported the isolation of a testicular hormone, melting point 155°, (&)D + 109°, to which they gave the name testosterone. (VIII). About 10mg of the pure hormone was isolated from 100kg of steer testis tissue. The testicular hormone was found to be an &B-unsaturated hydroxyketone with maximum absorption at 240mu. It was found to be 10 times as potent as androsterone in promoting comb growth (Gallagher and Koch (1935); and in the rat test was about 7 times that of androsterone (Callow and Deanesly 1935). Its structural formula was established by oxidation with chromium trioxide to androst-4-ene 3, 17, dione, which is readily obtained from androstanolone by Oppenauer oxidation, and which can be reconverted to testosterone by sodium propanol reduction of the 3-ethyl-enol ether by Meerwein-Pondorf reduction (Kissher and Fischer (1939) by lithium aluminium hydride reduction of the 3-N-Pyrroli-dine enamine (Huyl and Herr (1953), or best by reduction

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with sodium borohydride (Morynbeski and Woods 1954).

Improved partial synthesis of testosterone from androst-4-ene, 3, 17-dione have been described by sodium propanol reduction of the 3-ethyl enol ether-17-cyanohydrin (Ercoli and De Ruggieri (1953); by Raney nickel reduction of the derived D^5-3:3 dioxolane (Daubon et al (1954); and by lithium aluminium hydride reduction of the 3-benzyl thioenol ether. (Rosenkranz et al 1949; Romo et al 1951).

The simplest partial synthesis is probably that of Sondheimer et al (1953) by reduction of androstenedione with lithium aluminium hydride to the mixture of 3-epimerides which by oxidation with manganese dioxide in chloroform at 20° (Mongler et al 1961), gives a 90% overall yield of testosterone. Testosterone has also been obtained from sarsasapogenin and diosgenin (Marker 1940); radiotestosterone (4-^{14}C) has been prepared (Turner (1950); Steunwhite and Samuels 1956).

The total synthesis of (±) testosterone has been accomplished by Johnson (1956); Johnson et al (1956) (1960). The tetracyclic -ketone, as the dioxolane, was stereospecifically reduced, hydrogenated, and isomerised at C_{13} with base to the (±) - 18-nor-D-homo-17a-Ketone, which as the furfurylidene derivative was

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subjected to angular methylation. Oxidative fission, Diecmann cyclization, gentle pyrolysis of the resulting β-Ketoester, reduction of the 17-carbonyl group and acid hydrolysis of the Δ^5-dixolene gave (±) testosterone.

In the mammals plasma testosterone [illegible] primarily from the testosterone synthesised and secreted by the interstitial (Leydig) cells of the testis; only a small portion of the circulating hormone being due to the peripheral conversion of precusor steroids secreted by the adrenal cortex. (Migeon 1960). "In vitro" and organ perfusion studies suggest that the pathways of androgen synthesis are similar, but the amount formed vary in both the gonads and the adrenal cortex (Dorfman and Ungar 1965). While the experiments on steroid balance and on steroid absorption have been important in establishing the overall occurrence of steroid synthesis in animals (Fukushima et al (1952) they have been incapable of providing details of the synthetic process.

With the introduction of isotopic tracer technique to this study, a tremendous amount of information on the biological synthesis of steroids has been obtained. The first experiments which contained evidence to indicate the possible nature of cholesterol synthesis in animal

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tissues were carried out by Rittenberg and Schoenheimer (1937), who studied the incorporation of isotopic hydrogen into various tissues constituents from a heavy-water medium. The biogenesis of testosterone has been established in experiments using radioactive substances. Slaunwhite and Samuels (1956) incubated labelled progesterone with rat testis tissue from hypophysectomised and immature rats, after the tissue had been stimulated with chorionic gonadotropin and obtained androstenedione and testosterone.

The sequence of dehydrogenation, hydroxylation at C_{17}, elimination of the side chain, and reversible reduction at C_{17} of progesterone with conversion to androstenedione and testosterone has also been demonstrated with the testis (Savard et al (1956); Lynn and Brown (1956) (1958) ovarian tissue (Solomon 1956), and in the adrenal cortex (Kass and Kuriel 1962). The synthesis of testosterone from labelled acetate has been observed in testicular tissue slices (Brady 1951). Hellman et al (1952) observed that cholesterol is an obligatory intermediate in the conversion of labelled acetate to steroid hormone in the human (Fig. 6) (Cardwell et al 1951).

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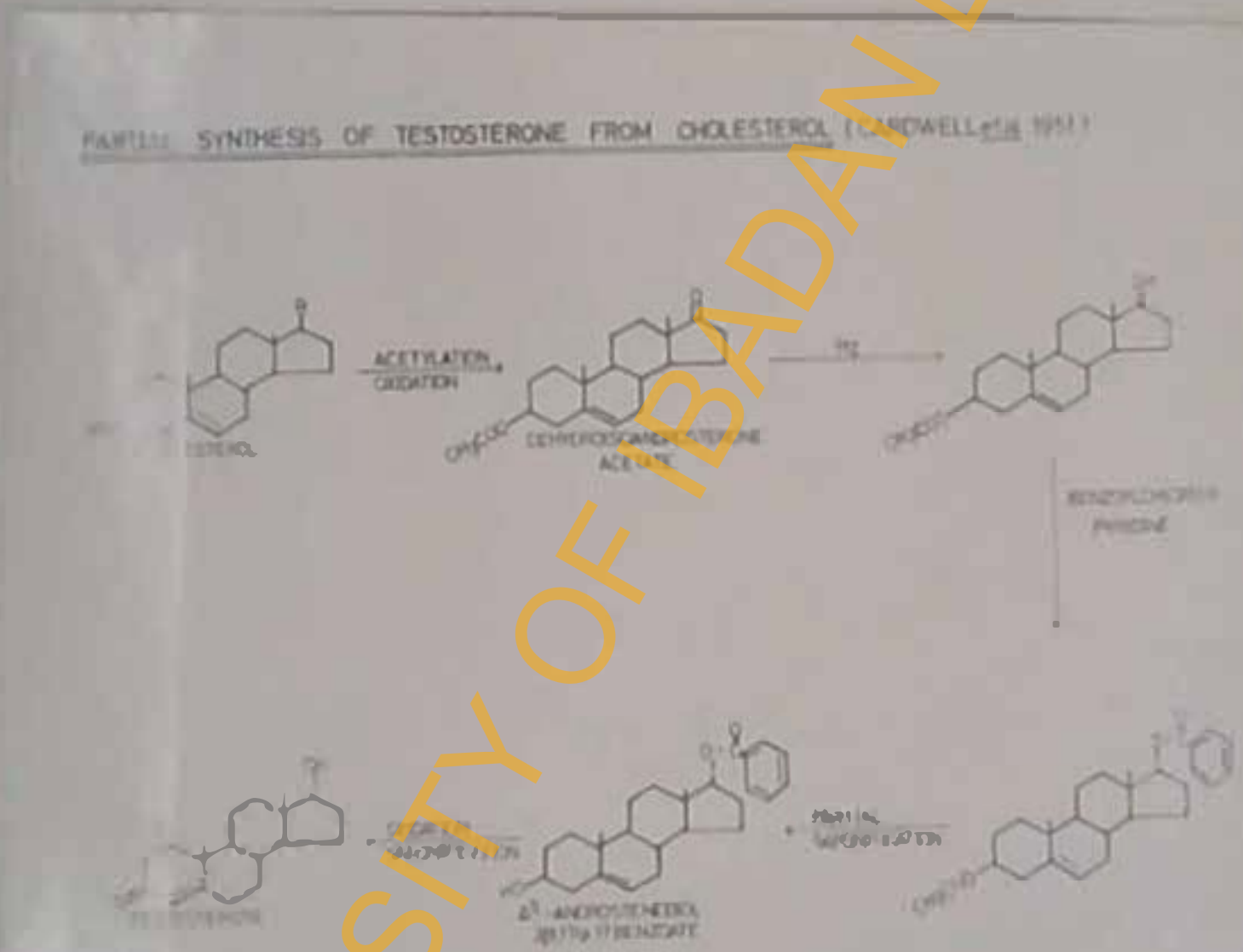
FIGURE 6.



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The "invivo" conversion of cholesterol to androsterone has been demonstrated by administration of C^{14} or tritium-labelled cholesterol to a human subject and isolation of these C_{19} urinary steroids (Werbin et al 1957). The androstenolone of human urine has long been regarded as arising in the adrenal gland, and work by Bloch et al (1956) has demonstrated the presence of the substance in human adrenal tissue and its biosynthesis from labelled acetate.

In the adult testis, the synthesis of testosterone has been shown to proceed via (Fig. 7) either the progesterone (Glaumwite and Samuels (1956); Block et al 1962) or pregnenolone pathways (Fig.7); it is uncertain which is the major route (E ik-Nee and Kekre (1963); Ellis and Berliner 1965). A third pathway via androstenediol has been demonstrated both "in vivo" (Baulieu 1963), and "in vitro" (Payne and Mason 1966). It is probable however, that this pathway accounts for less than 1% of the testosterone secreted by the testis.

The testis apparently secretes small amounts of free androstenolone and androstenedione, since the production rates of both steroids rise upon the administration of chorionic gonadotropin to the

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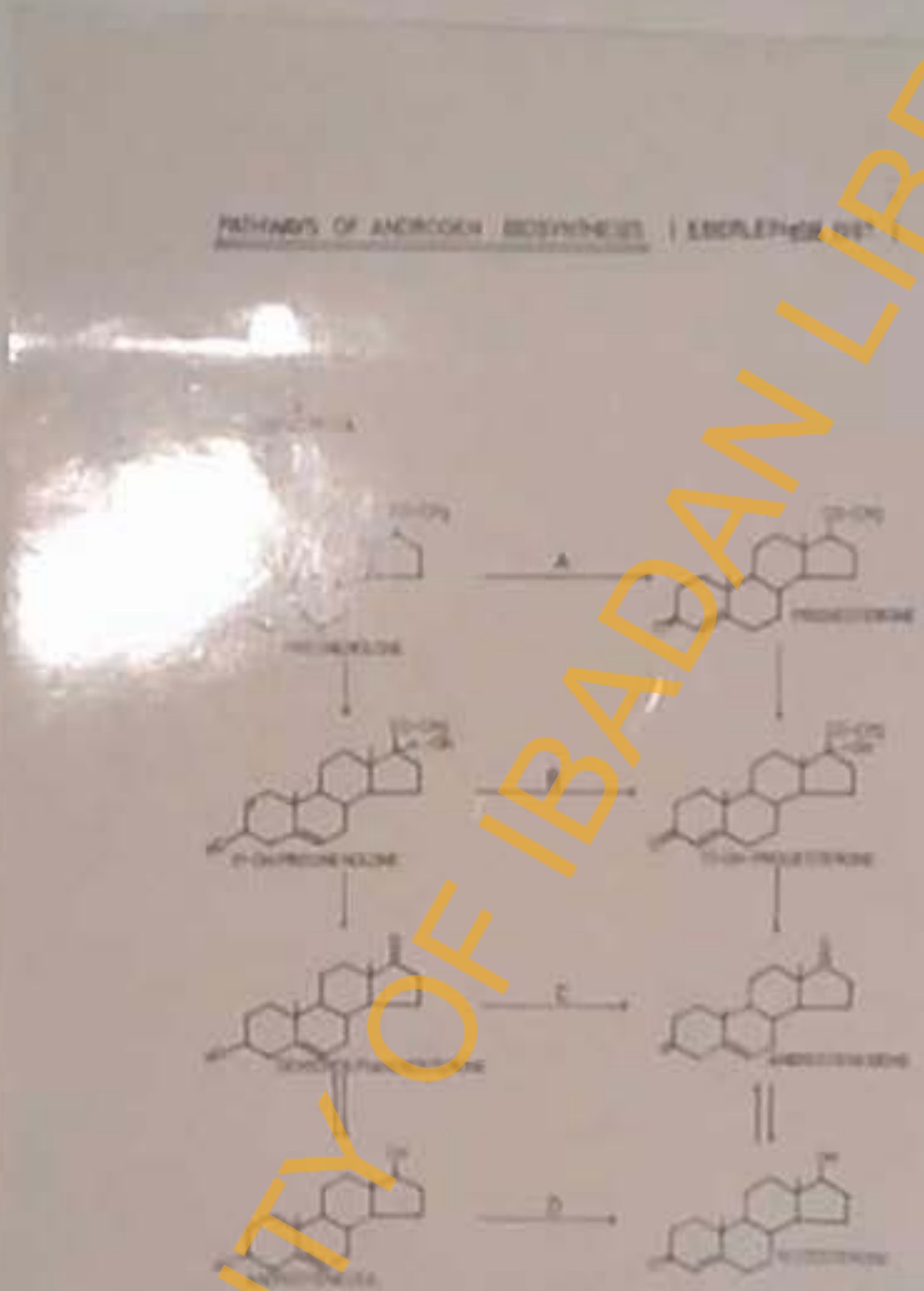
FIGURE 7.



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adult male (MacDonald et al 1965). Comparison of ovarian venous levels of certain C_{19} steroids with peripheral plasma levels has demonstrated that the major androgen secreted by the ovary is androstenedione, although small amounts of androstenolone are also formed. (Horton et al 1966).

Although the ovary is capable of synthesising testosterone under "in vitro" conditions (Solomon et al (1965); West and Neville 1962), under normal circumstances it does not secrete significant amount of testosterone (Horton et al 1966). Androstenolone is the major C_{19} steroid secreted by the adrenal cortex, and is released from the gland into the blood system both as the sulphate ester and as the free steroid (Baulieu 1963).

It has been suggested by Calvin et al (1964c), that the steroid conjugate may arise from sulphated precursors such as pregnenolone or cholesterol sulphate (Calvin et al (1964b) Roberts et al 1964). Although it has been suggested that androstenolone might arise here directly from cholesterol, via an intermediate such as 17, 20-dihydroxy cholesterol, Shimazu (1965), did not find this pathway to be functional in either the rat's adrenal or testis and seems limited to man.

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Kase and Kowal (1962) have shown by "in vitro" studies that the adrenal cortex is capable of converting progesterone to androstenedione and testosterone. Although it is now known that the synthesis or production of androgens in the testis is regulated by hormones produced in the anterior pituitary gland, "in vivo" effect of these hormones has not been widely studied. The hormones include follicule stimulating hormone (FSH) and luteinising hormone (LH).

"In vitro" experiments with hormones, with luteinising hormone like effect, such as chorionic gonadotrophin, stimulate the interstitial cells of the testis to secrete testosterone (Lipsett et al (1966); Rivarola et al, 1966) and possibly small amounts of androstenedione and androstenolone (Chapdelaine et al (1965). Lipsett et al (1966) also injected androgens and oestrogens into animals and found a fall in the plasma level of testosterone. This has been interpreted as due to inhibition of pituitary gonadotropin stimulation of the testis.

Since the amount of androgen normally produced by the ovary is very small, there are not many reported studies of the "in vivo" regulation of C_{19} steroid secretion by this gland. Adrenocorticotrophic hormone (ACTH) produced by the anterior pituitary gland is known to stimulate only the adrenal cortex and leads in both adult men (Migeon

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ox Klager 1955) and women (Short 1962) to an increased rate of secretion of free and esterified androsterone as well as of androstenedione (Rivarola et al 1966)

When testosterone or its propionate is given to human subjects there is an increased excretion of 17-ketosteroids. androsterone (Callow (1939); Dorfman et al 1939), isoandrosterone (Dorfman 1940)and etiocholanolone (Callow 1939); Schiller et al 1945) have been identified as metabolites of testosterone which are excreted in the urine. Their relative proportion does not vary much with sex (Baulieu 1964).

(d) Chemical determination of androgens

Paradoxically as it may seem, more information is available about the catabolic fate of the steroid hormones than about any other phase of steroid biochemistry. Some tentative inferences concerning the metabolism of androgens emerged from data on the excretion of neutral 17-ketosteroids in the urine. Estimation of these ketosteroids in the urine, blood and tissues is normally used as an index of the amount of androgens (Perlman 1942). Studies with intravenously administered

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testosterone and other steroids have proved that the hormones disappeared quickly from the circulation (Hoskins, 1950); West (1951); West et al 1951).

West et al (1951) have shown that the liver and kidney are both sites of androgen metabolism in the animal. Greene and Burrill (1941) had earlier presented evidences to indicate that the liver and kidney are both the sites of testosterone metabolism in the animal. It appears that the kidney is not as active as the liver towards the androgens although its metabolic reactions are the same. "In vitro" experiments by Samuels et al (1947), Clark and Kochakian (1947); Sweet and Samuels (1948); Schneider and Kosen (1948); West and Samuels (1949); Kochakian et al (1949), also confirmed the early observations.

As early as 1934, Zondek had demonstrated that enzymatic factors in the liver destroyed the biological activity of the oestrogens. Since that time, attention has been centred on that organ and indeed the liver is now considered to be the most important organ for the catabolism of the androgens, although other organs, such as the kidney, has been found. The role of the liver and the kidney in the metabolism of the steroid hormones has been reviewed by

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by many workers. Samuels (1950) on androgens Samuels and West (1951) on androgens, progesterone and adrenal cortical steroids.

The application of isotopic method have confirmed that non-endocrine tissues such as the liver, kidney, skin and skeletal muscles are capable both of androgen inactivation and of transformations of steroid procursors to androgens (Eberlein et al 1967).

What is known concerning the "in vivo" catabolic fate of the steroid hormones in humans has been derived for the most part, from detailed studies of the urinary metabolites. The metabolism of testosterone has been studied extensively. Beginning with early demonstrations that the administration of testosterone resulted in increased excretion of biologically active material in the urine (Dorfman and Hamilton (1940) Mason and Kepler (1945); (1947); this work has led to the isolation and identification of some of the transformation products (Engel et al (1941); Robinson and Goulder (1949); Elliott et al 1952).

That testosterone administration, either orally or by injection, gives rise to increased excretion of androgens and 17-ketosteroids in the urine, has been

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shown for man by Dorfman and Hamilton (1940); Callow et al (1939); Hoskins and Koch (1939); McCullagh et al (1933) McCullagh (1939). This conversion is also true for the rat (Dorfman 1938), the guinea-pig (Dorfman and Fish, 1940), the monkey and the chimpanzee (Fish and Dorfman, 1944).

The findings of Callow (1939), Dorfman et al (1939) that at least a portion of the administered testosterone may be accounted for by the conversion to the biologically active androgen-androsterone - showed the relationship between the tissue hormone and the urinary content. In addition to androsterone, it was also shown that two other isomers result from the metabolism of testosterone, namely, etiocholanol-3α-17-one and iso-androsterone. The inference that androsterone and etiocholanolone are metabolites of testosterone, received support from the finding that, administration of testosterone to hypogonadal men (Callow, 1939); to women (Schiller et al 1945) and to chimpanzees (Fish and Dorfman 1944) results in the production of these metabolites.

The possibilities that androstanediones, androstanediols and androsterone-3α - 17β-diol are intermediates in the metabolism of testosterone was suggested by the observation

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that, administration of such substance results in increased androsterone excretion. Here occurs evidence became available with the introduction of tracer techniques which have the obvious advantage of correlating substrate with specific excretory products. Fukushima and Gallagher (1952) administered deuterium-labelled testosterone and established its conversion to androsterone and etiocholanolone, two of the major urinary 17-ketosteroids which are conjugated either with sulphuric acid or glucuronic acid (Fukushima et al, 1954).

Almost all the radioactive label appeared in the urine within 48 hours (Sanchez and Slaunwhite, 1956). Experiments using isotopically labelled steroids have also pointed to the bile (Rashkis et al (1944), Berliner and Carcoo (1948); Rubin et al (1952), as an important excretory route for the metabolites of the steroid hormone. Martin et al (1965) recovered about 10% of the label from the bile and of this amount about one-half was re-absorbed in the gut. The inference that testosterone is first metabolised to androsterone was supported by the observation of Liberman et al (1950), that deuterium-labelled androstenedione is metabolised by man to androsterone and etiocholanolone, isolated from urine in the same amount as

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after administration of testosterone Direct reduction of testosterone to 5α and 5β - androstane-3α, 17-β-diol may account for as much as 30% of total testosterone catabolism (Stylianou et al (1961); Baulieu and Mauvais-Jarvis, 1964).

The androstanediols in turn may undergo oxidation to androsterone and etiocholanolone. The urinary androstanediols may also be derived directly from androstanolone sulphate without prior conversion to testosterone (Mauvais-Jarvis and Baulieu, 1965). The work of Sweat and Samuels (1948) has shown that there are two major enzymes in the liver which act on the testosterone molecule, one requiring citrate as a co-factor which acts on the conjugated system in ring A without effecting the alcohol on C_{17}, the other requires NADP for its action which first oxidises the alcohol to a 17-ketone and then, by further enzymic reactions reduces ring A to a saturated alcohol.

Pratt et al (1951) from "in vitro" experiments concluded that the latter route which will lead to the production of androsterone and its isomers was the preferred route in the human body. Practically all the steroid metabolites excreted in the urine occur in conjugated form, either as sulphates, glucuronides, or other forms as yet unidentified

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(Schneider and Lombart, 1959). A completely satisfactory method for the hydrolysis of any class of the conjugates of urinary steroids has still to be described.

Of the many known steroid metabolites in the urine, only a limited number have been isolated in the form of their sulphates - dehydroisoandrosterone (Munson, et al 1944) androsterone (Venning et al, 1942). The sulphate steroid conjugates are much more easily cleaved by acid hydrolysis than the glucuronides. In fact, it is merely necessary to extract continuously an acidified solution of these conjugates with ether to liberate the free steroids (Liebermen and Dobriner, 1948).

The glucuronides represent the second major mode of conjugation by which the steroids are known to be excreted in the urine. Both androsterone and eticocholanolone have been isolated from the urine in the form of their glucuronides and have been liberated to the free from by the use of the enzyme B-glucuronidase (Migeon and Plager, 1955; Camacho and Migeon, 1964).

For the hydrolysis of glucuronides by acid, elevated temperature are required and this may lead to the production of artifacts (Jensen and Totterman, 1951a, b).

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The chemical or colourimetric methods of estimating the urinary steroids are usually less sensitive than the bioassay methods. They are generally cheaper, simpler, more rapid and more precise. Hence colourimetric assay have all but replaced biological methods (Gold, 1964). It must be pointed out, however, that chemical analysis does not measure a biological activity and, in those instances where a biologically active material of unknown structure is sought the bio-assay techniques are indispensable (Zieler, 1964).

The colourimetric methods that have been used most widely for the determination of 17-ketosteroids are based on the reaction of the group - CH_2CO - with m-dinitrobenzene in alkaline solution. Zimmermann (1935) demonstrated the usefulness of this reaction for the qualitative determination of pure ketonic steroids. Hence the reaction is usually known as the Zimmermann reaction when it is applied to steroids. Steroids with a ketone group at C_{17} yield a red colour with an absorption maximum at 520mu (Frazer et al (1941); Holtorff and Koch (1940); Schneider and Mason (1948). Ketone groups at other positions of the steroid nucleus or in a side chain may also yield coloured product. Thus 3-keto steroids give

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a yellow colour and 20-ketosteroids a red colour clearly different from the typical colour of the 17-ketosteroids (McCullagh et al (1940), Pincus and Pearlman, 1943).

However, under the conditions of assay described by Callow et al (1938) and Holtorff and Koch (1940), these ketones may be ignored in so far as the ordinary neutral extract is concerned (Hanson et al 1943). However, in some special cases, such as pregnancy, in which relatively large amount of other ketones may be excreted, the effect of other ketones may not be ignored (Venning 1946). Various workers, including Neustadt (1938), applied the method described by Zimmermann to urinary extracts. The essential detail of the procedure is as follows: To 0.2 ml. of test solution in absolute alcohol is added 0.2 ml. of a 2% solution of m-dinitrobenzene and 0.2ml. of 2.5 ± 0.02N potassium hydroxide, both in absolute alcohol. The colour is allowed to develop at 25 ± 0.1 °C for one hour with the solution shielded from all out diffused light. After dilution with 10ml of absolute alcohol, the intensity of the colour is determined. A blank is prepared in the same manner except for substitution of absolute alcohol for the test solution (Hamburger, 1952).

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Many modifications and refinements have been made. The variable factors which influence the colour reaction have been studied by many investigators, including the purity and the concentrations of the reagents, the relative amounts of the reactants, the amount of water in the alcohol solutions and the duration of colour development. All these have facilitated the manipulations involved and enhanced the reliability and sensitivity of the method.

Callow et al (1940) emphasised the importance of purification of the m-dinitrobenzene and anaqlute alcohol and described methods for their purification. The use of the 2.5N potassium hydroxide presents a problem since the alcoholic solution deteriorates after a short time and a yellow colour develops even when it is stored at a low temperature. However, Wilson and Carter (1947), added ascorbic acid to the potassium hydroxide and found that it would then keep for at least three months if stored under nitrogen. Other contributors include Holtorff and Koch (1940); Kincus and Poorman (1943), Robbie and Gibson (1943); Hansen et al (1943).

The presence of chromogenic materials, other than 17-ketosteroids contained in the urine extracts constitute

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another problem. These give a brown colour which absorbs appreciably at 520mu and a blank of extract and alkali without dinitrobenzene will not wholly compensate for its presence. If the concentration of 17-ketosteroid is low, the intensity of the brown colour may be sufficient to interfere seriously with the determination. Three methods are available for eliminating in large part the effect of this interfering chromogenic material.

Cahon and Salter (1944) used a relatively simple method of extracting the final red colour with chloroform, the undesirable pigments then remaining in the aqueous layer. Kusuda and Thulin (1953) suggested the use of amyl acetate as a solvent for the final Zimmermann colour. Another means of correcting for the presence of chromogenic material in crude extracts involves the chemical purification of the extract. It has been shown by Talbot et al (1940) that the interfering chromogens occur largely in the non-ketonic fraction of urinary extracts and that the pigments derived from them absorb maximally at about 410 mu. The non-ketonic fraction was separated from the ketonic fraction with the aid of Girard reagent. The use of Girard's reagent, when used, results in a chemical separation, which eliminates the

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chromogenic fraction, but this is a time-consuming procedure.

The possibility of using a colour correction equation for eliminating the errors caused by the interfering chromogens in the crude extracts was suggested by Frazer et al (1941) and studied by Talbot et al (1942). The use of a correction equation has been criticised on statistical grounds by Pincus (1943). However, Talbot et al (1942) and Engstrom and Nason (1943) found good correlation between values determined with the aid of the correction equation and values obtained with the isolated ketonic fractions of the same extracts.

Other methods used for the removal of urinary pigments include chromatographic adsorption and subsequent elution of the steroids. (Dingemanse et al (1946) (1952); Rubin and Rosenkrantz (1952); Lakshmanan and Lieberman (1953); decolourisation of steroids with Norite (Nathanson and Wilson (1943); Wilson (1950). None of these procedures have offered a completely satisfactory solution to the problem of pigments in the urinary extracts. Drekter et al (1952), however, suggested the use of sodium hydroxide pellets instead of the customary aqueous sodium hydroxide solution in the Zimmermann reaction.

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Pincus (1943) found that 17-ketosteroids react with antimony trichloride in acetic acid solution to give a greenish-blue colour which is proportional to the amount of 17-ketosteroids. The reaction was applied to crude extracts of urine and appeared to be rather specific.

The chemistry and biochemistry of steroids have been well reviewed by various workers including Koch (1937) Miller et al (1950); Dorfman et al (1950) Pincus (1950) Lieberman and Dobriner (1948); Serio and Reich (1952); Rosenkrantz and Szego (1953); Hirschmann (1955) Klyne (1957); Dorfman (1957); and Hertzman and Wosettig (1960).

III. Biological properties of androgens

The organs and characters associated with sex and reproduction in mammals, as in other vertebrate groups, consist essentially of the testes, accessory sex glands, and the secondary sexual characters. The secondary sexual characters are associated with the sex of the individual but not directly concerned with the reproductive process. When the gonads become functional at puberty, there is a corresponding development of the accessory reproductive organs and secondary sexual characters. The reverse is seen at

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the menopause in women, the 'climateric' in men, or at the end of castration or breeding season in the lower animals.

Characters of mammals that may be influenced directly or indirectly by the sex of the individuals are the body size, relative size of parts, metabolism, activity, temperament and longevity (Parkes, 1966). Dorfman (1938) defined androgens as "substances which posses characteristic biological activity affecting the secondary sex characters of various male animals". The two major functions of the testis are the production of spermatozoa and the production of the male sex hormones, the androgens.

Enough evidence has accumulated to show that the androgenic hormone of the testis is produced by the interstitial tissue (Moore and McGee (1928); Koerlein et al (1967). The interstitial cells are found distributed among the seminiferous tubules and arise from the mesenchymal cells present in the interstitium. Recent "in vitro" studies by Christenson and Mason (1965) has shown that the seminiferous tubules are also capable of androgen production just like the interstitial tissue.

Spermatogenia, the male germ cells, line the basement membrane of the seminiferous tubules. These cells give rise through a series of divisions to adult

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spermatozoa, a process referred to as spermatogenesis. Mann (1964) described the process of Spermatogenesis as "occuring in waves". Both functions of the testis are under the control of the pituitary gonadotropins - follicular stimulating hormone - (spermatogenesis and luteinising hormone (androgen production). (Lipsett 1966).

The androgens are mainly produced in the testis (Savard et al 1952), but evidences have also been presented to show that they are also produced in smaller amounts in the Ovary (Parkes (1937); Hill and Strong, 1940); adrenal cortex (Reichstein (1936); Pfiffner and North, 1940), and the placenta (Cunningham and Kuln, 1941). The androgens or male sex hormones stimulate the development of the male reproductive organs and secondary sex characteristics (Roberts and Szego, 1953). The extent of their influence and the nature of the response depend on the species. The general effects of androgens may be observed in the growth, stimulation of sexual and non-sexual tissues.

Effects on male sex organs include growth responses of the seminal vesicle, prostate gland, vasa deferentia, epidiymis and penis (Hamilton, 1937); Hamilton and

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Hubert, 1938) Feiner and Rothman, 1939), Bowerman and Lenz (1961); stimulation of spermatogenesis (Ludwig, 1950); Mann (1954). In male rodents, stimulation of growth of the coagulating glands, which furnish the enzyme responsible for the coagulation of a protein to form the vaginal plug. Other effects of the androgens are, induction of ovulation in amphibians and stimulation of growth of the oviducts in birds and of the ovary in rodents (Parkes and Bowens (1934) Turnill and Feltwell, 1947), stimulation of the growth of the clitoris, the female prostate and the development of the mammary glands (Folley and Malpress (1948); Guyton (1961).

The effects of the male sex hormones on non-sexual tissues include, increased vascularity of the tissues. Like the corticosteroids, the androgens are partly responsible for flushing of the skin in the human. They also cause an increase in both red blood cell count and the haemoglobin content of the blood (Danowski, 1962), stimulation of the sebaceous secretion in the skin, and they may also promote tanning of the skin in man, and characteristic pigmentation in fish (Pickford and Atz, 1957), as well as the pigmentation of the plumage and beaks in birds (Noble and Wurm, 1940); (Andrews, 1940).

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The effect on the growth of the comb and wattles in the chicken is one of the bioassay materials for androgens (Gallagher and Koch, 1935). They are also responsible for the growth and distribution of facial and body hair in man. Castration or complete removal of the testes lead to hypertrophy of the adrenals in the male rat which therefore increases the adrenal activity. Other effects of castration include increase in size of the anterior pituitary and increase in Gonadotropin content (Feiner and Rothman (1939); Boach and Wolz, 1946), decrease in size of seminal vesicle and prostate gland (bioassay test) (Kochakian (1938), kidneys, heart, skeletal muscle mass and accumulation of subcutaneous fat (Nelson and Gallagher 1936) (Richter, 1933).

Androgens also affect the enzyme activity and metabolism (Soskins and Levine, 1950). Serum cholinesterase, succinic dehydrogenase and cytochrome oxidase activity in the seminal vesicle, prostate gland decrease after castration (Everett and Sawyer, 1946) Davie et al, 1949) Butler and Shade, 1958): Kalman (1952); Tepperman and Tepperman (1960). The renotropic activity of androgens also involved an increase in the

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arginase activity and decrease in the alkaline phosphatase activity in the kidneys. Castration increases the kidney alkaline phosphatase concentration without affecting the acid phosphatase concentration (Price et al, 1949).

The androgens promote nitrogen retention by increasing protein synthesis and by decreasing the rate of amino acid breakdown. "In vitro" studies have demonstrated that testosterone produces an increased rate of amino acid incorporation into protein of various tissues (Dodds (1957); Litwack and Kritchevsky, 1964). Androgens are also very active in decreasing urea excretion (Eisenberg and Gordan (1950); Shipley and Meyer (1955); Saunders and Drill (1957); Kochakian and Murlin (1935); Hershberger et al, 1955). Androgens inhibit oxygen uptake of liver, kidney, brain and muscle tissues (Eisenberg et al (1959); Hayano et al (1952); Gordan and Elliott (1947); Dorfman (1952). Hayano and Dorfman (1950) suggested that it is the inhibition of the D-amino acid oxidase enzyme system by the steroids that is responsible for the low oxygen uptake of these tissues (Dorfman, 1952).

Castration also leads to reduction of the amount of citric acid and fructose in the semen. Administration of androgens normally brings these to the normal level. The

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quantitative estimation of those substances in the semen had been used as a "chemical indicator test" both for the functional state of the testis and the human prostate. (Grant and Taylor (1950); Beutler and Yeh (1959).

Klaiber et al (1967) demonstrated "in vitro" that the inhibition of mono amino oxidase (MAO) activity by androgens, which results in an elevation of brain norepinephrine levels was responsible for heightened state of behavioural reactivity which facilitates the repetition of behaviours (Beach, 1946, 1947).

(a) Biological assay

In studies of substances or extracts containing androgenic material, the biological assay method is obviously indispensable. Biological methods are needed for the characterisation of a new androgen which must include its physiological action (qualitative), and its relative activity (quantitative). In studies on urinary samples, it was shown by early investigators that a reasonable parallelism exists between the quantity of androgenic material (biological assay) and the amount of 17-ketosteroids (chemical assay) present (Callow and Callow, 1940).

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Ousting (1937) has demonstrated that relatively good correlation exists between the androgenic and 17-ketosteroid titer of normal children's urine. The work of Callow (1938) indicates a close relationship between the two methods in a variety of urines. Holtorff and Koch (1940) have studied this relationship, and although their correlation appears to be poorer than that found by their earlier mentioned workers, it appears to be adequate.

Two types of biological assay methods for androgens based on their ability to stimulate sex characteristics are presently in the use (i) In the comb growth, chicks or capons are injected with the test material, the length, height or lateral area of the comb are used as a measure of androgenic activity. (ii) Barraclian assay. This depends on the weight increase and histological changes in the seminal vesicles and prostate glands of immature or castrated male rodents, which have been repeatedly injected with the test material (Dorfman, 1957).

The capon's comb has served as a test object for the evaluation of androgenic activity since the time of Berthold's (1849) classic experiments. It was served not only as a qualitative measure of androgenic activity

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but has also been used for quantitative studies. As a quantitative tool, many variables had to be discovered and controlled. Such factors as the age at which cocks are caponised, breed of bird, influence of light, initial comb size, route of administration, volume and amount of solvent used as carrier for injections, have been subjected to critical analysis. Methods that have been used for measuring the comb size include direct measurement of the comb size with a millimeter rule and photograph of the comb with subsequent measurement of the areas.

Detailed studies on the capon method have been reported particularly by Gallagher and Koch (1935); Greenwood et al (1935) later modified by McCullagh and Cuyler, 1939). The capon comb growth test is the best known "Hormone indicator test" (HIT) developed for the detection of male sex hormone activity. It depends on the growth response of the comb of a capon following subcutaneous injection or local application of androgens.

The method of Gallagher and Koch (1935) is representative of the capon method, except that white leghorn capons are now preferred since they are more sensitive to androgens than any other breed of cocks (Dorfman, 1948). The birds having been castrated at 6

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to 8 weeks old, and two weeks after, before the administration of the test material, the length and height of the comb was obtained by direct measurement with a millimeter rule. The capons were injected subcutaneously once daily for a period of days (usually 5 to 7 days) and the comb again measured daily and one day after the last injection. Each dose was contained in 0.2ml. of oil (Greenwood et al 1935).

The increase in the length (L) plus the increase in the height (H) was taken as the 'comb size'. The comb size or response was plotted against dosage and the characteristic curve determined. When this increase was related to log dose, straight regression lines were obtained from which relative potency may be obtained by standard methods. Very much greater sensitivity is gained by direct application of the androgen to the capon's comb in an otherwise similar test. This is called the injunction method. Studies of these methods by Deanesly and Parkes (1937); McCullagh and Cuylor (1939), have indicated that this method is more sensitive than the subcutaneous or intramuscular injection methods, although the injunction method is less accurate, (Womack et al, 1931).

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The early observations of Ruzicka (1935); Burrows et al (1937); Danby (1940); Dorfman and Greenblatt (1937), and Frank and Klempner (1937); indicated the advisability of using the chick's comb as the test object of androgen assay. In all two studies mentioned, the test materials may be by injection or applied to the comb daily over a period of days. The end-point consists of the weight of the comb which perhaps represents an advantage over the less exact methods of measurement of capon comb size.

The methods of Hollander (1941) and Frank et al (1942) is the most precise of the chick methods suggested and has been demonstrated to be an adequate method for the determination of androsterone and other urinary androgens. However, the capon comb method has the advantage that each animal serves as its own control, since comb measurements are made before and after hormone administration.

Various mammalian tests usually on rodents, have been employed for the assay of androgens, such as the weight or histological change of the seminal vesicles or the prostate (Moore and Gallagher, 1929), the clitoriod ejaculation test (Price, 1936) the ductus deferens tests

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and the Cowper's gland test (Heller, 1930, 1932) and a pharmacological ejaculation test (Loewe, 1937). Among the various mammalian tests employed, the most important from the standpoint of sensitivity and accuracy has been the weight change of the seminal vesicles and the prostate glands. The studies of Korenchevsky and Dennison (1934) Hisaw et al (1936), Callow and Deanesly (1935), Dulbring and Burns (1935), Greene and Burrill(1940), (1941) are important in the development of these methods.

Mathieson and Hays (1945) have reinvestigated the use of the seminal vesicles of the castrated rat for the assay of androgens. These workers used testosterone propionate and the experimental designs of Bliss (1944). They suggested the use of castrated rats rather than the use of immature rats. Greene and Burrill (1940, 1941) had described a method for the assay of androgens using immature male rats. The disadvantages of using juvenile rats include the use of large numbers of rats since it is difficult to distinguish accurately between different concentrations when the seminal vesicles are removed. Also a large number of rats would be bred in order to have enough immature rats between the ages of 15 to 20 days to carry out the assay. May and Mathieson (1945) also

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found that rats castrated at 26 to 29 days of age can be used for assay 14 to 300 days after castration.

The method of Moore and Gallagher (1929) forms the basis of the mammalian assay tests. Rats are castrated between 21 to 28 days of age and administration of androgens or test substances are started 14 days after castration. Twenty-four hours after the last injection, the rats are killed, the seminal vesicle and the prostate glands are dissected out and weighed fresh to the nearest milligram.

IV. Physical properties of androgens

In the processing of tissues and body fluids for the isolation and identification of steroid hormones and their metabolites, partition between two liquid phases play a predominant role. They are employed in the preliminary separation of these substances from other classes of compounds and also in the more delicate procedures involved in the resolution of mixtures of closely related substances by counter-current distribution and liquid-liquid partition chromatography (Martin (1955) Bush (1961; Heftmann, 1963).

During the past thirty-five years, increasing use has been made of spectroscopic methods for the

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identification, quantitative determination and elucidation of the structure of steroids (Dorfman (1953); Bitman et al (1957). The early acceptance of ultraviolet and visible spectroscopy has been followed by the adsorption infrared spectroscopy, nuclear magnetic resonance, mass spectrometry and more recently gas chromatography. Each of these techniques can offer certain structural information that the others cannot give and for the complete study of a new steroid the joint application of all the techniques has to be considered.

Ultraviolet absorption spectroscopy provides information on olefinic conjugated and aromatic systems and is, where applicable, the most reliable procedure for quantitative measurements. Infrared absorption spectroscopy reveals the nature of the carbon-oxygen, carbon - halogen, aromatic and olefinic groups (Jones et al, 1948, 1952) . Since it was first recognised that optical activity varies with the wavelength of light used, the rotatory dispersion (α) D, over a range of wavelength has been used for the identification of steroids.

Nuclear magnetic resonance (nmr) technique gives information on the character and number of proton containing

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groups. The use of x-ray crystallographic analysis for study of structure and stereochemistry of steroids has also been demonstrated. X-ray diffraction powder pattern of a steroid is highly characteristic and can be used to supplement melting point determination and infrared analysis. Mass spectrometry at a low voltage (10 - 15 v.) affords a method for accurate determination of molecular weight and molecular formula of a sufficiently stable compound with 1 mg. of steroid (de Mayo and Reed, 1956).

Cox et al (1958), have reported a method for the characterisation of steroid side chain based on pyrolytic fission of the side chain and characterisation of the volatile fragment by gas-liquid chromatography. The interdependence of these methods for the study of the physical properties of androgens have been well treated in several recent texts (Brand and Eghinton (1965); Problem (1965); Mathieson (1965); Fleming and Williams, 1966).

Steroids are unique in providing a vast number of different compounds based on the same fairly rigid tetracyclic ring system. These compounds differ from

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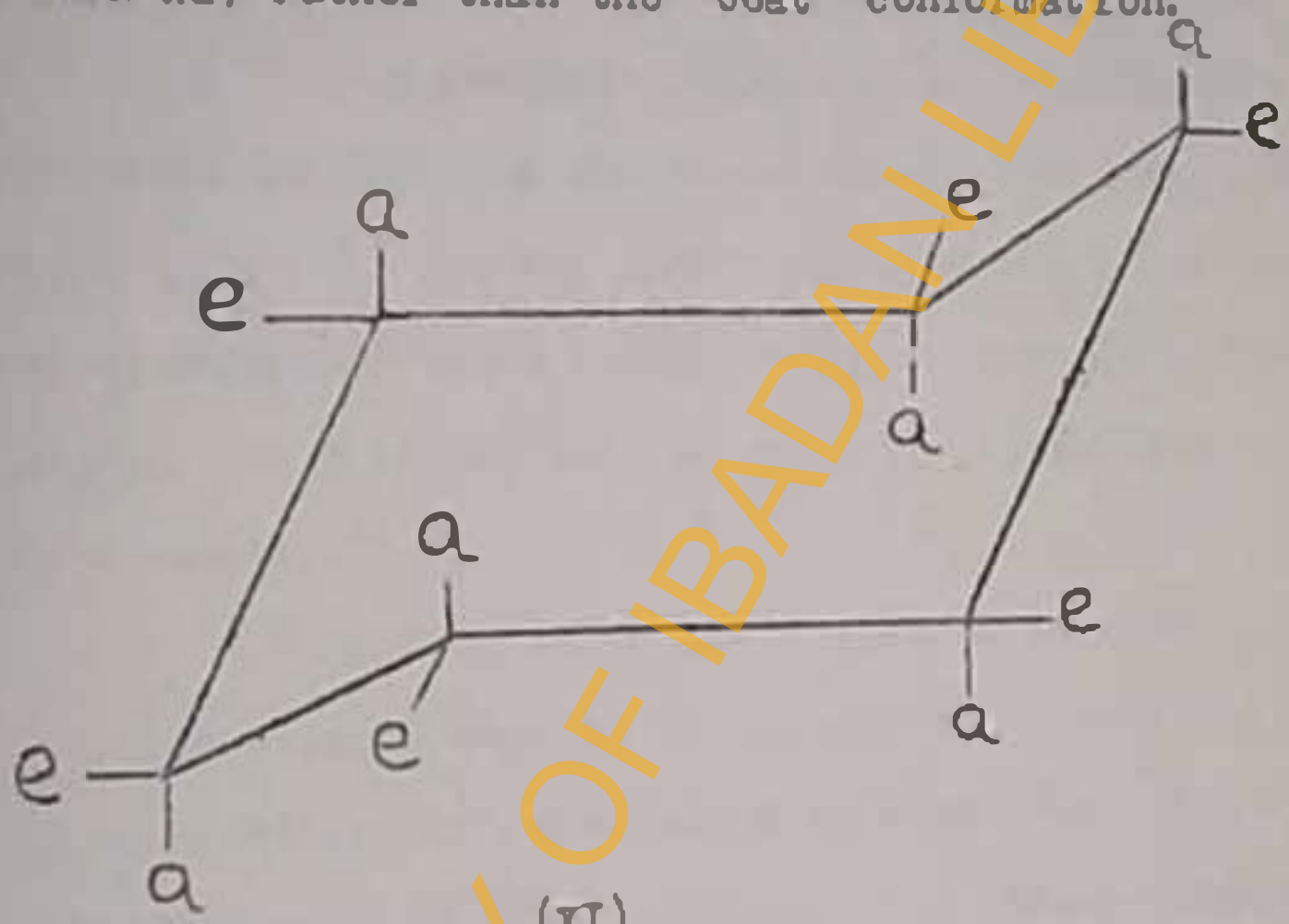
each other mainly in the number, position and stereochemical configuration of their substituents. The basic steroid skeleton consists of a tetracyclic ring system (formula I) in which the fused cyclohexane rings favour the 'chair' (formula xi) rather than the 'boat' conformation.



(XI)

Of the two substituent linkages from each carbon atom in a cyclohexane ring, one is parallel to the three-fold symmetry axis of the ring and is termed "axial" and the other is approximately in the plane of the ring and is termed "equatorial", (a = axialic = equatorial). An equatorial substituent is generally more stable than the axial one.

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The position of a spectral band is given by the wavelength (λ) of the radiation expressed in angstrom (A^{o}, 10^{-8} cm), millimicrons (mu, 10^{-7} cm) or microns (u, 10^{-4} cm). The fraction by which the intensity of the incident radiation is reduced by an absorbent is given by the Beer-lambert law $\log_{10} Io/I = E$, where I_o and I, are the intensities of the incident and transmitted light respectively at the given wavelength, and E is the optical density (Extinction or absorbance).

The absorption curve for a steroid is obtained by plotting optical density or some other absorption coefficient against wavelength or frequency. The infrared spectrum for a solution of a steroid normally shows a series of relatively sharp peaks.

(a) Infrared spectroscopy

Infrared radiation promotes transitions in a molecule between rotational and vibrational energy levels of the ground (lowest) electronic energy state. Infrared spectrophotometers for the examination of steroids are usually double-beam instruments and able to record spectra over the 4,000 - 400 cm^{-1} region.

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The introduction of simplified double-beam infrared spectrophotometers has led to the wider application of infrared methods in biochemical laboratories.

The simpler instruments are adequate for the identification of steroids in fractions obtained by chromatography. Cross (1964), Martin (1966) have surveyed the many commercially available infrared spectrophotometers. Steroids may be examined in the solid state, either as mixtures with a mineral oil (Nujol), or as pressed discs with alkali halides, or in solution in solvents, such as carbon disulphide, chloroform or carbon tetrachloride. Compensation for solvent absorption is obtained by placing a matched cell containing pure solvent in the reference beam of the spectrophotometer.

Since the infrared spectrum of a steroid depends to some extent on the physical state of the steroid, Page (1967), suggested that steroids should, whenever possible, be examined as dilute solutions in carbon disulphide, chloroform or carbon tetrachloride. Normally about 1 to 5mg of a steroid are needed for an infrared measurement, but by using microcells (Jones and Nadeau, 1958), ordinate scale-expansion, a microscope attachment or a beam condensing unit (Kendall (1966);

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Martin (1966), reliable spectra were obtained for 0.01mg. samples.

Infrared spectroscopy is used for the quantitative analysis, the identification and the elucidation of the structure of steroids. The application of infrared spectroscopy in quantitative analysis, depends on the same general principles as those employed in quantitative ultraviolet spectroscopy. In contrast to the relatively few absorption peaks observed in the ultraviolet region for most organic compounds, the infrared spectrum provides a rich array of absorption bands (Jones, 1963).

The identification of individual steroids by comparing their infrared spectra utilises the concept that the infrared spectrum of a steroid is unique. A small change in the stereochemical configuration of one substituent can produce a big change in the spectrum particularly in the so called "finger-point" region between 1450 and 650 cm^{-1} (Rosenkrantz and Zablow, 1953). If the steroid under study has been made previously its identity can be established by comparing its spectrum with that of the authentic sample. In order to facilitate such comparisons, several collection of reference spectra of steroids have been published (Dobriner et al (1953),

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Neudert and Ropke (1965); Dyer (1965).

The importance of infrared spectrometry in the identification and following the metabolism of steroids, ketosteroids and steroid esters has been demonstrated by Purchgott et al (1946); Jones and Dobriner (1949); Jones et al (1950); (1953).

V. Sex hormones in plants

Steroids of some kind are apparently present in all living organisms, with the possible exception of bacteria. A good deal has been learned about the nature and distribution of those steroids that occur in relatively large amounts. Many steroids have by now been found in both plants and animals. Biochemical work on plant steroids has had a rather late start. This was to some extent due to the feeling that steroids could not be very important to plants.

Realizing that minute amounts of many of the steroids in plants have very drastic effects on animals, investigators started to inquire whether they may not serve some functions in plants. That steroids possess physiological activity for the higher plant was in fact demonstrated only after the discovery of oestrogenic

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principles in plants in 1926. The main bulk of our knowledge of the influence of the animal steroid hormones on the vegetative growth and reproductive development of plants has been extensively reviewed by Love and Love (1945).

In animals, the main pathway of steroid hormone synthesis leads from cholesterol via pregnenolone to the androgen then to the oestrogen series (Fig.7) (Berlein et al, 1967). Synthesis of steroids in plants is believed to lead also from cholesterol hence a possibility for the synthesis of androgens (Bergmann (1953); Heftmann (1963). Many plant extracts have been examined (Stoll and Jucker 1955) and a large number of these have been reported to show some oestrogenic activity (Heldtamp and Bonner (1953); Bonner et al, 1963).

Two steroidal oestrogens have been isolated from plants, Betrone (xii) from palm kernel residues, (Butenandt and Jacobi 1933), and Estriol (xiii) from Pollo Catkins and flowers (Skarzynski, 1933). In addition Bradbury and White (1954) have extracted androne, a C_{18} steroid from plants.

The only androgen known to have been isolated from plant so far is 5α - Androstane - 3β, (6 x - 17x-triol (xiv), from the weed Aplopappus heterophyllus -

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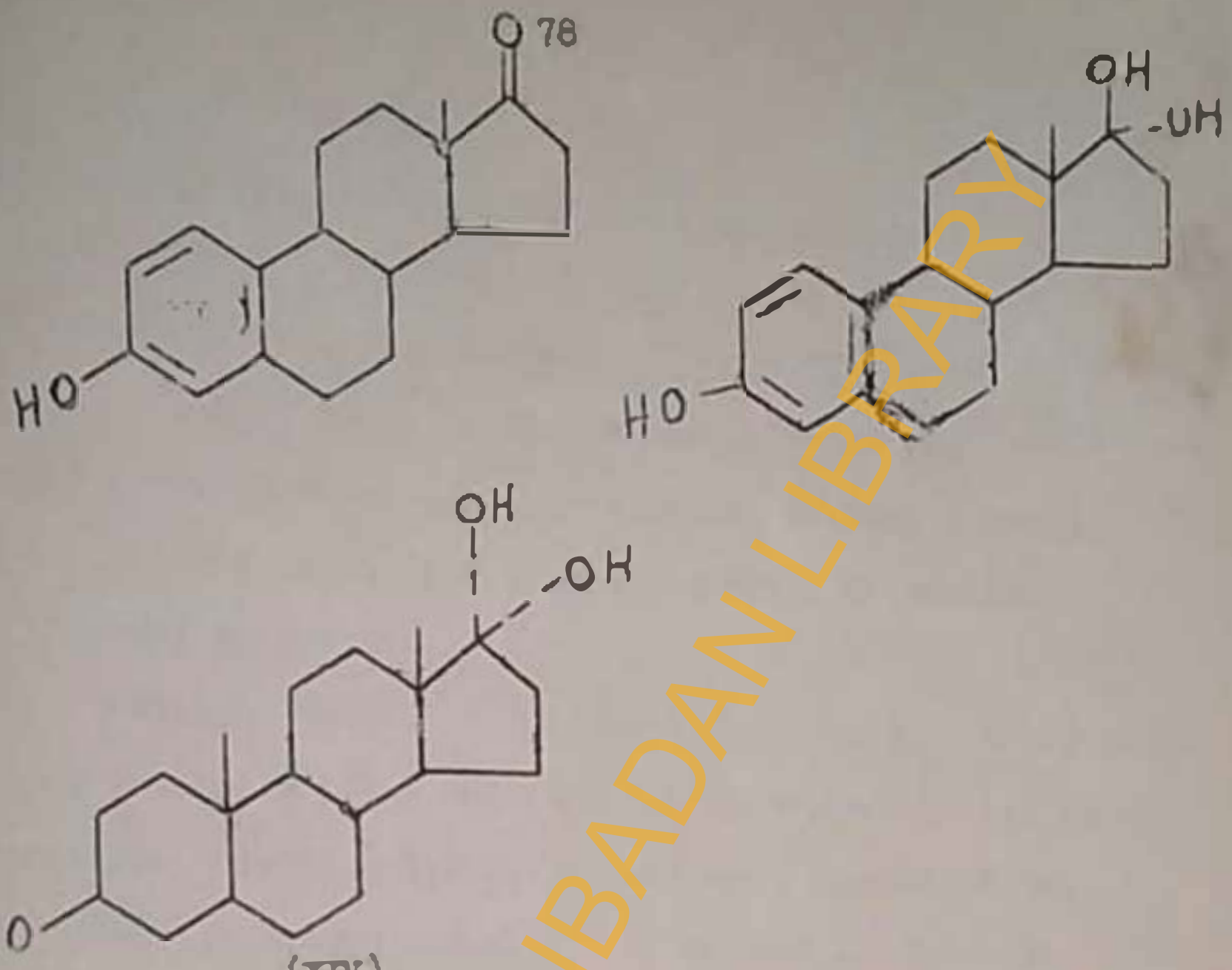

(XIV)

blake by Zalkow et al (1964). The weed is indigenous to the South Western United States. It behaves just like any other androgen although no bioassay experiments were reported by the authors to show its relative potency.

VI. Mechanism of androgen action

It is only in recent years that the nervous control of organs of internal secretions has been studied. It has also become increasingly evident that the latter, through their hormones act on the nervous system. Indeed there are not many functions which are under neural

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or hormonal control exclusively, the great majority are under the overlapping authority of both the endocrine and the nervous system. Thus, in tracing neural pathways, we often find hormonal links and hormal feedback which often lead to the central nervous system and through it rather than directly to an organ of internal secretion.

Berthold (1849), in the first conclusive demonstration of the action of hormones, implicated the nervous system as a target organ, but this thought was not pursued until later years. Brown-Sequard a contemporary of Berthold, never seems to have become interested in possible connections between the nervous system and the endocrine glands although he was one of the pioneers in the investigation of both (Olmsted, 1946).

The central nervous system possesses its own endocrine apparatus which produces and releases hormones (Releasing factors). The production of these releasing factors from the sensual centres in the central nervous system is under the control of both internal and external stimuli. The external stimuli include sight, smell, auditory and others like, salinity or pH of water as in some fishes and amphibians. The internal stimuli is

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represented by the amount of gonadal hormone circulating in the blood.

It is now known that the two distinct but related functions of the testis are under the direct control of the pituitary gland. The pituitary gland produces two types of hormones (gonadotropins) which control the dual function of the testis. The gonadotropins are (i) follicle - stimulating hormone (FSH) and (ii) interstitial cell-stimulating hormone (ICSH) or lutenising hormone (LH). It is clear that these two functions of the testis are not entirely divorced, since ICSH stimulates the interstitial cells of Leydig to produce androgens, which in turn stimulate the germinal epithelium, in collaboration with FSH to produce spermatozoa (Greep et al, 1936; Greep and Fevold, 1937).

The mechanism for release of gonadotropins in mammals was originally conceived as a simple feedback of gonadal steroids to the pituitary gland (Moore and Price, 1932). The detail process involved in the production of androgens has been worked out and is based on a simple feedback system (Fig. 8). Such feedback mechanism can be demonstrated by castration experiments, in which the lack of afferent stimuli alters the normal pattern

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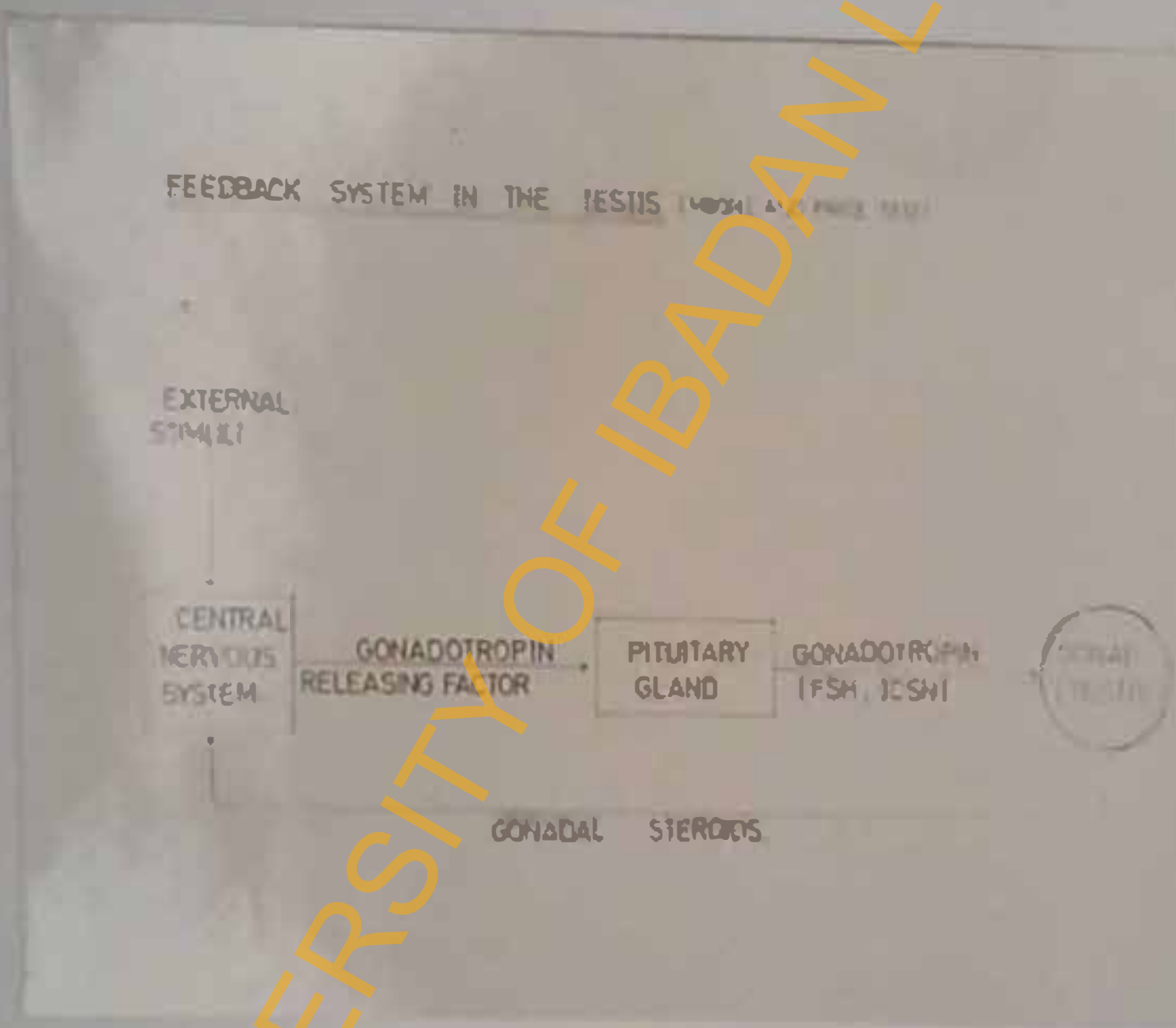
FIGURE 8.



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of increaoc and dccreasc in gonadotrophic activity.

In its simplcat form, thic self controlling systcm nccds to consist of only two componcnts, a hormonol stimulus in thc oppoaitc direction, thc two holding each other in balancc. Thc cxistcncc of such clementary mechanism first proposed by Moore and Pricc (1932) has by no means been disproved (Flerko and Bardos, 1960; Flerko, 1963). Direct evidence that tho ccntral nervous systcm is capable of rccording hormonal atimuli is provided by the fact that castration causes an increased output of gonadotropins while administration of androgens restoro thc balance (Hamilton and Wolfc, 1938); Holler et al 1943). A comparablc dcpression of gonadotrophic potency of tho pituitary is associated with the testicular inhibition accured in normal rats injected with androgen (Moore and Pricc, 1937, 1938). Tho inhibition action of the sex hormones on the testis is probably mediated through their suppression of the production of the pituitary gonadotropins. By inhibiting tho secretion of gonadotrophic hormonca from the pituitary the androgens serve to injure the seminiferous tubules in which spermatogenesis occur in the normal man.

Recent studies of Neumann et al (1970) using some compounds denoted as "antiandrogen" have confirmed the

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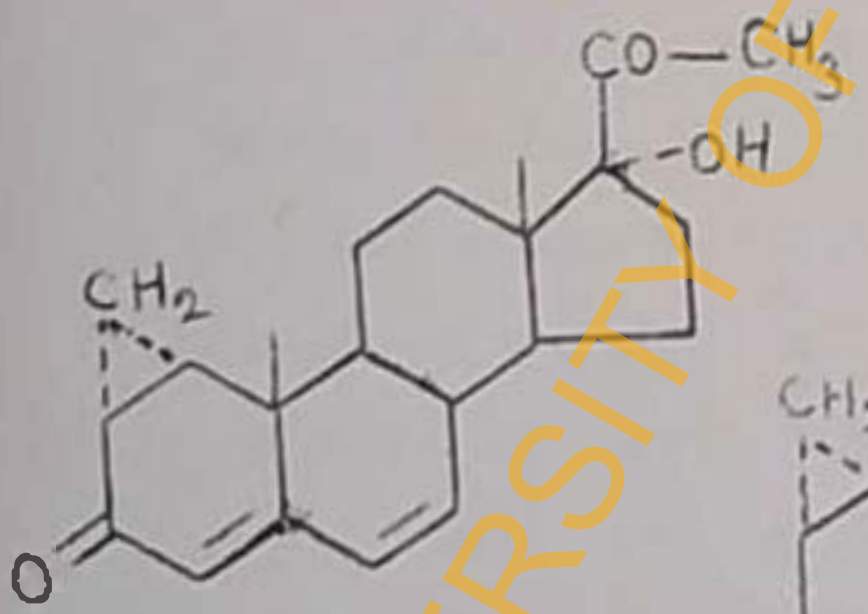
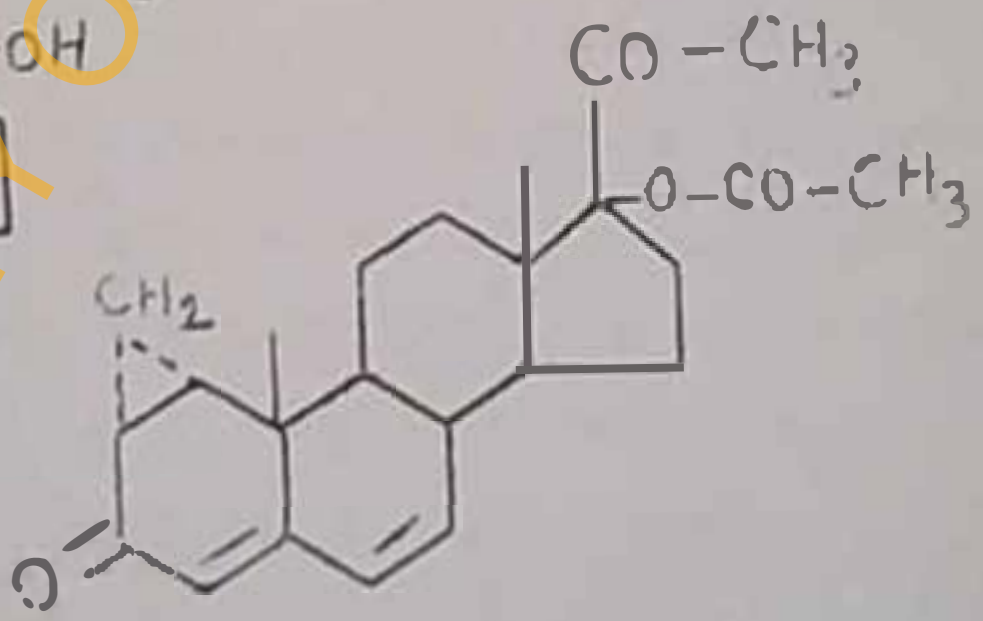
early observations that there is only one feedback mechanism responsible for the production of androgens in the testis. They believed that administration of antiandrogen stimulates an androgen deficit at the sexual centers that regulate the secretion and synthesis of releasing factors for gonadotropin. As after castration, the consequence was an increased secretion of releasing factors for gonadotropin and increased gonadotropin secretion. The two "antiandrogens" used were cyproterone and cyproterone acetate (formula xv) and xvi) Neumann and Elger, 1966).



(XV)



(XVI).

Chronic intrahypothalamic implantation of cyproterone in male rats resulted in specific stimulation of the testes, seminal vesicles and prostate. Bloch and Davidson (1957), concluded that decreases in the amount

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of testosterone reaching specific receptors in the median eminence or in nearby regions activate neural mechanisms whereby gonadotropin secretion is enhanced. The consequence of the increased secretion of gonadotropins was a stimulation of the organs mentioned. The cyproterone released from the implant was insufficient peripherally to inhibit the effect of the increased testosterone produced.

Having established the presence of such a feedback mechanism, the next problem was to determine at which point(s) do the gonadotrophins really act in the synthesis of androgens in the testis. Experiments with certain steroid-forming organs that are known to be able to convert cholesterol to the hormones secreted by those organs include corticosteroids in the adrenal (Long, 1947; Karaboyas and Koritz, 1965), testosterone, androstanediione in the testis (Hall et al, 1963; Hall 1966). Also, the conversion of cholesterol to these hormones is stimulated by the trophic hormones (Karaboyas and Koritz, 1965) and ICSH (Hall and Koritz, 1965. Hall, 1966). Furthermore, these two hormones deplete their target organs of cholesterol (Long, 1947; Bell , 1964). With such evidence it would seem that the source of

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Androgens could be ascribed to cholesterol with some confidence.

It has been proved beyond doubt that both acetate and cholesterol can be converted to steroid hormones (Brady 1951), Sevard and Casey 1964, Karaboyas and Koritz 1965). Before cholesterol is converted to steroid hormones, it must lose part of the side chain at C_{17}. This pathway involves the conversion of cholesterol to pregnenolone. This conversion takes place in the mitochondria from adrenal cortex (Halkerston, et al 1961 and testis (Toren et al 1964). Pregnenolone serves as the precursor of other steroids through pathways which lead eventually to the hormones which are secreted (Samuels 1960).

"In vivo" experiments by Shikita and Hall (1967 a, b) have demonstrated that the action of the gonadotropins is the stimulation of the enzyme 3β-hydroxysteroid dehydrogenase which increase the conversion of pregnenolone to progesterone.

One approach towards the elucidating the mechanism of steroid hormones has been the study of steroid-enzyme inter-actions either in crude cell-free systems or with purified enzymes. The multiplicity of levels

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at which hormones act on biological systems makes them versatile tools for elucidating the regulatory mechanisms involved in the maintenance of homoeostasis.

One of the earliest observed effects of steroid hormones "in vitro" upon enzymatic systems was a general inhibition of oxygen uptake brought about by a variety of steroids (McShan et al (1947); Hayano and Dorfman 1952). It is clear now that pharmacological concentrations of steroids, and perhaps lower and more physiological concentrations of steroids, as well, can induce permeability changes in the mitochondrial membrane, and that part of the decreased oxidative metabolism in the presence of steroids can be explained by such changes. Apart from changes in permeability, androgens specifically inhibit electron transport between $NADH_2$ and cytochrome C (Jensen (1959, Yielding et al 1960; Lindahl and Kochakian, 1961).

A great deal of interest and much intensive work has been focused on steroid stimulated trans-hydrogenase reactions (Ville et al 1960) Kurlock and Talalay (1958) found that partially purified preparation of 3x - hydroxysteroid dehydrogenase

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with dual pyridine nucleotide specificity from rat liver catalysed the transfer of hydrogen between $NADPH_2$ and NAD when androstenedione was added. Baron *et al* (1960) have reported the presence of an androgen activated transhydrogenase in rat prostate. Marks and Banks (1960) reported that glucose - 6 - phosphate dehydrogenase from a variety of mammalian sources is inhibited by 10^{-6} M pregnodione, dehydroisosndrosterone and certain related steroids. Two distinct molecular mechanisms by which steroids can control enzymic activity have emerged from 'in vitro' studies. In some cases steroids behave as co-factors and actively participate in the reaction they control. In other systems steroids control the rate of enzymic reactions without directly participating in the reaction. Steroids appear to regulate enzymic activity by reversibly altering the secondary or tertiary structure of the enzymic protein.

The early demonstration of Bischoff and Pilhorn (1948) that androgens are highly soluble in serum albumin has been shown to produce an increase in the helical content of the protein.

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The existence of some tissues especially the secondary sex organs, depend on the presence of the steroid hormones. There is no doubt that withdrawal or injection of androgens produce effects on protein synthesis within these tissues. Although not usually considered as a step in protein synthesis, the entry of exogenous amino acids into the cell when these amino acids are not themselves biosynthesised intracellularly, is obviously important and, furthermore can be regulated by steroid hormones. Metcalf and Gross (1960) showed that androgen administration increased the rate of x-amino isobutyric acid penetration into the levator ani muscle of the rat.

Hence, it appears that steroids influence the entry of amino acids into cells. One of the theories proposed to explain this hormonal effects was by Christensen (1960) who found that the transport sites for the amino acids are part of a matrix in the cell membrane and that their specificity is determined by the three-dimensional configuration of specific macromolecules in the matrix. He suggested that the hormones alter transport processes by affecting the structure of these transport sites.

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Whether amino acids enter cells from the outside, or are biosynthesised, the first reaction they undergo before polymerising to become proteins is combination with a specific transfer RNA (sRNA) which is catalysed by the amino-acyl-sRNA synthetases. Although this ATP-requiring process has not yet been found to be influenced by addition of hormones "in vitro", several reports indicate that the levels of these synthetases can be regulated by the steroid hormones (McCorquodale and Mueller, 1958; Smith et al, 1961).

On the incorporation of amino acids into proteins Liao and Williams-Ashman (1962) measured the ability of ribonucleoprotein (RNP) particles prepared from rat ventral prostate to incorporate valine from ^{14}C-valine-sRNA into acid precipitable material. It was found that RNP particles from castrate adult rats were much less active than those from normals and that testosterone injection completely restored the incorporating ability to normal. Furthermore, it was found that a synthetic polynucleotide, poly UG, which codes for valine, (Nathans et al 1962 Speyer et al 1962, Maxwell 1962), stimulated valine incorporation

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in the castrate preparation to the extent that it equaled that seen with the preparation obtained from the castrate-androgen treated animals.

Liao and Williams-Ashman (1962) also found that the direct addition of testosterone to either normal or androgen-deficient preparations did not stimulate the incorporation of amino acids into proteins. They concluded that the action of testosterone is on the synthesis or utilisation of messenger RNA. The idea that hormones can regulate protein synthesis by controlling messenger RNA is now an accepted fact. That androgens affect cellular RNA has been shown by Kochakian and Harrison (1962) who showed that castration of male mice decreased the RNA content of kidney, prostate and seminal vesicles and androgen injection restored those values to normal. Earlier evidence by Butler and Achado (1958) had also shown that either castration or stilbestol (androgen inhibitor) administration produced a depletion of RNA content of the ventral prostate of the rat.

The effect of hormones on messenger RNA synthesis has been studied more specifically by Hancock *et al* (1962) who studied the ability of the

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nuclear "aggregate enzyme" of rat ventral prostate to incorporate labelled CTP into RNA. Hancock et al (1962) demonstrated that the rate of incorporation of labelled nucleotide triphosphates into RNA by the aggregate enzyme was greatly decreased in castrated rats but restored to normal in similar animals injected with testosterone.

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CHAPTER TWO

MATERIALS

I. Composition of "Drug" A_1

(a) Boar's penis	1.1gm
(b) Cat's penis	1.7gm
(c) Lion's penis	4.3gm
(d) Horse's penis	7.9gm
(e) Testis of cow	2.5gm
(f) Testis of squirrel	3.2gm
(g) Testis of hedgehog	1.8gm

II. Composition of "Drug" A_2

(a) Gum of Combiphora spp.	5.3 gm
(b) Gum of Acrolobium macrophylla	6.1gm
(c) Tail of the Boa constrictor	1.8gm
(d) Penis of the Ram	1.6gm
(e) Penis of the Rat	0.8gm
(f) Penis of the Elephant	10.3gm
(g) Testis of the Bush-Cow	8.4gm
(h) Root of Carpolobia lutea	15.6gm
(i) Root of Desmodium adscendens	10.1gm
(j) Root of Sarcocephalus esculentus	6.5gm.

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III. Composition of "Drug" A_3

(a) Root of Hanniophyton africanum 10.3gm

(b) Root of Stylosanthes orocta 12.6gm

(c) Root of Paullinia pinata 11.2gm

(d) Root of Cassia siberiana 8.5gm

(e) Root of Phyllanthus Floribundus 8.9gm

IV. Composition of "Drug" A_4

(a) Dried Okro

(b) Musa sapientum var paradisiaca

(c) Tubor of Boerhaavia diffusa

(d) Tuber of Paullinia pinata

(e) Testis of the Boar

(f) Penis of the Antelope

(g) Penis of the Elephant

(h) Penis of the Baboon

(i) Testis of the Bush-cow.

"Drug" A_4 was bought in a powder form, hence the exact amount of the various components is not known.

V. Standards

The following sex steroids were supplied through the department:-

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(a) Testosterone (B.D.H.) 1 1gm

(b) Testosterone propionate (B.D.H.) 1gm

(c) Androsterone (B.D.H.) 1 gm

The following were supplied by courtesy of Mr. A. Sogbesan of the Chemical Pathology Department, University College Hospital, Ibadan:

(d) Dehydro-epi-androsterone (B.D.H.) 0.5gm

(e) Dehydro-iso-androsterone (B.D.H.) 0.5gm

(f) Epitestosterone (B.D.H.) 0.2gm

(g) 5B-Androsten - 3, 17 - dione (B.D.H.) 0.2gm

(h) Androstenedione (B.D.H.) 0.2gm

(i) 11B-OH - Androstenedione (BD.H.) 0.1gm

(j) 5A - Androsten - 3B-17-diol (B.D.H.) 0.1 gm.

VI. Rotary Evaporator

A Gallenkamp rotary evaporator which is thermostat controlled was used to remove alcohol from the various local drugs.

VII. Operating Tables

The operating tables for the bird and the rat were constructed in the department.

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VIII. Olive Oil

All injections were prepared in olive oil (B.D.H.) suspensions.

IX. Absolute ethanol

Since the recommended Ethyl alcohol for 17-ketosteroid determination was not available, the normal 99/100% absolute alcohol (R.R.) was used after purification by the method of Callow et al. (1938). This was used for all 17-ketosteroid determinations.

X. Syringes

1ml. tuberculin disposable syringes (BD.H.) were used for all injections.

XI. Oven

A Gallonkamp B.S. oven which is thermostat controlled was used for drying the thin-layer plates and for developing the plates after spraying with the detection reagents.

XII. Sudan black

The sudan black supplied gave four spots on the thin-layer chromatogram. The spot corresponding to Rf 0.62 was scraped off

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and eluted with chloroform. The chloroform eluate was concentrated and was used as a marker for the location of the positions of the steroids on the chromatogram.

XIII. Chloroform (B.D.H.)

It was redistilled from potassium carbonate immediately before use.

XIV. Ultraviolet lamp

A Hinuvis ultraviolet lamp, pre-set at 365nm, was used to locate the position of steroids on the chromatogram after spraying with the detection reagent, and heating.

XV. Chromatographic equipments

(a) Silica gel

Silica gel G "Merck" was used all through the experiments for thin-layer preparations for the separation of the steroids.

(b) Chromatography column.

A 'quick-fit' column of borosilicate glass (400 x 24mm) fitted with a 2-liter reservoir at the top, with adaptors to a nitrogen source was used for eluting the extracts from the silica gel.

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(c) Solvents.

(i) Chloroform as above.

(ii) Cyclohexane (B.D.H.)

(iii) Ethyl acetate (B.D.H.)

(iv) Carbon tetrachloride (Analar).

(d) Zone Extractor

This was constructed in the department and was used to scrape off spots or zones on the chromatogram under suction.

(e) Spray reagents:

(i) Concentrated sulphuric acid (B.D.H.)

(ii) Methyl alcohol (B.D.H.)

The reagent was a 50% sulphuric acid in methanol. The reagent was sprayed on the plates for the detection of the steroids from a 'quck-fit' spray gun.

XVI. Spectrophotometers

(a) Unicam spectrophotometer S.P. 500.

This was used for all colorimetric estimation of 17-ketosteroids in the samples.

(b) Infrared spectrophotometer.

This was a Perkin-Elmer 137 model. It was used

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for all infrared spectra determinations of all extracts using the sodium chloride cuvette.

XVII. Instruments for (i) Caponisation and (ii) Castration

(i) (a) A sharp scapel:- for making the incision through the skin.

(b) An adjustable retractor - which when inserted into the incision through the skin, between the ribs, spreads apart sufficiently to permit the operator to see clearly and to permit the removal of the testicles.

(c) A small probe with a hook at one end for tearing the skin membrane covering the intestines, the other end of the probe being used to push the intestines away from the testicles.

(d) A pair of forceps - for removing the testicles. Forceps with curved ends being preferred because they permit a clearer view.

(e) Rubber straps with fasteners for holding the legs and wings firmly to the operating board. These were made in the department from bicycle tyre inner tubes.

(ii) (a) A pair of scissors for making the incision through the skin of the scrotum.

(b) A pair of forceps for lifting the testis out of the scrotum while it is being ligatured.

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CHAPTER THREE.

M E T H O D S.

Section I:- Preparation of local virilising drugs.

(1) "Drug" A_1

All the materials listed under its composition in the Chapter under Materials, page 92 , were ground into a fine powder in a grinder. The powder was placed in a brown bottle and covered with 1 litre of absolute ethanol. It was left for 48 hours before use. A fresh preparation is made as soon as the alcohol is exhausted. This preparation is claimed to sustain erection longer during intercourse. It gives a light green colour to the alcohol.

(ii) 'Drug' A_2

The bark of the three roots listed under its composition in Chapter two under materials, page 92). were scrapped off and ground together with the other materials into a fine powder in a grinder. The powder was placed in a brown bottle and labelled "Drug" A_2. 1 litre of absolute ethanol was added to the powder in the bottle. It was left for two days before use.

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As with "Drug"A_1 above, a fresh preparation is made as soon as the alcohol is exhausted. A wine-glassful of this preparation (about 40 ml) represents a daily dose. This preparation is said to have a dual function. First, it serves to thicken the semen, thereby increasing the fertility rate, and secondly, it sustains erection of the penis during sexual intercourse. It has a yellowish coloured solution.

(iii) "Drug"A_3

This drug is made up of roots only. The roots (5 in all) are listed on page 93 . The roots were cut into very small bits and were placed in a brown bottle. The roots were covered with 1 litre of absolute alcohol. It was left to stand for two days before use. It has a deep red colour. A wine-glassful of this preparation represents a daily dose. A fresh preparation is made as soon as the alcohol is exhausted. This preparation is also claimed to sustain erection longer during intercourse but not as effective as "Drug" A_1 or A_2. It was labelled "Drug" A_3.

(iv) "Drug" A_4

Although the composition of this "drug" is known (page 93), it was bought in powder form. The powder

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weighed 15.3gm. It was placed in a brown bottle covered with 1 litre of absolute ethanol. It was left to stand for two days before use. It gave a light brown colouration to the alcohol. It was labelled "Drug" A_4. A wine-glassful of this preparation (about 40 ml.) represents a daily dose. A fresh preparation of this local drug is always made as soon as the alcohol is exhausted. This local drug is claimed to have a dual function like A_2 above, but is acclaimed to be more effective.

Section II - Operations on experimental animals

(1) Sterilisation of instruments

All the instruments were properly scrubbed with vim and rinsed with water. They were placed in a 5-litre beaker. The instruments were covered with about 1 litre of water. The beaker and its contents were placed in the autoclave and steamed for 20 minutes at 15 lb. per square inch pressure.

As soon as the beaker is removed from the autoclave, 10 ml. of antiseptic dettol is added to the contents in the beaker. A new set of sterile instrument is used after every 10 castrations.

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(ii) Animal husbandry

(a) Birds:- The birds on which these observation have been made were kept in a brooder on a floor space of 9.6 by 8.6ft (height 10ft.) It consists of 5 main compartments which are further divided into two by means of fine wire mesh, thus, giving a total of 10 compartments. Each division has its own food tray. All rations used were supplied by the University farm, poultry section. Fresh water is given ad. lib. in troughs. By limiting the number of birds to 4 in any one compartment we avoided overcrowding (which interferes with feeding and favours injury by trampling and thus keep the mortality to a very low level. Of 420 birds used during a period of two and half years, our loss was only 18 or 4.3 per cent.

Each compartment of the brooder contains only those birds which are being treated with the same solution of androgen or local drug material in olive oil. Our brooder is in a house which gets no direct daylight and only a minimum of artificial illumination and daylight during working hours.

The single-comb white leghorn bird is considerably more responsive to the administration of androgens

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than other broods of birds (Dorfman 1948). For this reason, we have adopted the single-comb white leghorn as a standard for our purposes. Our birds are obtained from the University Farm, Poultry Section, Ibadan University. All our birds were 8 10 weeks of age at the start of assay. They are caponised when they are 6 to 8 weeks old.

Since the 'total dose' of assay material is administered as a divided dose rather than in one single application, the duration of assay represents the number of days (seven) on each of which application of assay material is made. Hence, the first application is made, when the birds are 8 to 10 weeks of age and the last when they are 9 to 11 weeks of age.

(b) Rats: The rats were bred in the University pre-clinical animal house. All were the albino wistar breed. They were kept in the department in two batteries. Each battery was made up of 32 cages. Each cage had an area of 6" x 8" x 6"; and a rat was placed in each cage. Each rat was fed on the normal standard diet supplied by the animal house and was supplied with a water bottle.

Special perspex plates were made in the department

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and placed under each cage, to drain their urine into a beaker suspended by a piece of wire to the end of each cage. All the rats were 35 to 43 days old at the start of assay. They were castrated when they were 21 to 29 days old. Hence the first application of assay material was made when the rats were 35 to 43 days old and the last when they were 42 to 50 days old.

(iii) Caponisation

A capon is a normal male cockerel that has been castrated by the removal of its pair of testicles. The process of removing the sexual gland of the male chicken is known as caponisation.

The bird is placed on a sloping operation board under a 100-watt electric lamp. The operation board was made of two boards each 1" x 12" x 20" hinged together at one end. The top one was tilted at 45° to provide a good view and ease of operation.

2-inch nails were driven in each of the sides at 3-inch intervals so that about 1-inch of the nail was left standing out.

The nails were to permit the straps to be fastened for holding the legs and wings. Two rubber straps 1 inch wide and 18 inches long were made from a bicycle tyre

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inner tube. Each strap was provided with slots and screw eye. The birds were usually fasted for 24 hours before surgery. The fasting was necessary so as to reduce the amount of food in the intestine thereby aiding surgery.

The bird was firmly secured to the caponising table by means of the rubber straps. One strap was placed around the wings close to the body, the end was drawn through the ring and the strap fastened to the board by means of slots and the screw eye. The other strap was placed around the legs above the hook joint and when the bird was in position, it was fastened to the suitable slot. In securing the bird, to the board, the left side was placed next to the board, while the right side of the bird faced the operator.

Since the incision was made between the last two ribs of the bird, a few feathers were plucked off from this region, thus exposing these ribs. The surrounding feathers were soaked thoroughly so that they were held back off the operator's way and did not obstruct the view. The whole area was also cleaned with cotton wool soaked in antiseptic dettol to keep the area germ-free.

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Performing the operation: Julls Method (1943).

The scapel was held firmly between the thumb and forefinger of the right hand. The forefinger of the left hand was used to locate the last two ribs, which lie just in front of the hip joint. The skin was also drawn toward the hip and then an incision about 1 inch long was made by drawing the scapel towards the operator; care being taken to follow the curve of the ribs.

The retractors were inserted into the incision and the incision was opened as far as possible without tearing the flesh. The thin membrane surrounding the intestines were torn open with the hook, carefully avoiding the intestines. With the jaws of the forceps slightly open, the lower testicle was allowed to slip into the forceps. When the whole organ had been gripped, the forceps were slowly pulled out of the body cavity with a twisting motion until the organ was severed from the body.

The upper testicle was also removed in a similar manner, care being taken to avoid the posterior vena cava which runs between the two testes. Some of the birds (only 2) died on the table immediately this artery is punctured.

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The incision was closed up with skin sutures which were bought in a sterile form under cayl alcohol. The sutures are also fitted with the necessary needles. They were used once. The skin was also closed up with sterile threads. After the operation, the capons are kept singly in a cage until their incision is properly healed. This takes between six to seven days although the skin heals over in about two days.

However, some of those capons develop 'wind-puffs', which resulted from air escaping from the body cavity between the two ribs into the space beneath the skin which has healed over. Hence, the skin bulged out from the sides. It sometimes spreads to the head.

This was usually corrected by puncturing the "windpuff" with a sharp knife and all the air pressed out. Hence all the birds were checked everyday and proper remedy administered to affected birds. The birds were caponised between 6 to 8 weeks old (Bradley 1950, Sturkie 1954).

(iv) Castration of cats

A large piece of absorbent cotton wool was placed inside a 2-litre beaker. 10 ml of diethyl ether was

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poured into the beaker and quickly covered with the lid. The ether was replenished after every five castrations.

Each rat was weighed on the animal balance and quickly dropped into the beaker. After about twenty seconds, the rat was taken out and placed on its back on the operation board. The board was made of a flat wood (1" x 4" x 6") on four short stands at each corner. Four nails were also provided at the top end of each corner.

Each limb of the rat was firmly tied with a string to the nail at the corners of the board. A small piece of cotton wool soaked in ether and placed in a small funnel was used occasionally to keep the animal under anaesthesia by placing the funnel and its content over the rat's nose; care being taken not to over dose the animal.

With the ventral side of the animal facing the operator, the left hand is used to push the testes into the scrotal sacs. The testes tend to move up into the body cavity when the animal is being anaesthesised. The two scrotal sacs were thoroughly wiped clean with a cotton wool soaked in antiseptic dettol solution.

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Performing the operation - Griffith and Ferris (1942).

A single incision of about ¼ of an inch was made between the two scrotal sacs with the scapel. The forceps with the jaws open was used to grip the testis and the epididymis one at a time. The testis and the epididymis were carefully pulled out of the scrotum and a single ligature was placed around the internal spermatic vessels, the deferential vessels, and the ductus deference.

When the ligature was properly secured, the testis and epididymis were severed from the other parts just above the ligature. The same process was repeated with the other testis and epididymis.

The incision was closed by a suture using a sterile thread. Still under anaesthesia, the animal was carefully placed in its cage. Usually no post-operational problems were experienced with the rats. The rats were normally castrated when they were 21 to 29 days old.

Section III - Measurements

(i) Comb

The initial comb size of each bird was measured by the author throughout the period of all the

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experiments, with a colluloid scale graduated in millimetere. The rule was usually placed directly on the comb.

The measurement of the length of the comb did not usually present any major difficulty. But in the case of the height it was found necessary to use the third barble from the anterior end of the comb as an index of the height.

Gallagher and Koch (1935), defined comb size as the sum of the length and height of the comb. We have also used this as our index of measurement for all the experiments recorded. The comb size of each bird was also measured on the day of first application of estrogen and local drug solution. At the same time, each bird was branded with an individual number for identification to avoid confusion which might otherwise arise in handling a large number of birds. The comb size was also measured daily for the duration of the assay and 24 hours after the last application of the estrogen or local drug. Comb growth was expressed as the sum of the length and height (L + H) of comb in millimetors.

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(ii) Seminal Vesicle and the Prostate gland

24 hours after the last application of the androgen or local drug solutions, the rats were killed with ether. The ventral surface was opened to expose the male accessory organs and bladder. It was usually desirable to cut the body wall laterally so that the two flaps could be laid back, thereby allowing easier access to the seminal vesicle and the prostate gland.

With a pair of forceps and curved iridectomy scissors each of the vas deferens was cut and then the seminal vesicle and the prostate were removed by incising at a point near the base of the bladder. The organs were transferred into physiological saline and all the fats were teased off. They were dried on an analytical filter paper and the organs were weighed separately on a Roller-Smith balance, for each rat, to the nearest milligram.

Section iv - Analysis of urine samples

(i) Collection of urine samples

A special plate was designed in the department. This was placed below each rat cage. The plate drains the rat urine into a 100ml beaker placed at the apex

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of the plate. The beaker was secured to the front end of each cage by a piece of wire.

Since there is considerable diurnal variation in the excretion of 17-ketosteroids, and total 17-ketogenic steroids, the Medical Research Council's Committee on Clinical Endocrinology (1963), recommended a complete 24 hour collections of urine for the estimation of these 17-ketosteroids.

There is little or no reference in the literature to the estimation of 17-ketosteroids in the rat urine. Most of the estimations recorded relate to man and other lower animals. We have decided to estimate the ketosteroids in rats urine to corroborate our results on the weights of the seminal vesicles and the prostate gland.

We found it rather impossible to estimate the 17-ketosteroids in the 24 hour urine because, little or no urine was often obtained within this period, hence, all our estimations were based on the 48 hour urine samples.

(ii) 17-ketosteroids estimation

48 hour urine samples were used for this estimation. No preservatives were added but the samples are often stored in the refrigerator between 2^{o} to 4^{o}

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centigrade if they cannot be used immediately.

The estimations were performed simultaneously with the giving of the injections. The method used for the estimation was a modified form of King's method (1964). The volume of the urine was measured and this ranged between 4 to 7 ml. 4ml of the urine was pipetted into a test-tube. 0.5ml of concentrated hydrochloric acid was added and the tube heated on a boiling water-bath for 10 minutes.

The tube was cooled in an ice-bath and then shaken vigorously with 15ml of ether in a separating funnel. The ether layer was removed and washed with 3ml of 2N-sodium hydroxide followed by washing with two 3ml portions of distilled water. 5ml of the ether extract was evaporated to dryness in a clean test-tube. The dried residue was taken up with 0.2ml of ethanol (absolute).

Freshly prepared Zimmermann reagent (page 118) was added to this residue and allowed to stand in the dark, together with the blank and standard at 27°c for 1 hour. After 1 hour, 3ml of absolute ethanol was added to each tube and mixed well. The extinction of test at three wavelengths, 440, 520, and 600mu, were measured on an S.P. 500 spectrophotometer. Measurements

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at three wavelengths were necessary to correct for interfering chromogens (Medical Research Council Committee on Endocrinology, 1963). In each case, the blank was used to set the instrument to zero.

Correction for chromogens

For the test and the standard, the corrected extinction (E. corr.) was calculated by the relation.

Corrected Extinction

$$(E.\ corr.) = E520 - \frac{E440 + E600}{2}$$

Calculations

Then, the amount of steroid in the test

$$= \left(\frac{Eu}{Es} \times y\right) mg$$

Hence, the steroid in extract in mg per 100 ml

$$= \left(\frac{Eu}{Es} \times y\right) \times \frac{100}{x}$$

Where,

Eu = Corrected extinction for test

Es = Corrected extinction for standard

y = amount of steroid in standard.

x = volume of ether extract used.

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Section v - Volume and preparation of oily solution for assay.

One of the failures in the traditional African way of dispensing medicinal preparations is that there are no standard dosage procedure. However, for the four preparations analysed, the recommended dose was a wine-glassful every day.

The capacity of the wine glass used was about 40ml. As stated earlier, all the four preparations were soaked in 1 litre of ethanol each, on the day of preparation. A fresh prepration was always made as soon as the ethanol was exhausted.

Since the preparation of the doses of all the local drugs follow the same procedure, the method described below applies to each one of the local drugs. The preparation of doses could be described in two sections.

(i) Whole crude "drug":- Since it appears ridiculous to inject the whole of 40ml of crude drug into the animal, the volume of local drug required for a particular experiment was first calculated for the duration of experiment at the rate of 40ml. of local drug per day per rat.

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According to Greenwood et al (1935), 0.2ml of carrier (solvent) gives the best response in this type of assay. Hence, we had to remove the 40 ml ethanol from each dose in a rotary evaporator, the residue was then taken up in 0.2ml of olive oil.

If for instance, we had to inject 4 birds for 7 days with the whole crude drug, and since we now know that the residue from each dose of 40 ml would have to be taken up with 0.2ml of oil later, the total volume of oil which would be required would be 4 x 7 x 0.2 or 5.6 ml. But we normally prepare excess oily solution to provide for all contingencies.

(b) Extract of crude drug

The steroids were first extracted from each crude drug by the method described in section IX of this Chapter. As stated above, 40 ml. of each local drug was usually taken up with 0.2ml. oil for injection. Hence, 40ml. of each "drug" were extracted, and the steroids in each extract were separated and purified individually on a thin-layer chromatogram. Each steroid extract in each of the local drugs could then be taken up with 0.2 ml of oil and used for injection.

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Section VI - Administration of local drugs.

As stated in section V above, the dose required for the duration of an assay was usually prepared at once. It was stored in a brown specimen bottle and always kept in the refrigerator until ready for use. In both the capons, and the rats, injection was begun two weeks after castration (Gallagher and Koch 1935).

The technique for administering the test substances utilises an ordinary 1 cc tuberculin disposable syringe fitted with a disposable hypodermic needle. The solution of the test substance was applied in 0.2ml oil daily for the duration of the assay. The injections were given at approximately the same time each day and all groups of animals received the particular preparations alternatively from the left and right sides. All injections were given subcutaneously.

Section VII - Identification of steroids

Colourless sterols and steroids when treated with strong acids under dehydrating conditions give numerous colour reactions, which are useful for preliminary identification and for quantitative analysis although not completely specific.

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(i) Zimmermann's test

The most widely used colour test for steroids is the Zimmermann reaction, first proposed in 1935 by Zimmermann. The Zimmermann test has undergone a lot of modifications over the years but the basic principle still obtains. This test was used for the quantitative estimation of the steroid sex hormones in the urine of the rat as well as in the various local drugs.

Reagents:-

(a) m-Dinitrobenzene:- A well-crystallised and fairly pure material (B.D.H. melting point 89-89.5°c) was taken on further purified as follows:

20gm were dissolved in 750ml. of 95% ethanol. It was warmed to 40°c and 100ml of 2N-sodium hydroxide solution was added. After 5 minutes, the solution was cooled and 2,500ml of distilled water was added. The precipitated m-Dinitrobenzene was collected in a Buchner funnel, washed very thoroughly with distilled water, sucked dry and recrystallised twice in succession from 120ml and 80ml of absolute ethanol. The melting point was 90.5°c.

The reagent was a 2% w/v solution of m-dinitrobenzene in ethanol. It was stored in a brown stoppered bottle

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the refrigerator. It was stable for 10 days.

(b) Potassium hydroxide (Callow et al 1938)

The reagent solution was 2.5N potassium hydroxide in absolute alcohol. It was prepared as follows:

98g. of potassium hydroxide (B.D.H. 'analar' pellets) were dissolved with mechanical stirring in 500ml absolute alcohol and the solution filtered through a hardened filter paper (Whatman No.50) at the pump. The concentration was checked by titration of 0.5ml with 0.1N sulphuric acid using methylorange indicator. The solution was diluted whenever it was necessary to bring it within the limits of 2.48N and 2.52N.

The solution was stored in a brown stoppered bottle in the refrigerator. It was stable for only 2 days.

(c) Ethanol

The ordinary commercial "absolute" ethanol (R.R. 99-100%) was used. It was further purified by the method of Callow et al (1939) as follows:

The ethanol was treated with 4g. per litre of 2-Phenylene-diamine hydrochloride and allowed to stand in the dark for a week, with occasional shaking. It was then distilled, and the head and tail fractions were neglected. This purified ethanol was used for all

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purposes in connection with the estimation of 17-keto-steroids.

(ii) Sulphuric acid reagent test

Strong reagents have often been used with inorganic absorbents, so that all classes of steroid can easily be visualised through colour or fluorescence reactions. One of the best known is the sulphuric acid reagent which is prepared by mixing equal volume of concentrated sulphuric acid with methanol under a running tap.

The chromatogram was then sprayed with this reagent from a 'quick-fit' spray gun. The chromatogram was allowed to dry for 15 minutes in the air and then heated in the oven at 110°c until the colour had developed. The colour of the spots (zones) in daylight and under ultraviolet light (365nm) were recorded. The heating was for 15 minutes.

(iii) Infrared spectroscopy of steroid extracts

This analytical technique has recently been introduced as a confirmatory evidence for the presence of steroids in fluid and tissue extracts (Dobriner et al (1948); (1949), Rousey (1952); Torpley and Vitiello 1952).

1:1 of the chloroform extracts of steroids extracted from each of the local drugs was evaporated off in a Sol

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of chloroform (analar grade). This was transferred with a syringe into the sodium chloride cells which were then fixed in the instrument.

Infrared light produced by the instrument was split into two beams of equal intensity by a special device in the instrument. One beam was arranged to pass through the sample. The other beam was made to pass through another sodium chloride cell which contains chloroform only.

The spectrum was then scanned by comparing the intensities of the two beams after one had passed through the sample to be examined and the other through the solvent. The scanning was automatic and the finished spectrum consisted of a chart showing downward peaks. To allow for variation in the spectrophotometer, the spectrum was calibrated accurately with known bands of polystyrene whose peak was superimposed at 6.25μm.

Section VIII - Chromatographic methods

(i) Thin-layer chromatography.

Thin-layer chromatography were prepared as reported by Neher (1969). The thin-layer glass plates were rinsed clean with distilled water and dried on a drying rack at room temperature.

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6 (20 x 20cm) glass plates of equal thickness were placed on the Unoplan template. The spreader and the glass plates were cleaned with a piece of cotton wool soaked in acetone to free them completely from grease. The spreader was then placed on the end plate which was at the left hand end of the row of plates. To test whether there was any unevenness where the plates adjoin, the spreader was drawn across them before the suspension was added to the spreader.

50gm of Silica gel G "Merck" was evenly mixed with 100ml of distilled water by vigorous shaking in a stoppered 250ml conical flask for a minute using a mechanical shaker. The resulting slurry was quickly poured into the spreader, set at 275mu, with which it was applied over the 6 clean dry plates set on the Unoplan template.

The coated plates were allowed to dry up at room temperature (25 ± 2°C) for 1 hour in a dust-free atmosphere before being transferred into an air-draught oven maintained at 110°C for 1 hour. The dried plates were then stored in a dessicator cabinet (charged with anhydrous calcium chloride) until ready for use.

With a clean spatula, 2 cm portion of silica gel

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coating were removed along two parallel edges of the chromatoplate before spotting, to reduce solvent lag during development.

Applying the substances

The substances (extracts of local drugs) are usually applied to the plates immediately after the activation of the plates. 0.2 micro liter of the extract was usually applied to the starting line (1cm from the edges) of the chromatogram. The extracts were applied with a graduated micropipette as spots or strips. A hot-air blower was used for drying the chromatogram in between the multiple applications of the extracts.

Chromatographic tank

The Shandon Rectangular (10" x 12" x 10") chromatographic tank with ground-on lid was used. The atmosphere in the tank was made fully saturated by covering the inner walls as completely as possible with filter paper.

The filter paper was fully soaked with the developing solvent before the plates were placed in the tank. Up to twelve plates were usually stalked in a stainless steel holder and run simultaneously in the tank.

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Solvents

Since the rate of migration of a compound on a given adsorbant depends on the type of solvent used, a variety of solvents were tried to determine which are most suitable for the development of the extracts from the local drugs.

Chloroform alone was found to give the best separations followed by cyclohexane: ethyl acetate (1: 1v/v). Alternate use of these two solvents was employed in further purification of steroid extracts by two-dimensional chromatography.

(ii) Column Elution

As soon as the separated zones have been located and marked, the plate was held horizontally and the required zone of adsorbent was taken off with the zone extractor. The absorbant was then transferred into the borosilicate glass column (4.00 x 24 cm) containing chloroform. The absorbed compound was then eluted with 50ml chloroform under nitrogen at 10lb per square inch pressure.

Section II - Extraction of steroids from local drugs

There is a tremendous amount of report in the literature for the extraction of steroids from tissues of plants and animals as well as from body fluids

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and urine samples. Several of these extraction procedures were tried but none gave very encouraging results. Modification of the method proposed by Dobriner and Lieberman (1950), however gave relatively fair results.

This method is described below and was subsequently used for all extractions reported in this thesis. The required volume of the local drug was placed in the rotary evaporator to remove the alcohol. The residue was dissolved in 50ml of water. This was then acidified with 10ml of concentrated hydrochloric acid and boiled for 5 minutes. The hydrolysed solution was cooled quickly in an ice-bath and then transferred into a 250ml separating funnel.

30ml of diethyl ether was added to the solution and shaken vigorously for 3 minutes. The aqueous layer was removed, boiled for 30 minutes and re-extracted with 30ml of ether. This extract was labelled ether extract C.

The ether extract of the hydrolysed solution was washed with (i) 20ml of distilled water (ii) 10ml of 2.5N-sodium hydroxide solution (iii) 20ml of distilled water and (iv) 15ml of distilled water containing a pinch

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of sodium dithionite, labelled ether extract A.

The combined washings from above were placed in a container and acidified with hydrochloric acid and boiled for 1 hour. This was re-extracted and was labelled ether extract B. (Fig.9).

The three ether extracts (A.B.C.) were combined. The ether was evaporated off and the solute was dissolved in an equal volume of chloroform. A sample of this extract was spotted on the thin-layer chromatoplate for the separation of the steroids in the extracts.

Section X - Statistical analysis of results

One characteristic common to all biological material is that it varies, hence the biologist concerned with any quantitative assessments must use statistical methods. Biological assays are methods for the estimation of the nature, constitution, or potency of a material by means of the reaction that follows its application to living matter.

The typical bioassay involves a stimulus applied to a subject to produce a response. The relationship between dose (stimulus) and response is not usually exact and is often obscured by random variations between

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replicate subjects. However, the relationship can be used to indicate the potency of a dose from knowledge of the responses it produces.

The validity of an assay needs to be checked by means of various significance tests, without which the assay is worthless. In order to test whether or not the difference between the mean response of the control animals and that of each of the groups of experimental animal was significant, the student's t-test has been used.

The t and P values are given along with the other measurements as shown in the tables for the comparison of each experimental group with their control. They are considered statistically significant if P is less than 0.05; where P is greater than 0.05, the values are designated as not significant (N.S.). (Fisher 1954; Bailey 1959).

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CHAPTER FOUR

EXPERIMENTS AND RESULTS

Experiment I

Comparative effects of Testosterone and various local drugs on the weights of accessory sex glands in the castrated rat.

Rats from 18 litters were used. A randomised block design (Fisher 1954) was adopted and one animal from each litter, selected at random, was assigned to each of the eleven various doses and two groups of controls.

Altogether, there were thirteen groups of four rats each. All the rats were castrated by the method described on page 107 except one group which served as the normal control.

Two weeks after castration, each group of rats were injected daily for seven days with one of the 7 experimental doses, (0.2mg, 0.5mg, 1.0mg, 2.0mg, 4.0mg, 6.0mg and 8.0mg) of testosterone in 0.2ml of olive oil. Another four groups of castrated rats were also injected with each of the four prepared doses of local drugs (page 115) to be assayed. All injections were administered in 0.2ml of olive oil.

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The last two groups of rats which served as the controls were injected daily for the duration of the assay with 0.2 ml. of oil only.

24 hours after the last injection, all the animals were killed with ether. The entire reproductive tract of each rat was dissected out and the seminal vesicle as well as the prostate gland were weighed to the nearest milligram as described on page 111

Results

The response recorded was the weights of the seminal vesicle and prostate gland expressed as mg per 100 gm body weight. The data are recorded in Table I - III and Figs 10 to 13.

The most striking point in the figures (Fig. 12 and 13) was that with castration there was an immediate atrophy of the seminal vesicle and the prostate glands; especially when the two controls are compared. (Table I). This is in line with the early observations of Van Wagenen (1928), Richter (1933), Mathieson and Haye (1945).

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A quick look at Table II also revealed that the prostate glands tend to be heavier than the seminal vesicles. This confirmed the reports of Korenchevsky (1932 a,b); Butler and Schade (1958).

Table II further revealed that castrated rats injected with 0.5mg and 1.0mg testosterone daily had similar growth to the normal control. Extrapolation of graphs on Figs 10 and 11 showed that about 0.6mg of testosterone per day was required to maintain the prostate gland and approximately 0.9 mg daily application of testosterone was required for the maintenance of the seminal vesicle. (Figs. 10 and 11).

TABLE I

OBSERVATION OF GROWTH OF ACCESSORY SEX GLANDS IN RATS THREE WEEKS AFTER PRE-PUBERTAL CASTRATION.

CONDITION	PROSTATE GLAND MEAN ± S.E.M* (MG)	T	P	SEMINAL VESICLE MEAN ± S.E.M (MG)	T	P
Normal Control	115.0± 4.2	4.536	0.02	86.0± 3.7	4.542	0.02
Castrated Control	17.0± 1.8			10.0± 1.0		

S.E.M. = Standard Error of Mean.

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FIGURE 10.

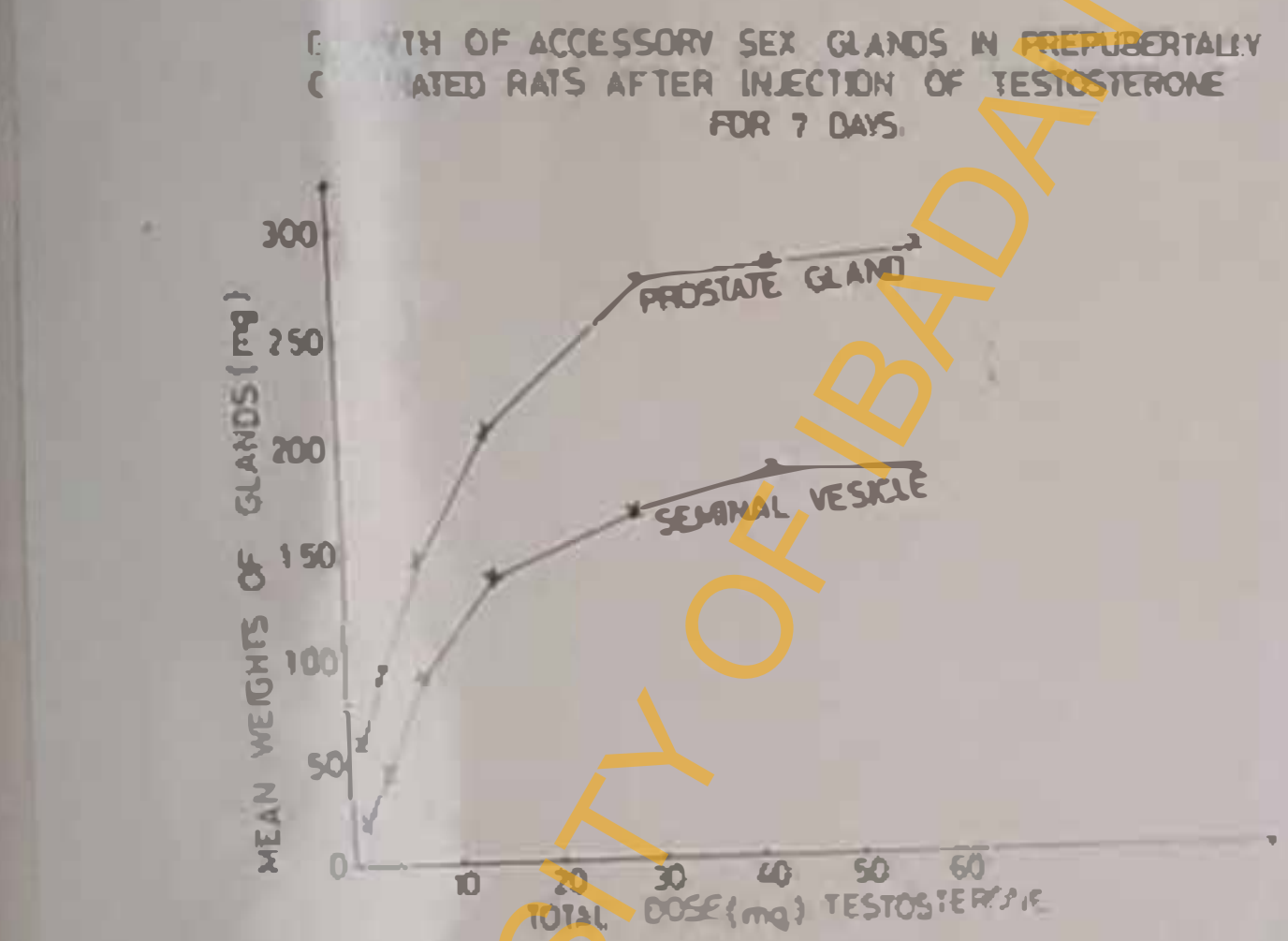

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FIGURE 11.

RELATION BETWEEN GROWTH OF ACCESSORY SEX GLANDS AND LOG DOSE OVER INJECTION OF TESTOSTERONE FOR DAYS.

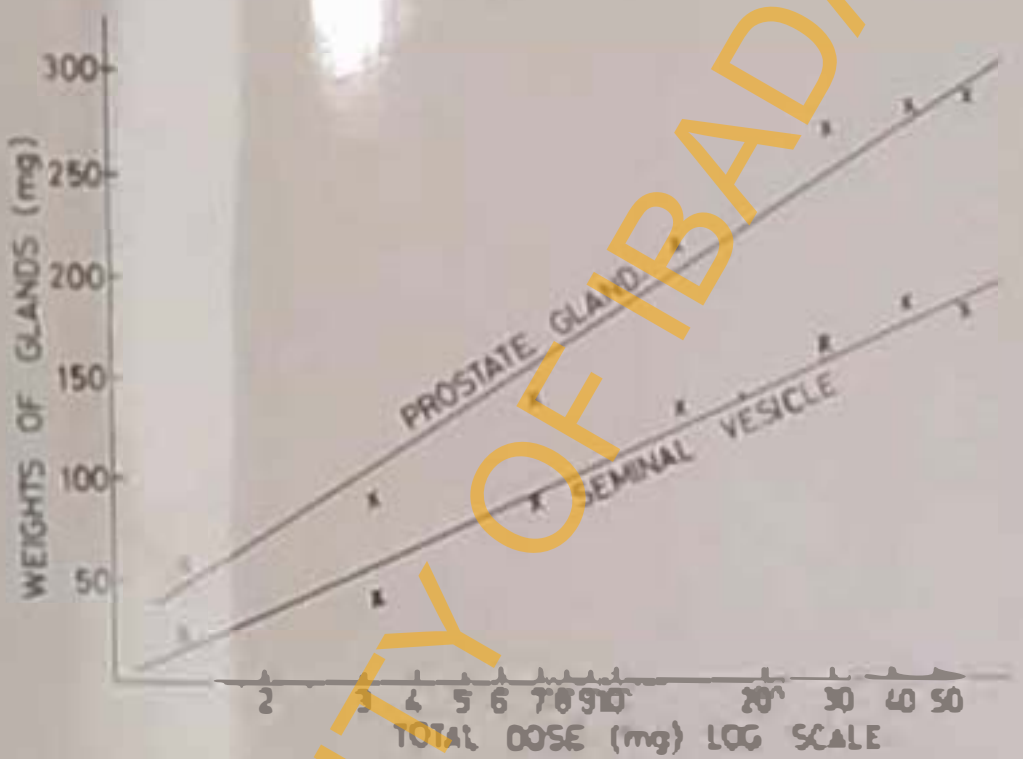

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FIGURE 12.

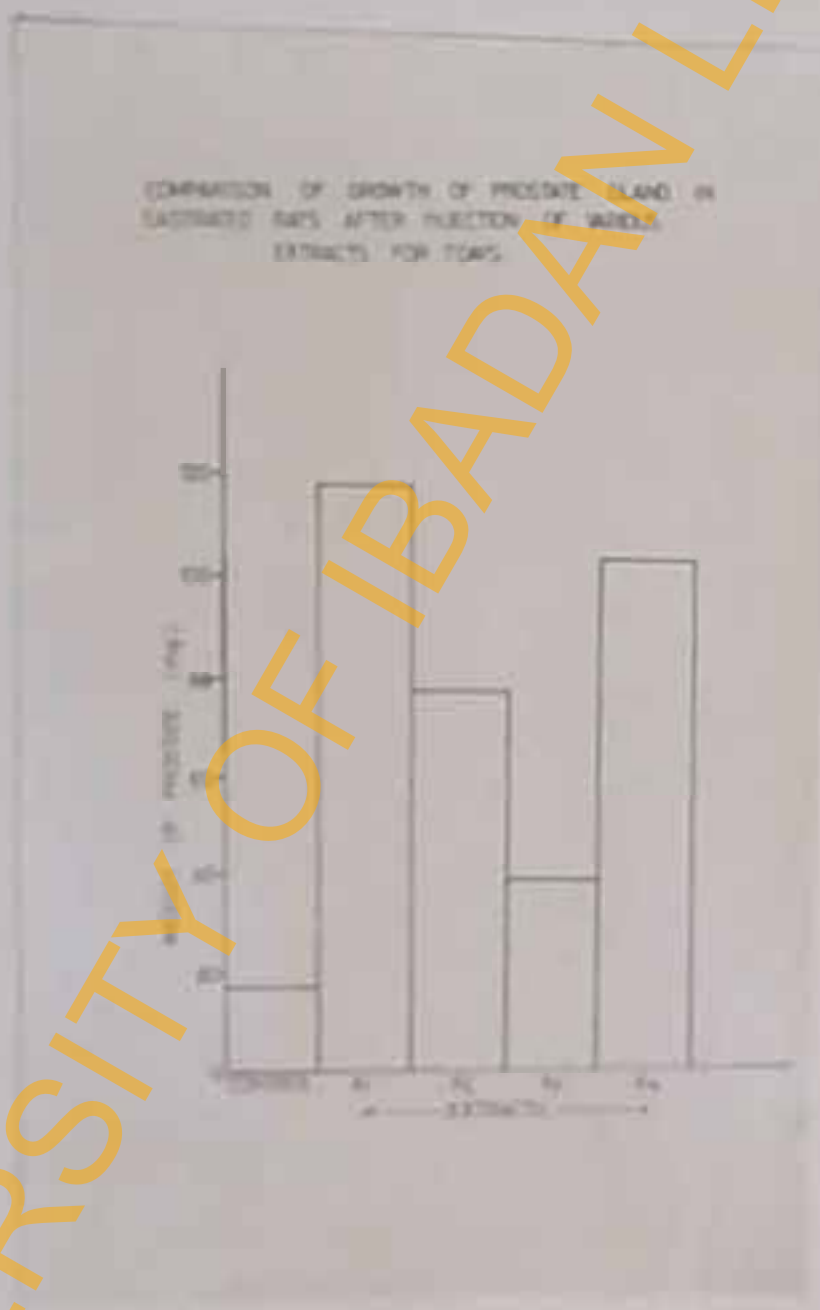

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FIGURE 13.

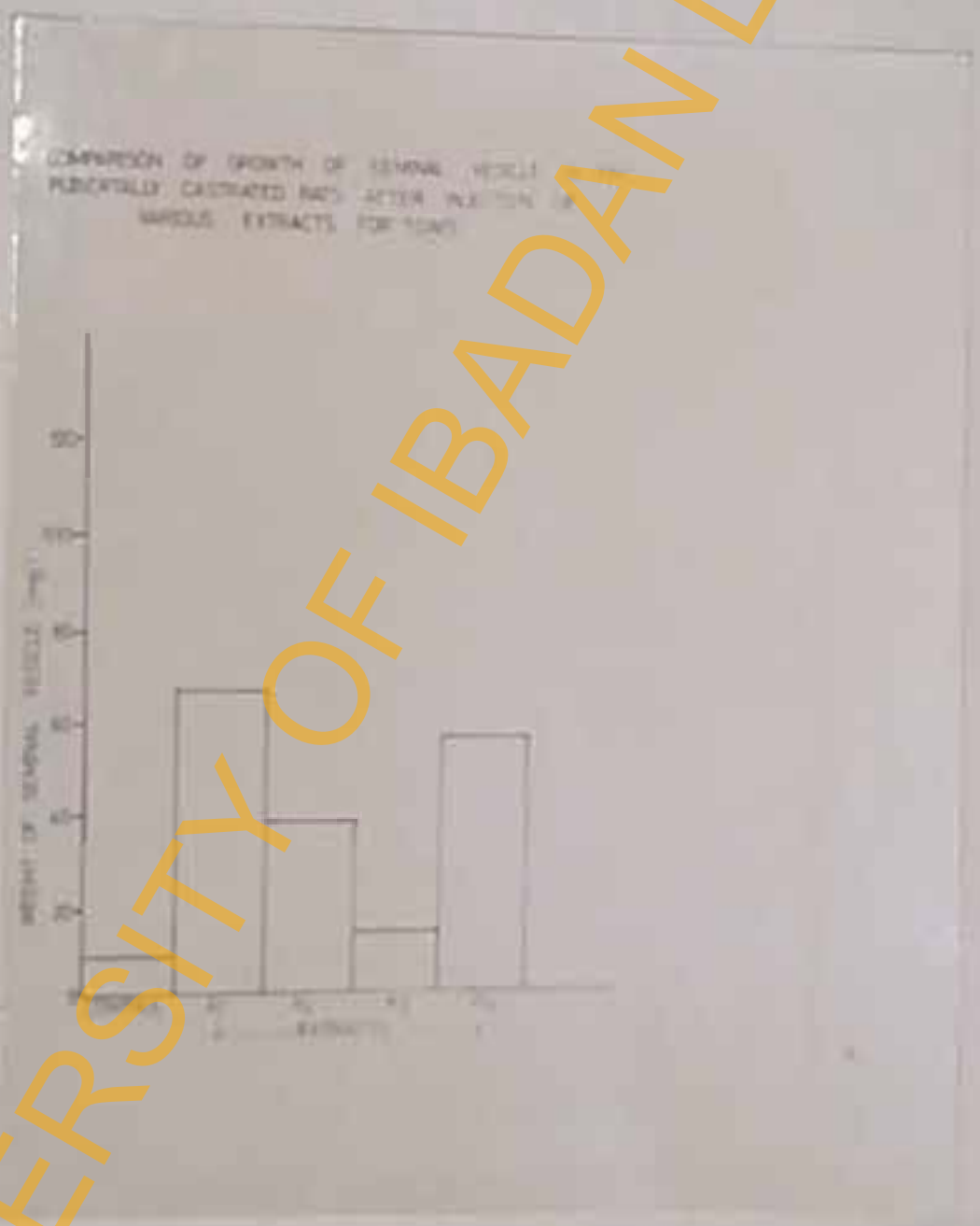

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TABLE II

EFFECTS OF TESTOSTERONE CONCENTRATIONS ON GROWTH OF ACCESSORY SEX GLANDS OF PRE-PUBERTALLY CASTRATED RATS.

(ME) DOSE IN 0.2ML OIL. DAILY	PROSTATE GLAND MEAN ± S.E.M.* (ME.)		A VERSUS B		SEMINAL VESICLE MEAN ± S.E.M. (MG.)		C VERSUS D	
	CONTROL (A)	TEST (B)	T	P	CONTROL (C)	(C) TEST (D)	T	P
0.2	17.0± 1.8	58.0± 2.0	4.547	0.02	10.0± 1.0	20.0± 2.1	4.003	0.02
0.5	17.0± 1.8	91.0± 3.2	4.000	0.02	10.0± 1.0	42.0± 2.2	4.967	0.02
1.0	17.0± 1.8	146.0± 2.2	4.557	0.02	10.0± 1.0	90.0± 1.8	4.613	0.02
2.0	17.0± 1.8	218.0± 3.1	4.662	0.02	10.0± 1.0	139.0± 1.7	4.826	0.02
4.0	17.0± 1.8	278.0± 3.0	4.894	0.02	10.0± 1.0	167.0± 4.5	4.719	0.02
6.0	17.0± 1.8	286.0± 3.6	4.916	0.02	10.0± 1.0	187.0± 3.3	4.976	0.02
8.0	17.0± 1.8	294.0± 2.9	4.942	0.02	10.0± 1.0	186.0± 2.6	5.213	0.02

± S.E.M. = Standard Error of Mean.

The results are mean values of 4 determinations.

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TABLE III

EFFECTS OF VARIOUS LOCAL VIRILISING DRUGS ON GROWTH OF ACCESSORY GLANDS IN PRE-PUBERTALLY CASTRATED RATS.

WHOLE LOCAL DRUGS IN 0.2ML OIL	PROSTATE GLAND MEAN ± S.E.M.* (MG)	T	P	SEMINAL VESICLE MEAN ± S.E.M. (MG)	T	P
CASTRATED CONTROL (0.2ML OIL ONLY)	17.0± 1.8			10.0± 1.0		
A_1	119.0± 4.1	4.548	0.02	66.0± 2.6	4.572	0.02
A_2	77.0± 2.3	4.163	0.05	38.0± 3.6	4.391	0.05
A_3	39.0± 2.5	3.058	N.S.*	12.0± 1.6	3.034	N.S.
A_4	103.0± 3.9	4.362	0.05	58.0± 2.8	4.594	0.02

*S.E.M. = Standard error of mean

*N.S. = Not statistically significant

Results are mean values of 5 determinations.

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The most interesting aspect of the assay was with the local medical preparations (Table III). All the local drugs except "Drug" A_3, gave statistically significant (compared with the castrated control) results when they were injected into the rats, although the responses were very pronounced in "Drugs" A_1 and A_4 (Figs 12and 13).

The results showed the presence of androgenic steroids capable of stimulating the growth of the accessory sex glands in castrated rats in the local drugs. The low response obtained with "Drug A_3 might be due either to the absence or presence of little androgenic hormone.

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EXPERIMENT II

Comparative effects of Testosterone and various local drugs on the growth of the capon's comb.

Cockerels of the pure white leghorn breed castrated between 6 to 7 weeks were selected at random and assigned to each of the eleven doses and two groups of controls. Each group contains 4 birds.

In all, there were twelve groups of four capons each, one of these serving as a castrated control group, and then, there was one group of four uncastrated birds which also served as the normal control. All castrated birds were caponised by the method described on page 106.

Two weeks after caponisation, the 7 experimental doses of standard testosterone were adminiatered to 7 groups of capons daily. Another 4 groups were injected with each of the 4 local drug respectively for 7 days. Injections were given as described on page 117. The two groups of controls were injected daily with 0.2ml of olive oil only; comb measurements were as stated on page 109 and the last measurement was taken 24 hours after the last injection.

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RESULTS:

The data obtained are recorded in Tables IV to VI and Figs. 14 to 16. Table IV shows clearly that there was a significant drop in the size of the capon's comb following castration. This follows the observations of Gallagher and Koch (1930); Greenwood et al (1935); Dingemanse et al (1937); and Dorfman (1948)

Table V shows a continous increase in size of comb with increase in dose of testosterone. The increase was linear (Fig. 14) up to 2.0mg per day. After this the growth rate ceased to be linear and gave a curve as shown in Fig. 15 when the log dose was plotted against the comb size. Tables IV and V compared showed that the growth of capons on daily dose of 1mg of testosterone were similar to those of the normal control. This is probably an indication that 1mg of testosterone is required to maintain the comb size in the white leghorn.

There was also significant increase in the comb size of the capons when they were injected with the 4 local drugs (Table VI). All the 4 local drugs gave some response compared with the controls. 'Drug' A_3 however, gave the largest response followed by 'Drug' A_1 and A_4 (Fig. 16). These responses clearly indicate the presence of androgenic substances capable of promoting comb growth in all the 4 local drugs.

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PIGURE 14.

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FIGURE 15.

RELATION BETWEEN INCREASE IN SIZE OF CAPONS COMB AND THE LOG TOTAL DOSE OF HORMONE GIVEN OVER 7 DAYS.

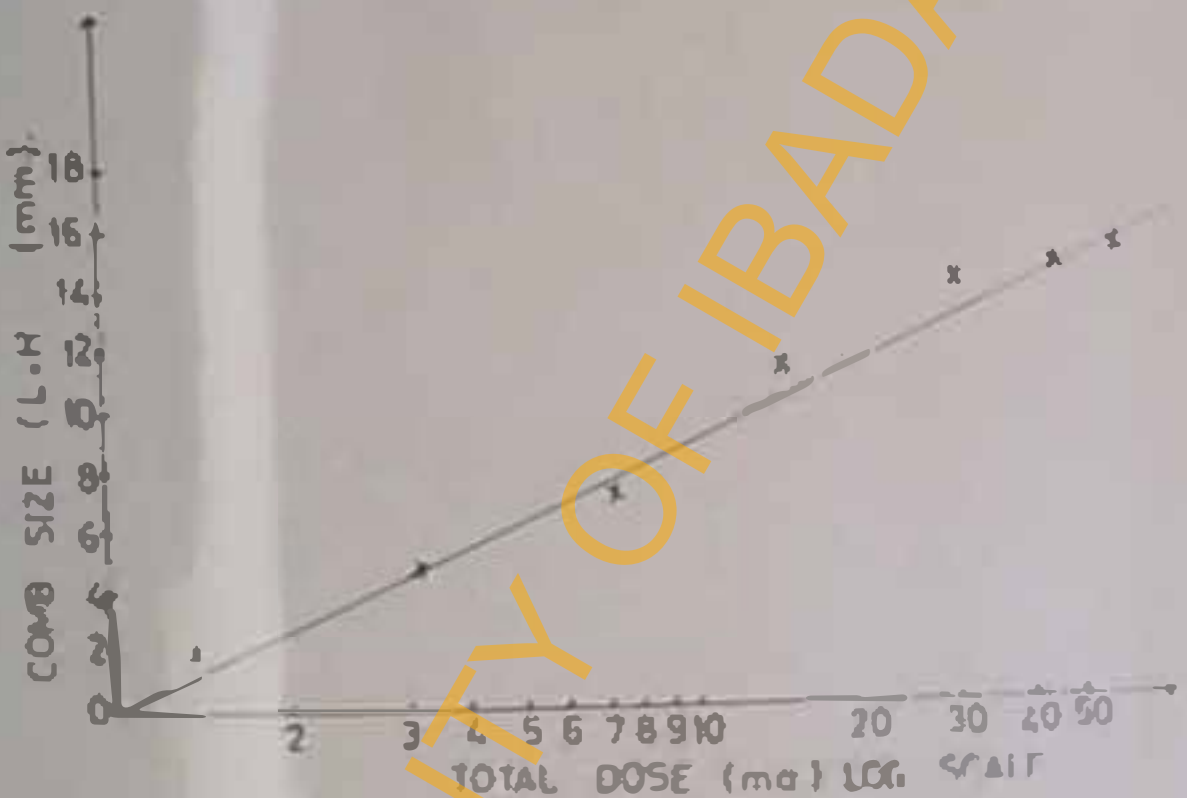

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FIGURE 16.

COMPARISON OF RESPONSE OF CAPONS COMB AND VARIOUS LOCAL DRUG EXTRACTS INJECTED OVER 7 DAYS

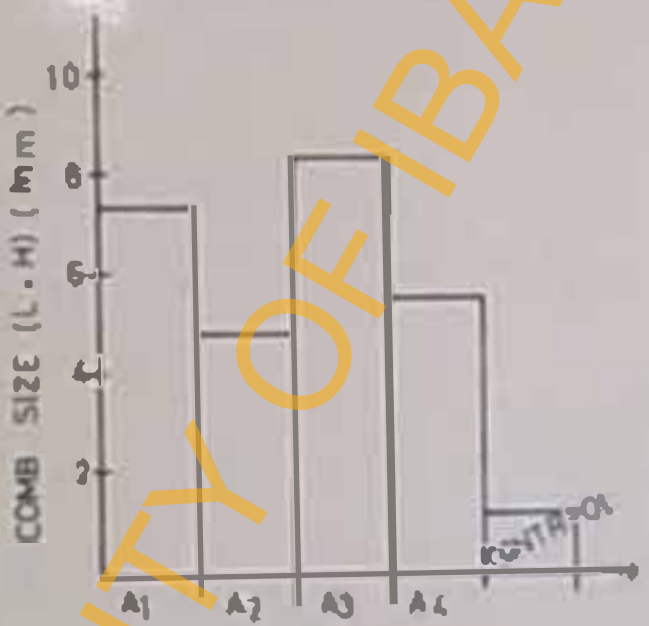

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TABLE IV

OBSERVATION OF GROWTH OF CAPON'S COMB IN COCKERELS THREE WEEKS AFTER PRE-PUBERTAL CASTRATION.

CONDITION	COMB SIZE MEAN ± S.E.M. (MM)	T	P
NORMAL CONTROL	7.93± 0.40	4.438	0.02
CASTRATED CONTROL	1.24± 0.03		

*S.E.M. = Standard Error of Mean.

TABLE V

EFFECTS OF TESTOSTERONE CONCENTRATIONS ON GROWTH OF CAPON'S COMB.

DOSE (MG) IN 0.2ML OIL DAILY.	COMB SIZE MEAN ± S.E.M.* (MM)		A VERSUS B	
	CONTROL (A)	TEST (B)	T	P
0.2	1.24± 0.03	2.23± 0.20	4.281	0.02
0.5	1.24± 0.03	4.87± 0.18	4.432	0.02
1.0	1.24± 0.03	7.39± 0.32	4.540	0.02
2.0	1.24± 0.03	13.84± 0.16	4.891	0.02
4.0	1.24± 0.03	14.41± 0.23	4.899	0.02
6.0	1.24± 0.03	15.03± 0.05	4.952	0.02
8.0	1.24± 0.03	15.62± 0.21	4.963	0.02

* S.E.M. = Standard error of mean.

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TABLE VI

EFFECTS OF VARIOUS LOCAL VIRILISING DRUGS ON THE GROWTH OF THE CAPON'S COMB.

WHOLE "DRUG" IN 0.2 ML.	COMB SIZE MEAN ± S.E.M.(MM)	T	P
CASTRATED CONTROL (0.2ML OIL ONLY)	1.24 ± 0.03		
A_1	7.62 ± 0.45	4.450	0.02
A_2	5.18 ± 0.23	4.429	0.02
A_3	8.25 ± 0.26	4.476	0.02
A_4	5.84 ± 0.17	4.446	0.02

*S.E.M. - Standard error of mean. Results are Mean values of 5 determinations.

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EXPERIMENT III

Comparative effects of testosterone and various local drugs on the excretion of 17-Ketosteroids in the urine of castrated rats.

17-Ketosteroids have been found in the urine of various species although no reference has yet been made to their presence in the rat urine. Karnofsky (1944), found that mice of strains having a high (C_3H) and low (C_{57}) incidence of mammary cancer excreted between 0.46 and 1.93 mg of 17-Ketosteroids per month in the urine. Dekoning et al (1948) found that adult female rabbits excreted between 1.5 and 4.0 mg of 17-Ketosteroids in 48 hours, while Kimeldorf (1948), showed that in male rabbits the average level of excretion was 2.2 mg per 48 hours.

The quantitative pattern of the urinary 17-ketosteroid is important in the understanding of some diseases and measurements of their quantity is often useful in clinical diagnosis and in the interpretation of changes in testicular function (Mason and Engstrom, 1950).

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Littermate rats 6 - 7 weeks old were used and were allocated at random to thirteen groups of 4 rats each. All the rats were castrated by the method described on page 109 , except one group which served as the normal control.

Two weeks after castration, seven groups were injected with one of the 7 experimental doses of testosterone each, for the duration of the assay. Another four groups of castrated rats were also injected with each of the four prepared doses of the local drugs (page 117) to be assayed. All injections were administered in 0.2ml of olive oil. The last two groups of rats which served as the controls were injected daily for the duration of the assay with 0.2ml of oil only.

Urine was collected as described on page 111 at 48 hours interval. The urine samples were analysed as stated on page 112 and estimated colourimetrically by the Zimmermann's test as described on page 118 .

R e s u l t s

It is known that castration, does not completely abolish the excretion of 17-ketosteroids in the urine (Mason and Engstrom 1950). This is because the testes are not the only source of ketosteroids found in the

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TABLE VII

LEVELS OF 17-KETOSTEROIDS PER 48 HOURS, EXCRETED IN THE URINE OF PRE-PUBERTALLY CASTRATED RATS.

CONDITION	17-KETOSTEROIDS (MG/48 HRS. MEAN ± S. E. M.	T	P
NORMAL CONTROL	0.016 ± 0.005	4.591	0.02
CASTRATED CONTROL	0.005 ± 0.001		

Results are mean values of 4 determinations with their standard error.

urine (Parkes, 1937 Hill and Strong, 1940); Pfiffner and North 1940). Table VII confirms these observations. It showed that although there was a sharp drop in the level of these compounds in the urine following castration, yet some were still present although in very low amount.

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TABLE VIII

EFFECTS OF TESTOSTERONE CONCENTRATIONS ON THE LEVELS OF 17-KETOSTEROIDS PER 48 HOURS EXCRETED IN THE URINE OF PRE-PUBERTAILY CASTRATED RATS.

MG TESTOSTRONE IN 0.2ML OIL DAILY	17-KETOSTEROIDS (MG/48 HRS.) MEAN ± S. E. M.*	T	P
CASTRATED CONTROL (OIL ONLY)	0.005 ± 0.001		
0.2	0.013 ± 0.001	4.463	0.02
0.5	0.027 ± 0.001	4.729	0.02
1.0	0.044 ± 0.008	4.736	0.02
2.0	0.058 ± 0.002	4.751	0.02
4.0	0.080 ± 0.009	4.803	0.02
6.0	0.087 ± 0.003	4.805	0.02
8.0	0.096 ± 0.005	4.827	0.02

* These results are mean values of 4 determinations with their standard error.

Table VIII shows a direct relation between increase in dose of testosterone and 17-ketosteroid excretion tending to a maximum after the 2.0 mg dose (Fig 17 and 18).

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FIGURE 17.

LEVEL OF 17-OXOSTEROIDS IN URINE OF PRE-PUBERTALLY CASTRATED RATS AFTER INJECTION OF VARYING DOSES OF TESTOSTERONE.

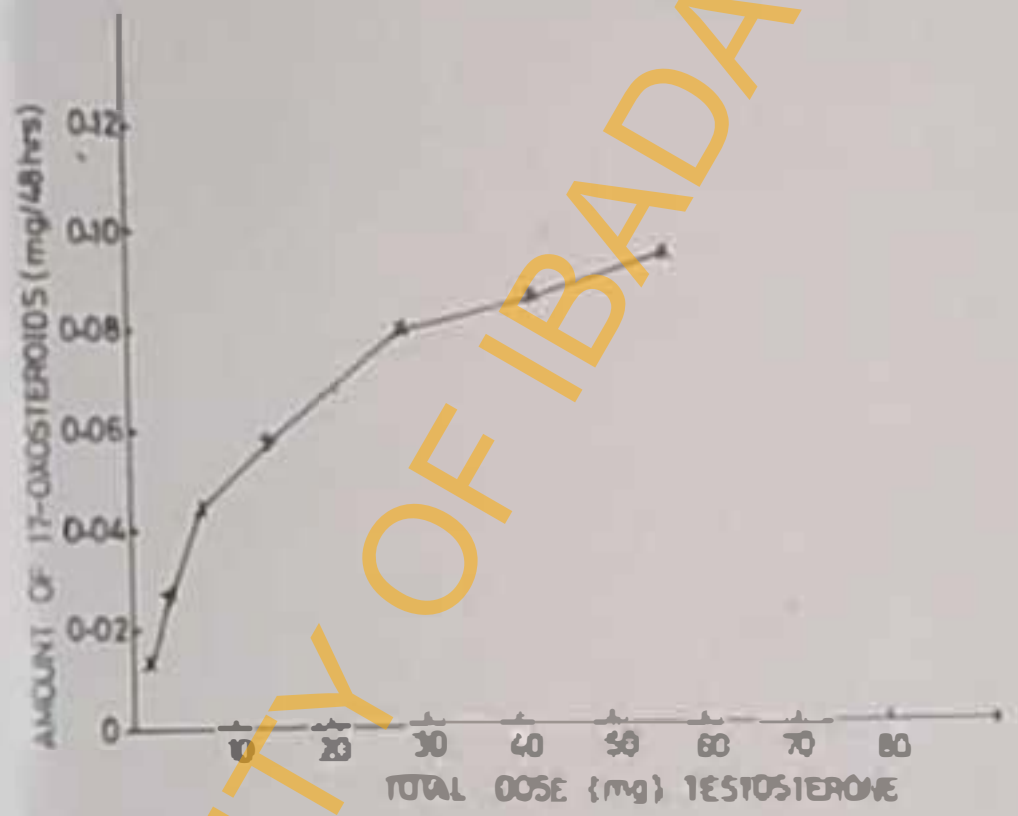

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FIGURE 10.

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This corresponds to the pattern of changes in the growth of the accessory sex glands in the rat (Table II) and increase in the capon comb (Table V). Comparison with Table VII shows that between 0.2 and 0.5mg of testosterone daily would be required to produce the normal 17-ketosteroids in the urine of immature rats.

Administration of the local drugs to the castrated rats also caused the excretion of substantial amounts of 17-ketosteroids in the urine of rats. Table IX indicates the presence of sex hormones in the three local drugs designated A_1, A_2 and A_4.

TABLE IX

EFFECTS OF VARIOUS LOCAL VIRILISING DRUGS ON THE LEVELS OF 17-KETOSTEROIDS PER 48 HOURS, EXCRETED IN THE URINE OF PRE-PUBERTALLY CASTRATED RATS.

"DRUG" I.M. 0.2ml	17-KETOSTEROIDS (MG/48 HOURS) MEAN ± S.E.M.	T	P
CASTRATED CONTROL (OIL ONLY)	0.005 ± 0.001		
CASTRATED CONTROL (OIL ONLY)	0.016 ± 0.004	4.591	0.02
A_1	0.015 ± 0.007	4.568	0.02
A_2	0.005 ± 0.001	2.941	N.S*
A_3	0.024 ± 0.006	4.716	0.02
A_4			

*N.S. = Not statistically significant.

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FIGURE 19.

COMPARISON OF LEVELS OF 17-OXOSTEROIDS IN URINE OF CASTRATED RATS AFTER INJECTION OF VARIOUS EXTRACTS OF LOCAL DRUGS.

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The response from A_3 was very low and compared with the castrated control was not statistically significant. This result compare favourably with those obtained in Experiment I (Page 137 Table III) and leads to the same conclusion that androgens are present in A_1, A_2 and A_4 with little or no androgens in A_3 (Fig. 19).

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Separation, purification, identification and estimation of steroids in the various local drugs.

Steroids were extracted from each of the 4 local drugs by the method described on page 124 (Fig. 9). The extracts were concentrated and spotted on thin-layer chromatoplates as stated on page 123).

Chloroform only was used for the development of the plates. The prepared sudan black (page 123) was used as a marker for locating the position of the various androgens on the chromatoplates.

The Rf and Rs (reference to sudan black) values were calculated for the standards (Table X and Table XVI), and the spots obtained with the extracts of each local drug (Table XI to XIV and Table XVII a, b, c and d).

"Drug" A_1 gave 5 spots; A_2 gave 5 spots, A_3 gave 9 spots and A_4 gave 4 spots. A large number of plates were prepared and each of the 4 local drug extracts was applied to the plate. About 200 scrappings were collected for each of the spots identified using their Rs values to determine their positions on the chromatoplate. The scrapping from each spot was eluted with chloroform in a colum

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under pressure (page 124). In all, 23 eluates corresponding to each of the separated spots were obtained and were numbered 1 to 23 (Figs. 20 to 24).

PURIFICATION.

The eluates were further purified by two dimensional chromatography as follows:

(a) All spots with Rf values between 0.10 - 0.70 were assumed to run fairly well in chloroform. The spots include 2 - 4(A_1); 7 - 9(A_2); 12 - 17 (A_3); 21 and 22 (A_4). The extract of each spot was applied to the chromatoplate and developed by two dimensional chromatography in the following solvents (i) Chloroform only (ii) Cyclohexane; Ethyl acetate (1:1). The position of each spot was located with reference to sudan black Figs 25 to 28). In order to find out if other separations were obtained from each spot, two plates were developed at the same time. One of this was sprayed with 50% sulphuric acid in methanol. Fortunately, only one spot could be identified for the extract of each spot. The Rf and Re values of each spot are shown in Table XVa. (b) All spots with Rf values between 0.71 - 0.99 (those running almost the solvent front) and these include spots 5 (A_1);

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FIGURE 20

THIN-LAYER CHROMATOGRAM OF THE FOUR LOCAL DRUGS SPRAYED WITH 50% SULPHURIC ACID IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

S = Sudan black
A_1 = Local drug labelled drug A_1
A_2 = Local drug labelled drug A_2
A_3 = Local drug labelled drug A_3
A_4 = Local drug labelled drug A_4.

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FIGURE 21.

THIN-LAYER CHROMATOGRAM OF "DRUG A"
SPRAYED WITH 50% SULPHURIC ACID
IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

EXTRACT FUNS

.5

.4

.3

.2

.1

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FIGURE 22.

THIN-LAYER CHROMATOGRAM OF "DRUG A_2"
SPRAYED WITH 50% SULPHURIC ACID
IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

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FIGURE 23.

THIN-LAYER CHROMATOGRAM OF "DRUG A"
SPRAYED WITH 50% SULPHURIC ACID
IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

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PLATE 24.

THIN-LAYER CHROMATOGRAM OF "DRUG A"
SPRAYED WITH 50% SULPHURIC ACID
IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

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FIGURE 25.

TWO-DIMENSIONAL CHROMATOGRAM OF SPOTS SPRAYED WITH 50% SULPHURIC ACID IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

EXTRACT NUMBER

.4

.3

.2

.9

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FOLIO 25.

EXTRACT NUMBER

TWO-DIMENSIONAL CHROMATOGRAM OF ROOT SPRAYED IN 50% SULPHURIC ACID

IN METANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

. 18

. 17

. 7

. 8

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FIGURE 27.

TWO-DIMENSIONAL CHROMATOGRAM OF SPOTS SPRAYED WITH 50% SULPHURIC ACID IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

EXTRACT NUMBER

.16

. 15

. 14

. 13

. 22

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FIGURE 28.

TWO-DIMENSIONAL CHROMATOGRAM OF SPOTS SPRAYED WITH 50% SULPHURIC ACID IN METHANOL AND VIEWED UNDER ULTRAVIOLET LIGHT.

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6 (A_2); 19 (A_3) and 20 (A_4), less polar solvents were used (i) Benzene; Ethanol 19: 1 (ii) Cyclohexane: Ethyl acetate (1:1). Their Rf and Rs values are recorded in Table XVb.

(c) For spots 1 (A_1); 10 (A_2); 11 (A_3); and 23 (A_4), those running at the origin (Rf 0.00 - 0.09), the solvents (i) Cyclohexane: Ethylacetate (2:8) (ii) Benzene: Ethanol (1:9) were used. Their Rf values are recorded in Table XVc.

IDENTIFICATION

Since there is no specific test for the identification of steroids, we have combined the use of two well known colour tests for steroids for their preliminary identification. The first was the Zimmermann's reagent. The plates were sprayed first with a 2% m-dinitrobenzene solution in absolute ethanol. The plates were left to dry so that the alcohol could evaporate off.

The plates were then sprayed again with a 2.5N alcoholic potassium hydroxide solution and heated in an oven at 70 - 100°C until maximum development of the colours were produced (usually 15-20 minutes. The colours obtained with the extracts are recorded in the tables along with their Rf and Rs values.

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(Tables XVI to XVIIId).

The second detection reagent was the sulphuric acid reagent which was prepared by mixing equal volumes of methanol and concentrated sulphuric acid together under ice-cold condition. The plates were sprayed with this reagent and the plates heated in the oven at 100°C for 10 minutes. A variety of colours were obtained in the daylight and when the plates were viewed under the ultraviolet light. The colours obtained with the standards and extracts are recorded in Table X to XIV).

Infrared spectroscopy was used to confirm the various local drug extracts (page 120). The infrared spectrum obtained for each of the extract was compared with those of the standards as well as with those recorded in the text by Dobriner et al (1953) and by Roberts et al (1958). The spectrum for each steroid in the extracts are shown in Figs 29 to 37 .

Estimation

Having identified the various steroids in each extract of the 4 local drugs, the amounts of these steroids were estimated colourimetrically using the Zimmermann's test.

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FIGURE 29.

INFRARED SPECTRA OF SPOT 2 IN "DRUG A."

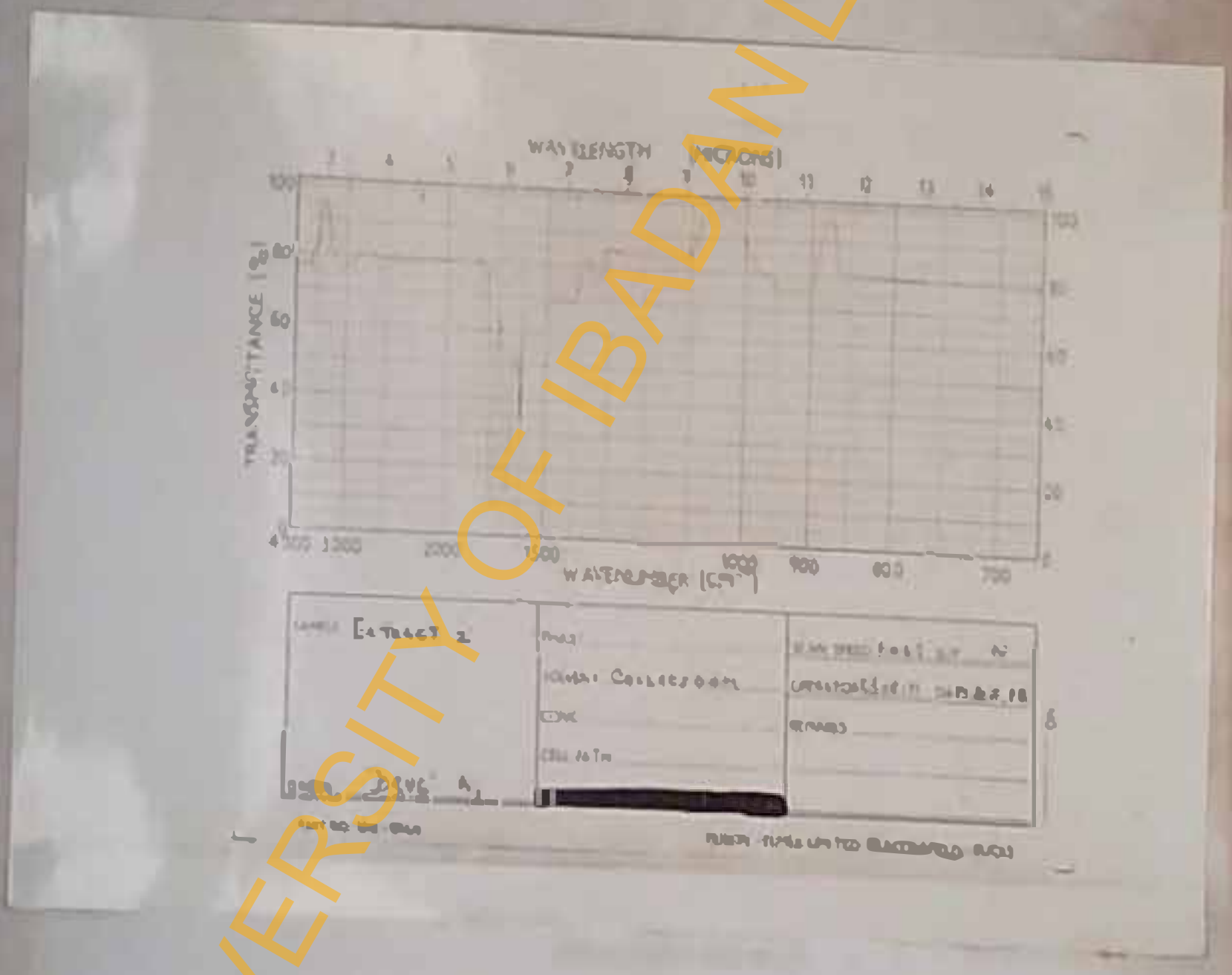

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FIGURE 30.

INFRARED SPECTRA OF SPOT 3 IN "DRUG A_1"

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FIGURE 34.

INFRARED SPECTRA OF SEPT 8 IN TRIS A2"

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FIGURE 12.

INFRARED SPECTRA OF SPOT 2 IN "TRIS A_2"

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FIGURE 33.

INFRARED SPECTRA OF SPOT 1L IN "DRUG A_3".

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FIGURE 3h.

INFRARED SPECTRA OF SPOT 15 IN "DRUG A_3".

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FIGURE 35.

INFRARED SPECTRA OF SPOT 16 IN "DRUG A_3".

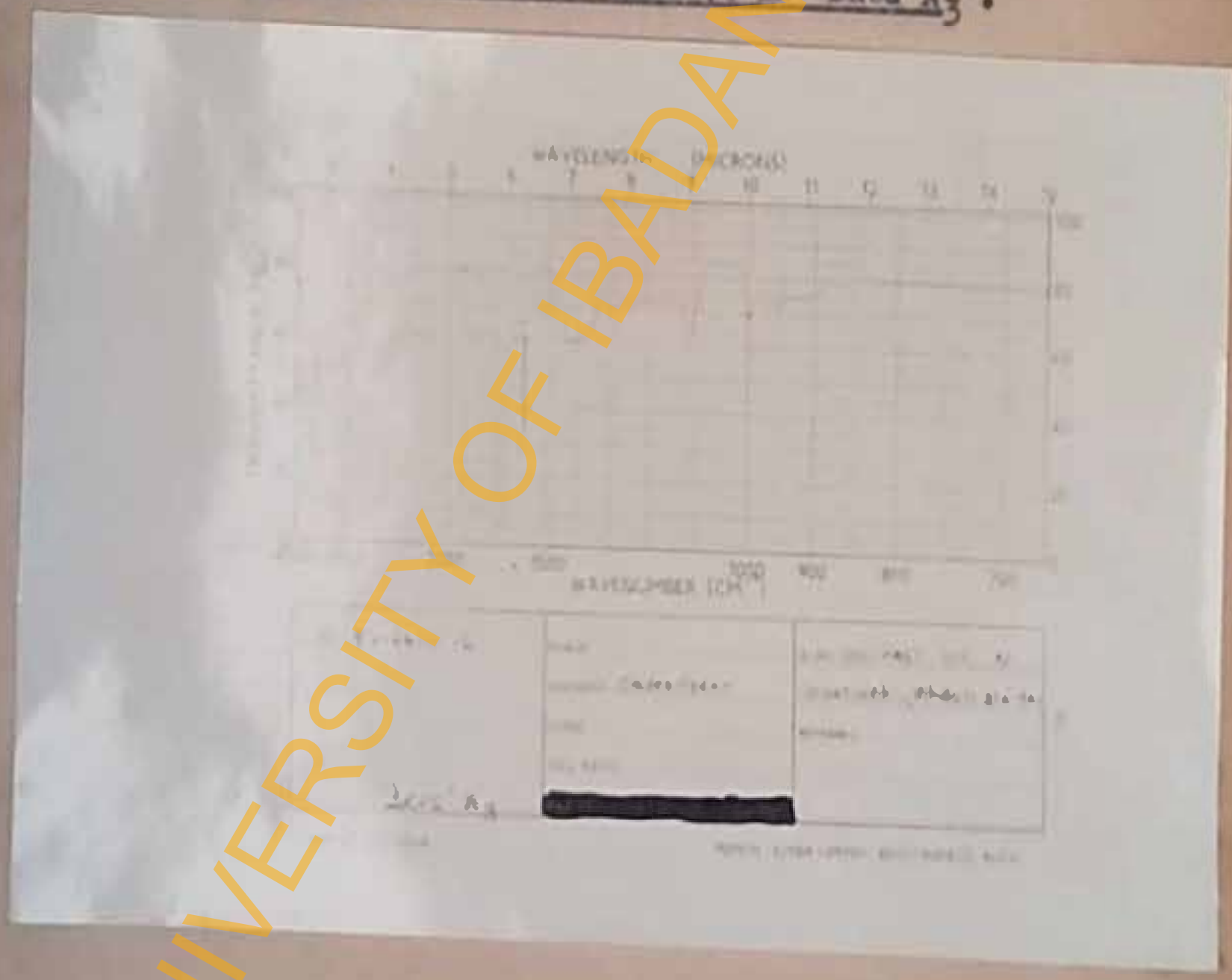

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FIGURE 36

INFRARED SPECTRA OF SPOT 21 "DRUG A4"

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FIGURE 37.

INFRARED SPECTRA OF SPOT 22 IN "DRUG A_{14}".

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All standards of the identified steroids were prepared as 10mg per 100 ml. Hence 0.2ml of the standard preparation would contain 0.02mg of the standard steroid.

5ml of each steroidal extract and 0.2ml of each of the standards was placed in individual test tubes and evaporated to dryness. To extracts, standards and a clean test tube for the blank, 0.2ml absolute alcohol, and 0.25ml of freshly prepared Zimmermann reagent were added.

The tubes were allowed to stand in the dark at $25^{\circ} \pm 2^{\circ}C$ for ooc hours, 3ml of ethanol, was later added, mixed well and the extinction of tests, and standards were measured at 440, 520, and 600 mu on the S.P.500 spectrophotometer. In each case, the blank was used to set the instrument to zero.

CALCULATIONS

For test and standard, the corrected extinction were calculated by the relation (King, 1964):

E corrected (E.Corr.) = E520 - ½ (E440 + E600).

Then the amount of steroid in extract is

$$\left\{ \frac{\text{E corr. for Extract}}{\text{E Corr. for Standard}} \times 0.02 \right\} \text{mg.}$$

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TABLE 7.

RF VALUES OF STANDARD STEROIDS AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL.

STANDARD STEROIDS	DESIG-NATION	NO. OF DETER-MINATION	RF VALUES MEANS ± S.E.M.	COLOUR AFTER HEATING FOR 10 MINUTES	
				DAYLIGHT	ULTRA VIOLET
TESTOSTERONE	A	5	0.24± 0.06	PURPLE	PINK
TESTOSTERONE PROPIONATE	B	5	0.54± 0.08	GREEN YELLOW	GREEN
ANDROSTERONE	C	5	0.26± 0.01	PURPLE	PINK
DEHYDRO-EPI - ANDROSTERONE	D	5	0.29± 0.05	PURPLE	PINK
DEHYDRO-ISO-ANDROSTERONE	E	5	0.35± 0.07	PURPLE	PINK
EPI-TESTOSTERONE	F	5	0.22± 0.02	LIGHT BROWN	GREEN
5B-ANDROSTAN-3, 17,-DIONE	G	5	0.53± 0.06	YELLOW	GREEN
ANDROSTENEDIONE	H	5	0.46± 0.04	GREEN	BROWN
11B-OH-ANDROSTENEDIONE	I	5	0.12± 0.02	PURPLE	BROWN
5 A - ANDROSTAN-3B - 17 - DIOL.	J	5	0.23± 0.03	PURPLE	PINK
SUDAN BLACK	S	5	0.52± 0.08	PURPLE VIOLET	

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TABLE XI

RF VALUES OF SPOTS OBTAINED WITH EXTRACT OF DRUG A_1 AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL

	SPOT WITH DESIGNATION	NO. OF DETERMINATION	RF VALUES MEAN ± S.E.M.	COLOUR AFTER HEATING FOR 10 MINUTES	
				DAYLIGHT	ULTRA-VIOLET
EXTRACT OF DRUG A_1	1	5	0.00	LIGHT BROWN	GREEN
	2	5	0.49± 0.03	GREEN	BROWN
	3	5	0.56± 0.05	YELLOW	GREEN
	4	5	0.70± 0.02	PURPLE	PINK
	5	5	0.95± 0.05	BROWN	BROWN

TABLE XII

RF VALUES OF SPOTS OBTAINED WITH EXTRACT OF DRUG A_2 AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL.

	SPOTS WITH DESIGNATION	NO. OF DETERMINATIONS	RF VALUES MEAN ± S.E.M.	COLOUR AFTER HEATING FOR 10 MINUTES	
				DAYLIGHT	ULTRA-VIOLET
EXTRACT OF DRUG A_2	6	5	0.94± 0.06	BROWN	BROWN
	7	5	0.64± 0.02	PURPLE	PINK
	8	5	0.55± 0.03	YELLOW	GREEN
	9	5	0.44± 0.03	GREEN	BROWN
	10	5	0.00±	LIGHT BROWN	GREEN

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TABLE XIII

RF VALUES OF SPO OBTAINED WITH EXTRACT OF DRUG A_3 AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL

	SPOT WITH DESIGNATION	NO. OF DETERMINATIONS	RF VALUES MEAN ± S.E.M.	COLOUR AFTER HEATING FOR 10 MINUTES	
				DAYLIGHT	ULTRAVIOLET
EXTRACT	11	5	0.00	BROWN	BROWN
	12	5	0.10 ± 0.01	PURPLE	BROWN
OF	13	5	0.18 ± 0.03	PURPLE	PINK
DRUG	14	5	0.37 ± 0.05	PURPLE	PINK
	15	5	0.44 ± 0.03	GREEN	BROWN
A_3	16	5	0.55 ± 0.02	YELLOW	GREEN
	17	5	0.66 ± 0.06	PURPLE	PINK
	18	5	0.87 ± 0.01	BROWN	BROWN
	19	5	0.95 ± 0.04	BROWN	BROWN

TABLE XIV

RF VALUES OF SPOTS OBTAINED WITH EXTRACT OF DRUG A_4 AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL.

	SPOTS WITH DESIGNATION	NO. OF DETERMINATIONS	RF VALUES MEAN ± S.E.M.	COLOUR AFTER HEATING FOR 10 MINUTES	
				DAYLIGHT	ULTRAVIOLET
EXTRACT	20	5	0.93 ± 0.06	BROWN	BROWN
OF	21	5	0.55 ± 0.02	YELLOW	GREEN
DRUG	22	5	0.43 ± 0.05	GREEN	BROWN
A_4	23	5	0.00	NO COL.	BROWN

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TABLE XV(a)

RF VALUES OF SPOTS BY TWO-DIMENSIONAL CHROMATOGRAPHY AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL.

	SPOTS WITH DESIG-NATION	NO. OF DETERMI-NATIONS	RF VALUES MEAN ± S.E.M.	COLOUR AFTER HEATING FOR 10 MINUTES	
				DAYLIGHT	ULTRAVIOLET
EXTRACT OF DRUG A_1	2	5	0.53 ± 0.06	GREEN	BROWN
	3	5	0.55 ± 0.03	YELLOW	GREEN
	4	5	0.71 ± 0.02	PURPLE	PINK
EXTRACT OF DRUG A_2	7	5	0.38 ± 0.04	PURPLE	PINKS
	8	5	0.58 ± 0.03	YELLOW	GREEN
	9	5	0.58 ± 0.04	GREEN	BROWN
EXTRACT OF DRUG A_3	12	5	0.18 ± 0.02	PURPLE	BROWN
	13	5	0.28 ± 0.01	PURPLE	PINK
	14	5	0.42 ± 0.05	PURPLE	PINK
	15	5	0.54 ± 0.04	GREEN	BROWN
	16	5	0.66 ± 0.06	YELLOW	GREEN
	17	5	0.79 ± 0.08	PURPLE	PINK
EXTRACT OF DRUG A_4	21	5	0.60 ± 0.03	YELLOW	GREEN
	22	5	0.56 ± 0.05	GREEN	BROWN

SOLVENTS: (i) CHLOROFORM ONLY
(ii) CYCLOHEXANE: ETHYL ACETATE (1:1)

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TABLE XV(b)

RF VALUES OF SPOTS BY TWO-DIMENSIONAL CHROMATOGRAPHY AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL.

	SPOT WITH DESIG-NATION	NO. OF DETERMI-NATIONS	RF VALUES MEAN ± S.E.M.	COLOUR AFTER HEATING FOR 10 MINUTES	
				DAYLIGHT	ULTRAVIOLET
DRUG A_1	5	5	0.25 ± 0.04	BROWN	BROWN
DRUG A_2	6	5	0.36 ± 0.02	BROWN	BROWN
DRUG A_3	18	5	0.48 ± 0.05	BROWN	BROWN
DRUG A_4	19	5	0.62 ± 0.01	BROWN	BROWN
	20	5	0.73 ± 0.07	BROWN	BROWN

SOLVENTS (i) BENZENE: ETHANOL (19.1)

(ii) CYCLOHEXANE: ETHYL ACETATE (1:1)

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TABLE XV(C)

RF VALUES OF SPOTS BY TWO-DIMENSIONAL CHROMATOGRAPHY AND COLOUR PRODUCED WITH 50% SULPHURIC ACID IN METHANOL.

	SPOTS WITH DESIGNATION	NO. OF DETERMINATIONS	RF VALUES MEAN ± S.E.M.	COLOUR AFTER HEATED FOR 10 MINUTES	
				DAYLIGHT	ULTRAVIOLET
DRUG A_1	1	5	0.20 ± 0.03	LIGHT BROWN	GREEN
DRUG A_2	10	5	0.40 ± 0.08	LIGHT BROWN	GREEN
DRUG A_3	11	5	0.59 ± 0.03	BROWN	BROWN
DRUG A_4	23	5	0.72 ± 0.06	NO COLOUR	BROWN

SOLVENTS: (i) CYCLOHEXANE: ETHYL ACETATE (2:8)

(ii) BENZENE: ETHER (1:9)

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TABLE XVI

RS VALUES OF STANDARD STEROIDS AND COLOUR PRODUCED WITH THE ZIMMERMANN REAGENT.

STANDARDS	NO. OF DETERMINATION	RS VALUES MEAN ± S.E.M	COLOUR
TESTOSTERONE	5	0.43 ± 0.05	BLUE
TESTOSTERONE PROPIONATE	5	1.10 ± 0.10	BLUE
ANDROSTERONE	5	0.45 ± 0.09	VIOLET
DEHYDRO-EPI-ANDROSTERONE	5	0.50 ± 0.06	VIOLET
DEHYDRO-ISO-ANDROSTERONE	5	0.71 ± 0.03	VIOLET
EPITESTOSTERONE	5	0.42 ± 0.08	BLUE
5B-ANDROSTAN-3, 17- DIONE	5	1.12 ± 0.09	VIOLET
ANDROSTENEDIONE	5	0.17 ± 0.04	VIOLET
11B-HYDROXY-ANDROSTENEDIONE	5	0.85 ± 0.11	VIOLET
5 & - ANDROSTAN - 3B - 17 - DIOL.	5	0.41 ± 0.07	NO COLOUR

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TABLE XVII(a)

RS VALUES OF SPOTS OBTAINED WITH EXTRACT OF DRUG A1 AND COLOUR PRODUCED WITH THE ZIMMERMANN REAGENT

DRUG A1	SPOTS WITH DESIGNATION	NO. OF DETERMINATION	RS VALUES M.S.N ± S.E.M.	COLOUR
EXTRACT OF DRUG A1	1	5	0.00	NO COLOUR
	2	5	0.89 ± 0.07	VIOLET
	3	5	1.22 ± 0.11	BLUE
	4	5	1.45 ± 0.08	BLUE
	5	5	1.80 ± 0.03	NO COLOUR

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TABLE XVII(b)

Rf VALUES OF SPOTS OBTAINED WITH EXTRACT OF DRUG A AND COLOUR PRODUCED WITH THE VANILLIN REAGENT

DRUG A2	SPOTS WITH DESIGNATIONS	NO. OF DETERMINATIONS	Rf VALUES MEAN ± S.E.M.	COLOUR
EXTRACT OF DRUG A2	6	5	1.81 ± 0.07	NO COLOUR
	7	5	1.25 ± 0.10	BLUE
	8	5	1.09 ± 0.03	VIOLET
	9	5	0.76 ± 0.08	VIOLET
A2	10	5	0.00	NO COLOUR

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TABLE XVII(c)

RF VALUES OF SPOTS OBTAINED WITH EXTRACT OF DRUG "3 AND COLOUR PRODUCED WITH THE ZIMMERMANN REAGENT

DRUG "3	SPOTS WITH DESIGNATIONS	NO. OF DETERMINATIONS	RF VALUES MEAN ± S.E.M.	COLOUR
EXTRACT OF DRUG "3	11	5	0.00	NO COLOUR
	12	5	0.14 ± 0.04	NO COLOUR
	13	5	0.39 ± 0.07	NO COLOUR
	14	5	0.74 ± 0.11	VIOLET
	15	5	0.78 ± 0.09	VIOLET
	16	5	1.13 ± 0.05	VIOLET
	17	5	1.30 ± 0.16	BLUE
	18	5	1.76 ± 0.03	BLUE
	19	5	1.83 ± 0.06	NO COLOUR

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TABLE XVII(c)

RF VALUES OF SPOTS OBTAINED WITH EXTRACT OF DRUG A_4 AND COLOUR PRODUCED WITH THE ZIRCONIUM REAGENT.

DRUG A_4	SPOTS WITH DISSIOCIATIONS	NO. OF DETERMINATION	RF VALUES MEAN ± S.E.M.	COLOUR
EXTRACT	20	5	1.79 ± 0.18	NO COLOUR
OF	21	5	1.15 ± 0.09	VIOLET
DRUG	22	5	0.76 ± 0.05	VIOLET
A_4	23	5	0.00	NO COLOUR

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TABLE XVIII(a)

EXTINCTION VALUES AND AMOUNT OF STEROIDS IN MILLIGRAMS PER 100ml IN EXTRACTS OF THE 4 LOCAL DRUGS

EXTRACTS OF LOCAL DRUGS	DESIGNATIONS OF EXTRACTS	EXTINCTION 440 mu	EXTINCTION 520 mu	EXTINCTION 600 mu	CORRECTED EXTINCTION	STEROIDS IN MG/100ml
EXTRACT A_1	2	0.30	6.59	6.04	3.42	68.40
EXTRACT A_1	3	1.70	9.25	8.00	4.40	29.22
EXTRACT A_2	8	1.06	2.86	2.08	1.29	10.32
EXTRACT A_2	9	0.28	5.37	4.48	2.99	59.80
EXTRACT A_3	14	1.57	3.98	3.65	1.37	7.83
EXTRACT A	15	0.89	3.84	3.69	1.55	31.00
EXTRACT A_3	16	1.10	4.79	3.08	2.70	21.60
EXTRACT A_4	21	1.05	3.59	2.15	1.99	15.92
EXTRACT A_4	22	0.22	8.92	8.02	4.80	96.00

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TABLE XVIII(b).

AMOUNT OF INDIVIDUAL STEROIDS IN 40 µl OF EACH OF THE 4 LOCAL DRUGS.

LOCAL DRUGS	DESIGNATIONS OF EXTRACTS	AMOUNT OF STEROIDS IN µg per 40 µl
A1	2	27.36 ± 0.46
A'1	3	11.68 ± 0.35
A"1	8	4.12 ± 0.49
A"2	9	23.92 ± 0.07
A'2	14	3.13 ± 0.02
A"3	15	12.40 ± 0.13
A'3	16	8.64 ± 0.39
A"3	21	6.36 ± 0.28
A'4	22	38.40 ± 0.18
A"4		

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Hence steroid in Extract = $\left(\frac{\text{\# corr. for extract}}{\text{\# corr. for Standard}} \times 0.02 \times \frac{100}{5}\right)$
(mg. per 100 ml.)

$$= \frac{\text{\# corr. for Extract}}{\text{\# corr. for Standard.}} \times 0.$$

The readings obtained at the three wavelengths and the calculated amount of steroid in each extract are recorded in Tables XVIII a and b.

R E S U L T S

Although, Rf values are not always a positive indicator of the type of substance or compound in a spot, we have compared the Rf and Rs values of some of the spots obtained with extracts of the 4 local drugs with the Rf and Rs values of standard steroids.

It was obvious that some of the spots might be steroids, in particular special note was taken of the following spots 1 to 4 (A_1), 7 to 10 (A_2), 12 to 12 (A_3), 21 and 22 (A_4), which in one form or the other correspond to some of the standard steroids in Rf, Rs values, colour produced after spraying the plates with Zimmermann reagent and 50% sulphuric acid in methanol Table X to XVIII b).

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Since no further spots were obtained during purification by two-dimensional chromatography, it was assumed that the spots are pure.

With the Zimmermann reagent, two types of colours were observed.

(i) Violet spots. The standard steroids which gave this were:

(a) Androsterone

(b) 5B-Androstan, 3, 17 dione

(c) Androstenedione

(d) 11B - OH - androstenedione

(e) Dehydro-iso-androsterone

(f) Dehydro-epi-androsterone.

Among the extracts of the 4 local drugs, the following gave violet colour:

(a) Spots 21 and 22 in Extract A_4

(b) Spots, 14, 15 and 16 in Extract A_3

(c) Spots 8 and 9 in Extract A_2

(d) Spot 2 in Extract A_1.

According to Dobriner and Liebermann (1950) only 17-keto steroids give violet colours to the Zimmermann reagent.

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(ii) Blue spots. The standards steroids which gave this colour were:

(a) Testosterone

(b) Epitestosterone.

Among the extracts, the following gave the blue colour:

(a) Spots 3 and 4 in extract A_1

(b) Spot 7 in Extract A_2

(c) Spots 17 and 18 in Extract A_3.

No colours were obtained for the standard steroid 5x-androstan, 3B - 17B diol and for the following spots of the extracts when treated with the Zimmermann reagent:

(a) Spots 1 and 5 in Extract A_1

(b) Spots 6 and 10 in Extract A_2

(c) Spots 11, 12, 13, and 19 in Extract A_3

(d) Spots 20 and 23 in Extract A_4.

Dobriner and Liebermann (1950) had suggested that only 3-ketosteroids give blue spots with the Zimmermann reagent.

From the foregoing findings and by comparing the values for the Rf and Ro as well as colour produced with the Zimmermann test and the 50% sulphuric acid in methanol, we concluded that:

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Although the infrared spectra, obtained with each extract as shown in Figs 29-37 do not exactly correspond with those listed in the text by Dobriner et al (1953) we feel that the differences might be due to the conditions under which our experiments were carried out.

We are therefore unable, at this stage to positively identify any of the steroids present in drugs A_1 A_2 A_3 and A_4 although there are some indications that :

(i) "Drug" A_1 might have contained Androstendione as spot 2 and probably a Testosterone sefor as spot 3;

(ii) "Drug" A_2 might have contained 5B - Androstan, 3, 17 dione as spot and Androstenedione as spot 9.

(iii) "Drug" A_3 might have contained Dehydro-iso-androsterone. as spot 14; Androstenedione as spot 15 and 5B - Androstan 17 dione as spot 16.

(iv) "Drug" A_4 might have contnined 5B-Andrstan. 3. 17 dione as spot 21 and Androstenedione as spot 22.

Further work will have to be done to finally identify these compounds. The amount of steroids in the various extracts were estimated colourimetrically and the amounts in mg/100ml are shown in Table XIIIa and in kg/40ml of crude local drug in Table XIIIb. The tables show that A_4 has the largest amount of steroid followed by A_1. A_2 and A_3.

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Although the infrared spectra. obtained with each extract as shown in Figs 29-37 do not exactly correspond with those listed in the text by Dobriner et al (1953) we feel that the differences might be due to the conditions under which our experiments were carried out.

We are therefore unable, at this stage to positively identify any of the steroids present in drugs A_1 A_2 A_3 and A_4 although there are some indications that :

(i) "Drug" A_1 might have contained Androstendione as spot 2 and probably a Testosterone esfor as spot 3;

(ii) "Drug" A_2 might have contained 5B - Androstan, 3, 17 dione as spot and Androstenedione as spot 9.

(iii) "Drug" A_3 might have contained Dehydro--iso-androsterone. as spot 14; Androstenedione as spot 15 and 5? - Androstan 17 dione as spot 16.

(iv) "Drug" A_4 might have contained 5B-Andreton. 3. 17 dione as spot 21 and Androstenedione as spot 22.

Further work will have to be done to finally identify these compounds. The amount of steroids in the various extracts were estimated colourimetrically and the amounts in mg/100ml are shown in Table XIIIa and in kg/40ml of crude local drug in Table XIIIb. The tables show that A_4 has the largest amount of steroid followed by A_1. A_2 and A_3.

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EXPERIMENT V

Comparison of the effects of individual extracts in each local drug with their individual equivalent pure steroids on the weights of the accessory sex glands of the castrated rats.

Since the recommended dose of the local drug is one wineglassful (about 40ml), the amount of presumed steroid in each of the extracts of the local drugs were calculated per 40 ml (Table XVIII b).

This amount taken up in 0.2ml oil was administered to castrated rats daily for 7 days (page 117). The same amount of the equivalent standard steroids were also injected daily into another set of castrated rats for 7 days.

The rats were killed 24 hours after the last injection and their accessory sex-glands dissected out and weighed to the nearest milligram (page 111).

The amount of 17-ketosteroids excreted in the 48-hour urine samples of the rats were also estimated, pages 113 to 114). Results obtained are recorded in tables XIX to XXIV.

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RESULTS

As with earlier experiments, there was a clear indication of atrophy of accessory sex glands in the rats after castration (Table XIX). This is in line with observations of earlier workers (Hoye and Mathieson, 1945).

There were similarities in the responses obtained when individual extracts were compared with their equivalent pure steroids although the equivalent pure steroids tended to give higher response than the extracts. However, extract A_2 (spot 9) gave a higher response than its equivalent standard steroid in both the weight of the seminal vesicle and the prostate gland although the difference is not significant. Extract A_4 (spot 22) also had high value for the weight of the prostate gland than that of the standard.

Very low responses were obtained with extract A_1 (spot 3), extract A_2 (spot 8), extract A_3 (spots 14, 15 and 16 and Extract A_4 (spot 21) as well as with their equivalent standard steroids. This shows that the steroids are either not potent enough to stimulate the growth of these organs or the amount injected was too low, or they could not be properly utilised by the body.

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The pattern of 17-ketosteroids in the urine (Tables XXII to XXIV was similar to those of the growth of the accessory sex glands. All the extracts mentioned above also gave low values for the 17-ketosteroids in the urine.

TABLE XIX

OBSERVATIONS OF GROWTH OF ACCESSORY SEX GLANDS IN RATS THREE WEEKS AFTER PRE-PUBERTAL CASTRATION.

CONDITION	PROSTATE GLAND MEAN ± S.E.M. (mg)	T	P	SEMINAL VESICLE MEAN ± S.E.M. (mg)	T	P
NORMAL CONTROL	139.0 ± 0.02	4.729	0.02	99.0 ± 0.02	4.672	0.02
CASTRATED CONTROL	21.0 ± 0.02			13.0 ± 0.01		

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TABLE IX

EFFECTS OF INTRAVENOUS PURIFIED EXTRACT OF VARIOUS LOCAL DRUG ON GROWTH OF ACCESSORY SEX GLANDS OF PRE-PUBERTALLY CASTRATED RATS.

EXTRACT	DESIGNATION	PROSTATE GLAND MEAN ± S.E.M.(mg)		A VERSUS B		SEMINAL VESICLE MEAN ± S.E.M.(mg)		C VERSUS D	
		CONTROL (A)	TEST (B)	T	P	CONTROL (C)	TEST (D)	T	P
$^{-}1$	2	21.0± 0.2	84.0± 0.5	4.543	0.02	13.0±0.1	45.2±0.7	4.481	0.02
$^{-}1$	3	21.0± 0.2	22.0± 0.1	1.146	N.S.	13.0±0.1	12.7±0.3	1.003	N.S.
$^{-}2$	8	21.0± 0.2	5.5± 0.6	0.018	N.S.	13.0±0.1	2.7±0.1	0.082	N.S.
$^{-}2$	9	21.0± 0.2	74.0± 0.2	4.393	0.02	13.0±0.1	47.5±0.2	4.496	0.02
$^{-}3$	14	21.0± 0.2	27.2± 0.2	1.148	N.S.	13.0±0.1	16.5±0.2	1.129	N.S.
$^{-}3$	15	21.0± 0.2	23.0± 0.1	1.136	N.S.	13.0±0.1	12.2±0.4	1.032	N.S.
$^{-}3$	16	21.0± 0.2	6.5± 0.1	1.007	N.S.	13.0±0.1	3.2±0.1	0.038	N.S.
$^{-}4$	21	21.0± 0.2	7.8± 0.3	1.013	N.S.	13.0±0.1	3.0±0.1	0.097	N.S.
$^{-}4$	22	21.0± 0.2	111.0± 0.8	11.584	0.02	13.0±0.1	54.2±0.3	4.363	0.02

N.S. = Not significant.

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TABLE XXI

EFFECT OF THE EQUIVALENT STANDARD STEROIDS OF INDIVIDUAL EXTRACTS OF LOCAL DRUG ON THE GROWTH OF ACCESSORY SEX GLANDS OF PREPUBERTALLY CASTRATED RATS

STANDARDS OF INDIVIDUAL EXTRACT	PROSTATE GLAND MEAN ± S.E.M.(mg)		A VERSUS B		SEMINAL VESICLE MEAN ± S. E. M. (mg)		C VERSUS D	
	CONTROL(A)	TEST (B)	T	P	CONTROL (C)	TEST (D)	T	P
A_1(2)	21.0± 0.2	97.2± 0.2	4.541	0.02.	13.0± 0.1	52.2± 1.4	4.431	0.02
A_1(3)	21.0± 0.2	24.5± 0.1	1.006	N.S.*	13.0± 0.1	17.5± 0.1	0.193	N.S.
A_2 (8)	21.0± 0.2	8.5± 0.1	0.964	N.S.	13.0± 0.1	5.7± 0.2	0.932	N.S.
A_2 (9)	21.0± 0.2	70.2± 0.2	4.142	0.02	13.0± 0.1	40.0± 0.2	4.263	0.02
A_3(14)	21.0± 0.2	37.5± 0.2	3.162	0.05	13.0± 0.1	18.0± 0.1	3.176	0.05
A_3(15)	21.0± 0.2	29.5± 1.5	1.186	N.S.	13.0± 0.1	15.0± 0.1	1.342	N.S.
A_3(16)	21.0± 0.2	11.0± 0.1	1.135	N.S.	13.0± 0.1	7.2± 0.1	1.128	N. S.
A_4(21)	21.0± 0.2	12.0± 0.1	1.146	N.S.	13.0± 0.1	6.5± 0.2	1.113	N.S.
A_4(22)	21.0± 0.2	03.5± 0.7	4.568	0.02	13.0± 0.1	56.2± 0.1	4.542	0.02

N.S.* = Not significant.

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TABLE XXLI

AMOUNT OF 17-KETOSTEROIDS PER 48 HOURS EXCRETED IN THE URINE OF PRE-PUBERTALLY CASTRATED RATS

CONDITION	17-KETOSTEROIDS (MG)48 HOURS MEAN ± S.E.M.	T	P
NORMAL CONTROL	0.018 ± 0.002	4.368	0.02
CASTRATED CONTROL	0.001 ± 0.0001		

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TABLE XXIII

EFFECTS OF INDIVIDUAL PUR- EXTRACT OF VARIOUS LOCAL DRUG ON LEVELS OF 17-KETOSTEROIDS EXCRETED IN THE 48 HOUR URINE OF PRE-PUBERTALLY CASTRATED RATS.

EXTRACTS	DESIG-NATIONS	17-KETOSTEROIDS (MG) 48 HRS. MEAN ± S.E.M.	T	P
CASTRATED CONTROL		0.001 ± 0.0001		
"1	2	0.009 ± 0.002	4.526	0.02
"1	3	0.005 ± 0.001	3.176	0.05
"2	8	0.001 ± 0.0001	0.000	N.S.*
"2	9	0.010 ± 0.003	4.532	0.02
"3	14	0.003 ± 0.001	3.114	0.05
"3	15	0.004 ± 0.002	3.163	0.05
"3	16	0.001 ± 0.001	0.000	N.S.
"4	21	0.002 ± 0.001	0.021	N.S.
"4	22	0.019 ± 0.006	4.691	0.02

N.S.* = Not significant.

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TABLE XXIV

EFFECTS OF THE EQUIVALENT STANDARD STEROIDS OF INDIVIDUAL EXTRACTS OF LOCAL DRUG ON THE LEVEL OF 17-KETOSTEROIDS PER 48 HOUR IN THE URINE OF PRE-PUBERTALLY CASTRATED RATS.

STANDARDS OF INDIVIDUAL EXTRACTS		17-KETOSTEROIDS (MG/48 Hours) MEAN ± S.E.M.	T	P
CASTRATED CONTROL		0.001 ± 0.0001		
μ_1	(2)	0.011 ± 0.006	4.546	0.02
μ_1	(3)	0.006 ± 0.001	3.182	0.05
μ_2	(8)	0.002 ± 0.001	0.924	N.S.*
μ_2	(9)	0.012 ± 0.002	4.551	0.02
μ_3	(14)	0.004 ± 0.002	3.163	0.05
μ_3	(15)	0.006 ± 0.001	3.182	0.05
μ_3	(16)	0.002 ± 0.001	0.924	N.S.
μ_4	(21)	0.002 ± 0.001	0.924	N.S.
μ_4	(22)	0.021 ± 0.004	4.734	0.02

N.S.* = Not significant.

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Exp.XI and XI: Comparison of the effects of individual extracts in each local drug with the individual equivalent pure steroids on the cockerel's comb size.

Having determined the amount of individual steroid extract in 40 ml of each local drug (Table XVIII(b), this amount was injected in 0.2 ml oil (Exp.117) daily for 7 days into cockerels.

The 4 groups prepared as described on page 106 had been distributed randomly into groups of 4 birds each. Two sets of cockerels made up of 8 groups each were prepared. One set was injected with the extracts of local drugs and the other set was injected with the equivalent standard steroids. Injections were administered as described on page 117. .

The length and height of the comb of these cockerels were measured (page 107) daily for the duration of the experiment. The comb size (length + height) of each bird was recorded in Tables XXV to XXVII.

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RESULTS:

As expected, there was a significant drop in the comb size following castration of the cockerels (Table XXV). (Gallagher and Koch 1930; Dorfman 1948).

In most cases, the responses of the comb to the individual steroids extracted from the local drug as well as those of the presumed equivalent standard steroids were comparable. This suggests further that our steroidal extracts from the local drug might be equivalent to their standards.

Fairly lower responses were obtained with extract A_2 (spot 8), extract A_3 (spot 16) and extract A_4 (spot 21) when compared with their presumed equivalent standard steroids. This discrepancy might have resulted from the fact that some of these individual steroid extracts might not be as pure as their presumed standard equivalent.

TABLE XIV

OBSERVATIONS OF GROWTH OF CAPON'S COMB IN COCKERELS THREE WEEKS AFTER PRE-PUBERTAL CAPONISATION.

CONDITION	COMB SIZE MEAN ± S.E.M.(mm)	T	P
NORMAL CONTROL	6.48 ± 0.43	4.431	0.02
CASTRATED CONTROL	0.15 ± 0.01		

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TABLE XXVI

EFFECTS OF INDIVIDUAL PURE EXTRACT OF THE 4 LOCAL DRUGS ON THE GROWTH OF THE CAPON'S COMB.

EXTRACTS	DESIGNATIONS	COMB SIZE (mm) MEAN ± S.E.M.		A VERSUS B	
		CONTROL (A)	TEST (B)	T	P
A_1	2	0.15± 0.01	2.22± 0.58	4.241	0.02
A_1	3	0.15± 0.01	3.20± 0.37	4.268	0.02
A_2	8	0.15± 0.01	1.12± 0.03	3.118	0.05
A_2	9	0.15± 0.01	2.00± 0.26	4.209	0.02
A_3	14	0.15± 0.01	2.72± 0.45	4.253	0.02
A_3	15	0.15± 0.01	2.17± 0.51	4.221	0.02
A_3	16	0.15± 0.01	1.60± 0.28	3.179	0.05
A_4	21	0.15± 0.01	1.10± 0.03	3.114	0.05
A_4	22	0.15± 0.01	2.10± 0.31	4.213	0.02

Results are mean values of 4 determinations with their standard error.

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TABLE XXVII

EFFECTS OF THE EQUIVALENT STANDARD STEROIDS OF INDIVIDUAL EXTRACTS OF LOCAL DRUGS ON THE GROWTH OF THE CAPON'S COMB

STANDARDS OF INDIVIDUAL EXTRACTS	COMB SIZE (mm) MEAN ± S.E.M.		A VERSUS B	
	CONTROL (A)	TEST (B)	T	P
A_1 (2)	0.15± 0.01	2.95± 0.32	4.276	0.02
A_1 (3)	0.15± 0.01	3.71± 0.47	4.380	0.02
A_2 (8)	0.15± 0.01	1.10± 0.61	4.245	0.02
A_2 (9)	0.15± 0.01	2.90± 0.01	4.270	0.02
A_3 (14)	0.15± 0.01	3.85± 0.26	4.429	0.02
A_3 (15)	0.15± 0.01	3.47± 0.55	4.363	0.02
A_3 (16)	0.15± 0.01	2.92± 0.03	4.273	0.02
A_4 (21)	0.15± 0.01	2.12± 0.20	4.249	0.02
A_4 (22)	0.15± 0.01	3.40± 0.41	4.359	0.02

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EXPERIMENT VII:- Investigations on the presence of other biochemical compounds in the various local virilising drugs.

A large variety of compounds are soluble in ethyl alcohol. Among these are the hydrocarbons, aldehydes and ketones, fatty acids, phenols, some amino acids, peptides and proteins, some sugars, some vitamins and biologically active quinones, alkaloids, amines and other basic compounds and steroids.

The 4 local drugs analysed in his work were usually soaked in alcohol (Ethanol). This alcoholic extract, after removal of the alcohol were injected into animals (page 117) in some of our experiments. It was therefore necessary to test this extract for as many of those substances as could be found in the alcoholic extract.

Biochemical tests were carried out as described by Bassir (1963). A summary of the results obtained are recorded in Table XXVIII.

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TABLE XVIII

SUMMARY OF SOME BIOCHEMICAL TESTS ON THE ALCOHOLIC EXTRACTS OF THE 4 LOCAL DRUGS.

TESTS	"1	"2	"3	"4
ALDEHYDES:-	-	-	-	-
KETONES:-	-	-	-	-
FATTY ACIDS:-	+	+	+	+
SUGARS:-	-	-	-	-
LIPIDS:-	+	++	+++	+
PROTEINS:-	+	+	+	+
PHENOL:-	-	-	++	-

- = Negative

+ = Positive

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RESULTS:-

Earlier experiments (Experiments I - VI) had indicated the presence of steroids in the four local drugs. From Table XXVIII, it was evident that apart from steroids, fatty acids, lipids as well as amino acids and proteins are present in the local drugs. Aldehydes and ketones could not be detected in any of the 4 local drugs while phenol was detected in A3 alone. The presence of phenol in A3 is not surprising since it is made from roots only.

This results indicate that when experimental animals are injected with the crude local drugs small amounts of other substances like lipids, fatty acids and proteins are also carried along.

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EXPERIMENT VIII:- Comparison of the effects of mixtures of extracts in each local drug with mixtures of the equivalent pure steroids on the weights of the accessory sex glands of the castrated rats.

Each of the 4 local drug was extracted after acid hydrolysis (page 116) and the various steroids in each of them were separated (by thin-layer chromatography) identified (by colour tests and infrared spectroscopy) and estimated (colourimetrically) as reported in experiment IV.

The individual steroids extracted from each local drug were then combined at the estimated amounts present in 40 ml. (Table XLIII(B)) of each crude drug. This combined steroid extract was then injected into castrated rats for 7 days.

Two sets of littermate castrated rats were used. Each set was made up of 4 groups of 4 rats each. Each rat was castrated (page 109) and injection was started two weeks after castration. One set of rats were treated with steroid extracts of crude local drug, each group of rat being given the same amount of the presumed equivalent standard steroids of the steroids extracted from each local drug. The same amount of steroids were injected daily for 7 days.

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The urine of the rats were also collected every 48 hours, (page 111) and analysed for 17-Ketosteroids. (page 112).

24 hours after the last injections the rats were killed, the whole of the accessory sex glands of each rat was dissected out, cleaned and weighed to the nearest milligram (page 111).

R E S U L T S

In all cases, the responses of the rats in terms of the weights of seminal vesicle and prostate glands were higher with the pre-pubertal steroids than the extracted steroids. However, the differences between the responses were low enough to give room for comparison. The slight difference in response might be explained in terms of relative purity of the steroids.

The pattern of responses were similar in both cases with mixture of extract A_4 giving the highest response and mixtures of extracts from A_3 giving the lowest response. This is because "Drug" A_4 has been found (experiment IV Table XVIII(b) to contain the largest amount of androgenic steroids and A_3 the least.

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The pattern of 17-Ketosteroid excretion also followed those of the seminal vesicle and the prostate gland. Results of this experiment combined with those of experiment V, indicate clearly that not only are steroids present in the extracts, but that the responses obtained so far were due to the presence of these steroids in the various extracts. (Table XXIX to XXXII).

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TABLE XXIX

EFFECTS OF MIXTURES OF PURE EXTRACTS OF EACH LOCAL DRUG ON GROWTH OF ACCESSORY SEX GLANDS OF PRE-PUBERTALLY CASTRATED RATS

MIXTURES OF PURE EXTRACTS IN	PROSTATE GLAND MEAN ± S.E.M.(mg)		A VERSUS B		SEMINAL VESICLE MEAN ± S.E.M.(mg)		C VERSUS D	
	CONTROL(A)	TEST (B)	T	P	CONTROL(C)	TEST (D)	T	P
DRUG A_1	21.0 ± 0.2	102.0 ± 0.3	4.529	0.02	13.0 ± 0.1	48.0 ± 0.3	4.432	0.02
DRUG A_2	21.0 ± 0.3	69.0 ± 0.1	4.447	0.02	13.0 ± 0.1	36.0 ± 0.3	4.421	0.02
DRUG A_3	21.0 ± 0.2	33.0 ± 0.2	4.113	0.05	13.0 ± 0.1	16.0 ± 0.2	4.101	0.05
DRUG A_4	21.0 ± 0.2	103.0 ± 0.1	4.529	0.02	13.0 ± 0.1	55.0 ± 0.2	4.448	0.02

TABLE XXX

EFFECTS OF MIXTURES OF EQUIVALENT STANDARD STEROIDS OF EXTRACTS IN EXTRACTS IN EACH LOCAL DRUG ON GROWTH OF ACCESSORY SEX GLANDS OF PRE-PUBERTALLY CASTRATED RATS

MIXTURES OF EQUIVALENT STEROIDS IN EXTRACTS OF	PROSTATE GLAND MEAN ± S.E.M.(mg)		A VERSUS B		SEMINAL VESICLE MEAN ± S.E.M.(mg)		C VERSUS D	
	CONTROL(A)	TEST (B)	T	P	CONTROL(C)	TEST (D)	T	P
DRUG A_1	21.0 ± 0.2	115.0 ± 0.3	4.536	0.02	13.0 ± 0.1	54.0 ± 0.8	4.449	0.02
DRUG A_2	21.0 ± 0.2	87.0 ± 0.6	4.516	0.02	13.0 ± 0.1	54.0 ± 0.2	4.430	0.02
DRUG A_3	21.0 ± 0.2	43.0 ± 0.1	4.216	0.05	13.0 ± 0.1	22.0 ± 0.1	4.411	0.05
DRUG A_4	21.0 ± 0.2	117.0 ± 0.9	4.540	0.02	13.0 ± 0.1	54.0 ± 0.3	4.449	0.02

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TABLE XXXI

EFFECTS OF MIXTURES OF EXTRACTS OF EACH LOCAL DRUG ON 17-KETOSTEROID EXCRETION IN 48 HOUR URINE OF PRE-PUBERTALLY CASTRATED RATS

MIXTURES OF PURE EXTRACTS IN	17-KETOSTEROIDS (mg/48 HOURS MEAN ± S.E.M.		A VERSUS B	
	CONTROL (A)	TEST (B)	T	P
DRUG A_1	0.001± 0.0001	0.012± 0.003	4.530	0.02
DRUG A_2	0.001± 0.0001	0.010± 0.002	4.516	0.02
DRUG A_3	0.001± 0.0001	0.007± 0.007	4.476	0.05
DRUG A_4	0.001± 0.0001	0.017± 0.004	4.543	0.02

TABLE XXXII

EFFECTS OF MIXTURES OF EQUIVALENT STANDARD STEROIDS OF EXTRACTS IN EACH LOCAL DRUG ON 17-KETOSTEROID EXCRETION IN 48 HOUR URINE OF PRE-PUBERTALLY CASTRATED RATS.

MIXTURES OF EQUIVALENT STEROIDS IN EXTRACTS OF	17-KETOSTEROIDS (mg/48 HOURS) MEAN ± S.E.M.		A VERSUS B	
	CONTROL (C)	TEST (D)	T	P
DRUG A_1	0.001± 0.0001	0.014± 0.004	4.538	0.02
DRUG A_2	0.001± 0.0001	0.013± 0.001	4.538	0.02
DRUG A_3	0.001± 0.0001	0.009± 0.002	4.502	0.02
DRUG A_4	0.001± 0.0001	0.016± 0.006	4.541	0.02

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EXPERIMENT IX:- Comparison of the effects of mixtures of extracts in each local drug with mixtures of the equivalent standard steroids on the capons combs.

Each of the local drugs was extracted with acid hydrolysis (page 45) and the various steroids in each of the local drugs were separated (by thin-layer chromatography) identified (by colour tests and infrared spectroscopy) and estimated (colourimetrically) as reported in experiment IV.

The individual steroids in each of the local drugs were then combined on the basis of their weight in 40 ml of the crude local drug and then injected into capons. The mixtures of these combined steroids were used to match out with made up of 4 groups of birds each. Each bird was caponized (page 106) and injection was started two weeks after caponization. One set of capons were treated with steroid extracts of the crude local drugs each group of capons being given one of the four local drugs. The other set of capons were treated with the same amount of the presumed equivalent standard steroids. The steroids were injected in 0.2ml oil daily for 7 days

The combs of the capons were measured daily throughout the period of the experiment (page 109).

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RESULTS.

The responses obtained are shown in Tables XXIII and XXIV. Comparison of the two tables showed that the results of both the presumed standard steroids and the steroidal extracts follow the same pattern although the responses of the former are higher. This again might be due to relative purity.

Response of A_3 was higher than the other three local drugs and indeed was very close to those of the presumed equivalent standard steroids. This response by A_3 shows that the birds in one way or the other are more sensitive to A_3 than the other three local drugs when the results of experiment VIII are compared.

The other three mixtures of extracts of the local drugs had lower responses compared with their presumed equivalent standard steroids but the differences are not statistically significant. The results showed clearly that the four local drugs might contain similar steroids to the standard steroids.

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TABLE XXXIII

EFFECTS OF MIXTURES OF PURE EXTRACTS OF EACH LOCAL DRUG ON THE CAPON COMB SIZE

MIXTURES OF PURE EXTRACTS IN	COMB SIZE (mm.) MEAN ± S. E. M.		A VERSUS C	
	CONTROL(A)	TEST (C)	T	P
DRUG A_1	0.15 ± 0.01	5.10 ± 0.05	4.449	0.02
DRUG A_2	0.15 ± 0.01	3.50 ± 0.21	4.265	0.02
DRUG A_3	0.15 ± 0.01	6.52 ± 0.35	4.538	0.02
DURG A_4	0.15 ± 0.01	3.72 ± 0.28	4.317	0.02

TABLE XXXIV

EFFECTS OF MIXTURES OF EQUIVALENT STANDARD STEROIDS OF EXTRACTS IN EACH LOCAL DRUG ON THE CAPON COMB SIZE

MIXTURES OF EQUIVALENT STEROIDS IN EXTRACTS OF	COMB SIZE (mm.) MEAN + S. E. M.		A VERSUS C	
	CONTROL(A)	TEST (C)	T	P
DRUG A_1	0.15 ± 0.01	6.07 ± 0.49	4.517	0.02
DRUG A2	0.15 ± 0.01	4.22 ± 0.63	4.321	0.02
DRUG A3	0.15 ± 0.01	6.70 ± 0.31	4.543	0.02
DRUG A4	0.15 ± 0.01	4.37 ± 0.07	4.376	0.02

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EXPERIMENT X

Comparison of the effects of the whole crude local drug with the mixture of extracts in each local drug on the weights of the accessory sex glands of the castrated rats.

Separation, purification and identification of the individual steroids in each local drug was achieved by the methods described in experiment IV. The amount of the steroids in 40 ml of each local drug was determined colourimetrically by the Zimmermann reaction test (Table XVIII b).

Mixtures of all steroids identified in each local drug were prepared on basis of weight per 40ml of whole crude drug and injected in 0.2 ml. of oil daily for 7 days into castrated rats (page 117).

Two sets of littermate castrated rats were used. Each set was made up of 4 groups of 4 rats each. Each rat was castrated (page 109) and injection was started two weeks after castration. Another two groups, one of these groups being castrated, served as controls. One set of rats were treated with mixtures of steroid extracts of the crude drug. The other set of rats were treated with the whole crude drug.

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24 hours after the last injection, the rats were killed and the seminal vesicle as well as the prostate gland were dissected out, cleaned, and weighed to the nearest milligram, (page 111).

Urine was collected from the rats at 48-hour intervals over the period of the experiment (page 111), and the amount of 17-ketosteroid estimated for each rat (page 112). The results obtained are recorded in Tables XXV to XXVII.

R E S U L T S

The responses of the seminal vesicle, prostate gland as well as the amount of 17-ketosteroids excreted were very much higher with the whole crude drug than with mixtures of steroidal extracts in each of the local drugs.

This results suggest that there are other substances in the whole crude drug which augment the actions of the steroids in the local drugs.

Since it has been established reasonably well in the early experiments reported in this thesis, that all the supposed steroidal substances in the local drug had been extracted and presumed identified then the disparity in the results must definitely be due to the presence of other non-steroid substances in the local drugs. Substances that could enhance the action of steroids include acetates, amino acids and proteins.

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TABLE XXXV.

COMPARISON OF THE EFFECTS OF WHOLE CRUDE LOCAL DRUG WITH THE MIXTURE OF EXTRACTS IN EACH LOCAL DRUG ON THE WEIGHT OF THE PROSTATE GLAND OF PRE-PUBERTALLY CASTRATED RATS.

WHOLE CRUDE LOCAL DRUG	PROSTATE GLAND MEAN ± S.E.M. (mg)	MIXTURES OF EXTRACTS IN	PROSTATE GLAND MEAN ± S.E.M. (mg)
CASTRATED CONTROL	21.0± 0.2	CASTRATED CONTROL	21.0± 0.2
A_1	126.0± 0.3	A_1	102.0± 0.3
A_2	87.0± 0.2	A_2	69.0± 0.1
A_3	47.0± 0.1	A_3	33.0± 0.2
A_4	116.0± 0.3	A_4	103.0± 0.1

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TABLE XXXVI

COMPARISON OF THE EFFECTS OF WHOLE CRUDE LOCAL DRUG WITH THE MIXTURE OF EXTRACTS IN EACH LOCAL DRUG ON THE WEIGHT OF THE SEMINAL VESICLE OF PRE-PUBERTALLY CASTRACTED RATS

WHOLE CRUDE LOCAL DRUGS	SEMINAL VESICLE MEAN ± S.E.M. (mg)	MIXTURE OF EXTRACTS IN	SEMINAL VESICLE MEAN ± S.E.M. (mg)
CASTRATED CONTROL	13.0 ± 0.1	CASTRATED CONTROL	13.0 ± 0.1
A_1	72.0 ± 0.1	A_1	48.0 ± 0.3
A_2	45.0 ± 0.1	A_2	36.0 ± 0.3
A_3	19.0 ± 0.3	A_3	16.0 ± 0.2
A_4	65.0 ± 0.1	A_4	53.0 ± 0.2

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TABLE XXXVII

COMPARISON OF THE EFFECTS OF WHOLE CRUDE LOCAL DRUG WITH MIXTURES OF EXTRACTS IN EACH LOCAL DRUG ON 17-KETOSTEROID EXCRETION IN 48-HOUR URINE OF PRE-PUBERTALLY CASTRATED RATS.

WHOLE CRUDE LOCAL DRUGS	17-KETOSTEROIDS MEAN ± S.E.M. (mg /48 HOURS)	MIXTURES OF EXTRACTS IN	17-OXOSTEROIDS MEAN ± S.E.M. (mg/48 HOURS)
CASTRATED CONTROL	0.001± 0.0001	CASTRATED CONTROL	0.001± 0.0001
A_1	0.019± 0.002	A_1	0.012± 0.003
A_2	0.016± 0.004	A_2	0.010± 0.002
A_3	0.009± 0.003	A_3	0.007± 0.001
A_4	0.021± 0.008	A_4	0.017± 0.004

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EXPERIMENT XI

Comparison of the effects of the whole crude local drug with the mixture of extracts in each local drug on the capon's comb size.

The method of separation, purification, identification, estimation of individual steroids in each local drug has been reported in experiment IV.

Since 40ml of the crude local drug is the recommended dose, the amount of the individual steroids in this dose was estimated (Table XVIIIb). All extracted steroids in each local drug were combined on weight basis per 40ml and injected in 0.5ml oil daily for 7 days into capons (page 117).

Two sets of 32 birds were selected randomly. Each set was made up of 4 groups of 8 birds each. Each bird was caponized (page 106) and injection was started two weeks after caponization. Another two groups, one of these groups being castrated, served as controls. One set of capons were treated with mixtures of the steroidal extracts from each of the 4 local drugs. The other set of capons were treated with the whole crude drug (page 115).

The comb of each bird was measured (page 109) daily throughout the period of the experiment. The data obtained are shown in Table XXVIII.

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TABLE XXXVIII

COMPARISON OF THE EFFECTS OF WHOLE CRUDE LOCAL DRUG WITH MIXTURES OF EXTRACTS IN EACH LOCAL DRUG ON THE CAPON COMB SIZE

WHOLE CRUDE DRUG	COMB SIZE (mm) MEAN ± S.E.M.	MIXTURES OF EXTRACTS IN	COMB SIZE (mm) MEAN ± S.E.M.
A_1	7.45 ± 0.52	A_1	5.10 ± 0.05
A_2	5.82 ± 0.23	A_2	3.50 ± 0.21
A_3	10.48 ± 0.07	A_3	8.52 ± 0.35
A_4	5.70 ± 0.48	A_4	3.72 ± 0.28
CASTRATED CONTROL	0.15 ± 0.01	CASTRATED CONTROL	0.15 ± 0.01

Results are mean values of 4 determinations with their standard error.

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R E S U L T S

As with the last experiment (X), the growth of the capon's comb was higher in the set treated with the whole crude drug than the set treated with mixtures of steroidal extracts.

The same argument also go for this results and that is, the presence of substances such as amino acids and protoins which are known to aid sex steroid hormones in the activation of the enzymes in their target organs (Ville et al 1960).

Comparison of table XXXVIII with tables XXXV to XXXVII also showed that whereas, A_3 Gave the least response with rats, it gave the highest response with the capons. This as suggested earlier might be due to the presence of certain substances which the rat cannot utilise or due to species differences.

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CHAPTER FIVE

DISCUSSION

It is widely accepted that variations in the state of secondary sex characters in the male are due to variations in the amount of hormone produced by the testis, the transformations of puberty being regarded as a manifestation of an abrupt and great increase in testicular hormone secretion and senile changes being attributed to dimunition or cessation of this secretory activity.

There are, however, few data on hormone production at different stages in the life cycle. Womack and Koch (1932a), without citing values for individual animals, report the highest concentration of testis hormone in fetal and young calves with a progressive decrease thereafter. Funk et al (1929b) report less comb growth promoting activity in the urine of old men than in the urine of young adults. Womack and Koch (1932b) state that no hormone occurs in the urine of boys under 10 years of age, while after 10 years, its concentration becomes as great as in the adults

Hooker (1937) reported a progressive increase in hormone content of bull testes up to 2 years after

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which there is a sharp increase which is continued to 5 years. After 5 years the hormone content gradually decreases. It is now an accepted fact that there is a natural decline in the general activity of the body with old age. Medical scientists, especially the biochemists are well aware of this situation and attempts have been made to correct or at least improve, if only temporarily, some of the disturbances that results from this reduced activity.

One of these reduced activities is that of decline in sexual arousal. It has often been assumed that senility in man is precipitated by failure of testicular function, and conversely that restoration or replacement of such function in old age would result in rejuvenation. In the past, attempts to effect rejuvenation have usually been based on methods alleged to stimulate the androganic tissue of the senescent tissue on transplantation of testes from a young individual. In modern medicine, it is based on the administration of the testis hormone-testosterone - or its ester-testosterone propionate.

Administration of androgen has been shown to stimulate the metabolism and restore to some extent the virility of old men. The literature is replete

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with information on the treatment of sexual decline in old men with daily injections of testosterone propionate doses (Hamilton, 1937), Kinsey et al (1948); McCullagh (1939); Nowakowski and Lenz (1961). The treatment was based on early observations that reduced sexual activity is accompanied by reduced androgen excretion in the urine (Callow, 1938). Castration studies by Kochakian (1938) and by various other workers confirmed this. The isolation and characterisation of the major hormone by David et al (1935) laid the basis for the treatment of reduced virility in old age with testosterone.

In Africa, organ magic has figured in the old superstition of many of its peoples. There are several types of cures such as hare's brains for nervous disease, wolf's liver for hepatitis, powdered elephant skull for epilepsy and animal sex organs for sexual incapacity.

The management of any case in general involves divination to diagnose the case and to prognosticate and then treatment of symptoms. Some basic steps of treatment are (a) propiation of offended spirits with the appropriate sacrifices (b) treatment of symptoms with extracts from plants or animals or mixture of both. Cure by suggestions based on psychological therapy, wearing of

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charms in form of amulets, scarification and local application of medicines and by attempting to transfer certain animal qualities to man. The heart of a lion may be prescribed for a weak and timid child, while the head of a tortoise is added to the prescription for curing mental retardation (Adesoye 1971); Ahonkhai (1971).

We have selected for analysis four local medicinal preparations which are claimed to have a virilising effect on the male. These preparations commonly called "agbo mokole" (penile erection drugs) were bought from a local market about 6 miles from the university. Since it has been established beyond reasonable doubt that the mating activities of many species are heavily dependent on testicular secretion; we decided to analyse for androgens in the locally purchased drugs which are claimed to have stimulatory effects on sexual activity.

In endocrine research, the most important thing is to find a test for whatever hormone one suspects to exist, - some sharp cut characteristic effect that is lost when the gland is removed and that can be restored by giving back the gland in implants or injections. In this case, it is known that the

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testis is responsible for the production of the hormone testosterone and that removal of this organ or fall in the level of the circulating hormone in the blood, could lead, among other things, to a reduction in the sexual arousal in man.

In studies of substances or extracts containing androgenic material, the biological assay method is obviously indispensable. Biological assays are method for the estimation of the nature, constitution, or potency of a material by means of the reaction that follows its application to living matter. The typical bioassay involves a stimulus applied to a subject to produce a response. The relationship between dose (stimulus) and response can be used to indicate the potency of a dose from knowledge of the responses it produces. Bioassay methods are needed for the characterisation of a new androgen which must include its physiological action (quantitative) and its relative activity (quantitative).

Two most important types of biological assay methods for androgens based on their ability to stimulate sex characterisation (qualitative) are presently in use. The rat assay test and the capon comb test. Biological assays

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Two most important types of biological assay methods for androgens based on their ability to stimulate sex characteristics (qualitative) are presently in use. The rat assay test and the capon comb test. Biological assays

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are usually comparative, the estimate of potency being obtained relative to a standard preparation of the stimulus In experiment I, seven experimental doses of standard preparations of testosterone were used. On the other hand, the whole crude "drug" prepared in dose form as described in chapter three, were also used. The weight of the seminal vesicle and prostate gland in the castrated rats were used as the index of response. In Table I, it was obvious that with castration there was a rapid atrophy of the seminal vesicle and prostate glands.

Among the several functions of the testis hormone is their ability to promote nitrogen retention by increasing protein synthesis and by decreasing the rate of amino acid breakdown in their target tissues. "In vitro" studies by Litwack and Kritchovsky (1964); Wilson and Loeb (1965); Bruchovsky and Wilson (1968); have demonstrated that androgens promote the effective synthesis of ribonucleic acid and increased incorporation of amino acid into protein within the nuclei of various target tissues of these hormones. Hence, castration, or removal of the testes results in the decrease of the rate of protein synthesis in these organs. This leads to an atrophy of these organs. Table I therefore

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confirms the work of various earlier investigators (Moore and Gallagher: 1930); Korenchevsky et al (1936); Mathieson and Hays (1945). Reversal of these changes is effected by androgen replacement.

Table II, revealed that the prostate glands tend to be heavier than the seminal vesicles. This confirmed the observations of Korenchevsky et al (1936). Explanation to this might lie on the fact that the seminal vesicle serves mainly as a storage organ for the spermatozoa with little secretory activity, while the prostate glands act mainly as a secretory organ. The milky fluid secreted by the prostate glands contain citric acid alkaline phosphatase and spermine which adds to the bulk of the semen. The alkaline characteristic of the prostate fluid may be quite important for the successful fertilisation of the ovum, for the fluid of the vas deferens is relatively acid owing to the presence of metabolic end products of the sperm, and hence inhibits sperm fertility. This very important function of the prostate might be responsible for its relatively large weight compared with the seminal vesicle.

Table II and Fig1 further revealed that there was a linear increase in the weight of the seminal vesicle and the prostate gland with increase in dose of testosterone.

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In Fig. 10 when dose was plotted against response, a curve was obtained. The curve gave a straight line from 0.2mg daily dose of testosterone to 2.0mg daily dose after which it was no longer linear. This means that the maximum dose required to obtain the maximum response in the rat is about 2.0mg of testosterone daily.

Comparing Table I and II, the weights of the two glands investigated showed that castrated rats injected with about 1.0mg testosterone daily had similar growth to the normal control. This suggests that about 1.0mg of testosterone is required by a normal rat of about 100 gm body weight to maintain its prostate glands and seminal vesicle. Exact figures could be obtained when Fig. 10 was extrapolated. It showed that about 0.6mg of testosterone daily was required to maintain the prostate gland and approximately 0.9mg testosterone daily wally was required for the maintenance of the seminal vesicle in rats of this size.

The responses obtained with the local medicinal preparations are recorded in Table III. All the local drugs except the one designated as A_3 gave significant responses when they were injected, compared with the castrated controls injected with oil only.

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Fig12 showed that the response was highest with A_1 and A_4.

Having established earlier that the response of the seminal vesicle and prostate gland in terms of their growth, is dependent on the testicular hormones, it is safe to assume that since there was a significant response in the two organs to the administration of the local drugs, then the local drug must at least contain some androgens which have initiated the responses obtained. The low response obtained with A_3 might be due either to the absence or presence of little androgenic steroid in the preparation. The word 'absence' is used here with some reservations, in that, it is possible that there might be some substances in the plant extract of A_3 which are inhibiting its action in the rat, or, that, androgens might be present in a form that could not easily be utilised by the rat. However, there was a slight growth, although not statistically significant, in the prostate gland and the seminal vesicle when compared with the castrated control.

In experiment II, we tried again to see the effect of the four local drugs on the growth of the capon comb using the seven experimental doses of testosterone as a standard preparation of the stimulus, and the four

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local drugs prepared in dose form as described in chapter three.

A capon is a castrated cockerel. One of the several castration effects in the bird is the atrophy of the comb. The capon's comb has served as a test object for the evaluation of androgenic activity since the time of Berthold (1849) classic experiments. Detailed studies on the capon method have been reported particularly by Gallagher and Koch (1935); Greenwood et al (1935); McCullagh and Cuyler (1939).

The method is based on the fact that when cockerels are castrated there is a gradual decrease in the comb size which can be reversed by administration of androgens. This method like the rat assay method is based on the physiological function of androgens in promoting protein synthesis in their various target organs. This as stated earlier is based on the ability of the androgens to stimulate the enzymes responsible for the incorporation of amino acids into proteins in the tissues.

Table IV showed clearly that there was a significant drop in the size of the cap on's comb two weeks after caponisation. This proves that the testis is responsible for the maintenance of the size of the bird's comb, and that its removal does results in the reduction of the

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capon comb size. This confirms the work of early investigators like Galloghor and Koch (1935), Deanesly and Parkes (1937).

Tables IV and V compared, showed that the growth of capon's comb on daily dose of 1.0mg of testosterone were similar to those of the normal control and that daily injection of about 1.0 mg of testosterone is equivalent to the comb growth-promoting activity of the normal testis. A plot of comb size against dose (Fig. 14) gave a curve. The straight line part of the curve was maximal at about 1.5 mg daily dose of testosterone. The linear increase in the size of comb with increase in the dose of testosterone is quite clearly depicted in Fig. 15 when log dose was plotted against response. Extrapolation of this graph showed that exactly 0.8mg of testosterone was required to maintain the combsize of the capon daily.

Table VI and Fig.16 indicated significant growth of comb for each of the local drugs when they were administered to the capons. The highest response was obtained with A_3 followed by A_1 A_2 and A_4 also gave significant results compared with the castrated controls.

Since we now know that only androgens could restore the comb growth promoting activity of the normal testis, it seems reasonable, to assume that all the local drugs

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contain significant amount of androgens capable of stimulating comb growth in capons.

In experiment III we tried to determine the effects of 17-ketosteroids in the urine of castrated rats. The 17-ketosteroids comprise a group of steroid compounds which, as the name indicates, have in common, a ketone group at position 17 of the steroid nucleus (Cyclopentanoperhydrophenanthrene). Because of this keto group, they are characterised by certain colour reactions. Some urinary 17-ketosteroids have androgenic properties while others are biologically inactive. The quantitative pattern of the urinary 17-ketosteroid is important for an understanding of certain diseases and measurement of their quantity is often an aid in clinical diagnosis and in the interpretation of changes in testicular and adrenal cortical function in the male. The urinary content of 17 ketosteroid is assumed to reflect the male hormone content of the blood or of actual androgens.

The colourimetric method that has been used most widely for the determination of 17 ketosteroids are based on the reaction of the group - CH_2CO - with m-dinitrobenzene in alkaline solution. Zimmermann (1935) first demonstrated the usefulness of this reaction for the quantitative

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determination of pure ketonic steroids, hence the reaction is usually known as the Zimmermann reaction when it is applied to steroids. 17-ketosteroids have been found in the urine of various species. It is clear from these studies that steroid output varies a lot in different species of animals.

In the estimation of these compounds in the rat's urine considerable difficulties were encountered especially with the tremendous amount of coloured compounds formed with the Zimmermann reagent. Again the values obtained were very low even with administration of testosterone, that one, is apt to suggest that the urine is not the most important excretory route for the excretion of 17-ketosteroids in the rat. The possibility of its excretion through the bile and the faeces should be investigated. However, a series of results were obtained after several efforts which were useful to some extent.

Although the amount of 17-ketosteroids in the urine were very low, it was clear that (Table VII) there was a drop in the level of these compounds in the urine following castration. The adrenal glands are also known to contribute a bit to the total amount of urinary 17-ketosteroid and this might account for the fairly

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high value obtained with the castrated control. As anticipated, there was a linear increase in 17-ketosteroid excretion with increase in testosterone injection (Table VIII, Figs. 17 and 18), although as stated earlier, there is reason to believe that the urine is not the major excretory route of 17-ketosteroids in the rat. Table IX and Fig 19 indicated the presence of androgens in the three local drugs designated as A_1 A_2 and A_4 with a rather low response from A_4. The amount of 17-ketosteroid in the urine is assumed to reflect the amount of the androgen in the local drugs. These results compare favourably with those obtained in experiment I and leads to the same conclusion that steroids are present in the local drugs designated as A_1 A_2 and A_4 with little or no steroid hormone in A_3.

With the strong suspicion based on the last three experiments that androgens are possibly present in these 4 local drugs, we decided in experiment IV to separate, identify and estimate the various androgenic steroids, if any, in the various local drugs.

There is no standard method for the extraction of androgens from tissues or other body fluid although it is known that androgens are generally soluble in organic

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solvent. All the 4:1 and the weight to volume
in methanol or gin. The recommended concentration
dosage of the ethanolic extract daily. In methanol
local drugs in absolute alcohol, considering the amount
of local in the ethanol, and a wine glassful of this
ethanolic extract (about 40 ml) was taken at the prescribed
daily dose.

Since endrogens are soluble in absolute alcohol, we
assumed that all the endrogens possibly present in each
of the local drug would have been involved in the ethanolic
extraction. The ethanol was removed with the rotary evaporator
and the residue, mixed with the equivalent amount of water,
was hydrolysed with acid. The hydrolysis was necessary to
make sure that all the endrogens in conjugated forms, if
any, were split into the free endrogen and the conjugated
form. The detailed method followed was as described in
Fig. 9).

Extraction of the endrogens in the hydrolysed local
drug was with ether and was done in three stages,
(Fig. 9). The exhaustive extraction of each of the
finely powdered local drug was a modified method of Lehrman
and Lipschutz (1950). The first stage of the extraction
is believed to lead to the extraction of the free endrogens
and endrogens partially combined with sulphate ion (A).

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catabolism in the animal. In experiment V we compared the effects of individual androgen in each of the local drugs with their individual equivalent pure steroids on the weights of the accessory sex glands of the castrated rats.

In our own case, it was not necessary to dissolve our androgen in serum albumin before they were injected into the experimental animals. This was because, the amount being injected were so low, such that they can easily diffuse into the blood system. Again, the anabolic effects of androgens on the accessory sex organs were used as an index of their activity.

As with earlier experiments, there was a clear indication of atrophy of accessory sex glands in rats after castration as well as a reduction in the amount of 17-ketosteroid excreted in the urine in 48 hours. This was due, as stated earlier, to the removal of the testis which is the main organ responsible for the production of the male sex hormone which in turn is responsible for the growth and maintenance of the accessory sex glands as well as maintaining the 17-ketosteroid level in the urine so as to reflect its amount in the circulating blood. (Tables XIX and XXII).

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When an androgen is injected into an animal, a large percentage of it quickly disappears from circulation and is carried into the tissues. The androgen might go directly to its target organ in the form that is injected, or is first metabolised in the liver or kidney, and the metabolite which might be more or less potent than the original androgen might then be transferred to the target organ. To a large extent, the androgens are also inter-convertible therefore, injection of an androgen may result in the circulation of other androgen whose potencies may be greater or less than the androgen injected.

There were similarities in the increase weights of the accessory sex glands of castrated rats when individual androgen identified in each of the local drugs were injected into a set of rats and their responses compared with another set of rats injected with their equivalent pure steroid.

Although the responses were not exactly equal, yet they were close enough to suggest that the steroids which were presumed present in each local drug were the same as the authentic steroids used for comparison.

Very low responses were obtained with six of the extracted androgens as well as with their presumed equivalent standard steroid.

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Further work will have to be done before any meaningful explanations of this observation can be made.

The pattern of 17-ketosteroid excretion was similar to the growth of the accessory sex glands. As mentioned earlier, considerable difficulties are normally encountered in the determination of these compounds in the urine. However, their estimations (Table XXII to XXIV) did prove of value, since they showed a decrease value after castration and their relative low values correspond with the six androgens which also gave low responses with the weight of the accessory sex glands of the rat suggest that the androgens are probably potent but are present in low amount or could not be effectively utilised by the rat. It is worthy of note that all the three androgens extracted from the local drug designated as A_3 are among the six identified androgens that are producing the low responses in the rat.

In experiment VI we compared the effects of the androgenic substances in each of the local drug with the presumed individual equivalent pure androgens on the growth of

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the capon comb. Although the capon comb test is based also on the anabolic effect of androgens yet it served other purposes in our experiments. First, it has served as a comparative test for the action of androgens on rats and capons. It has also served as a confirmatory test to the action of androgens on rats. As with the rat test, there was a significant reduction in the comb size following the castration of the cockerels (Table XXV). This as earlier stated confirmed the theory that the testis is responsible for the growth and maintenance of the capon comb size. When the individual androgens from each of the local drugs were administered to the capons, there was, in most cases a comparable responses of the comb, to that of the presumed equivalent standard steroids.

Fairly low responses were obtained with three individual androgens, one each from A_1 A_2 and A_4 when compared with their equivalent standard steroids.

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however it is worthy of note again that unlike with the rat test, all the three individual androgens extracted from A_3 gave comparative responses with the standard steroid (Table XXVII). Whereas in the rat test, steroid hormones from A_3 gave very low responses compared with steroids from the other local drugs (Table XXIII and XXIV).

It is known that certain variables such as nature and volume of oil injected, the period of injections, time of measurement and mode of application of androgens, among others tend to influence biological assay methods. Dorfman and Parker (1936) found that the presence of oestrogens in an injection of androgens into rats have an additive action on the seminal vesicle and prostate tests. Korenchevsky et al (1936) also found that the effect of testosterone on the seminal vesicle and prostate gland of castrated rats could be doubled if a physiologically impure extract, prepared from the tissues of animals or plants were injected simultaneously. Koch (1937) found that the presence of impurities such as fatty acids in the preparation which affect the action of testosterone in rats do not have

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this effect in capons treated by intramuscular injection. The capon test thus has the advantage that provided injection is subcutaneous, or intramuscular, the exact technique and the presence of impurities do not greatly influence the result.

In view of these reports, we decided to investigate the presence of other biochemical compounds, regarded as impurities in the various "drugs". The detailed analysis is reported in experiment VII. All the biochemical tests reported were carried out as described by Bassir (1963). Apart from androgenic steroids which have been separated and extracted, fatty acids, lipids as well as amino acids and proteins were also detected in all the local drugs. Phenols were present in A_3 alone (Table XXVIII).

The mechanism of action of those impurities on androgens in influencing their action is not yet known but there is the possibility of competition between the impurities and androgens for the enzyme active sites where the androgens begin their actions by forming an androgen - enzyme complex which in effect activate the enzyme.

In experiment VIII an attempt was made to compare the effects of mixtures of individual androgens in each

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"local drugs" with mixtures of the equivalent presumed pure steroids on the weights of the accessory sex glands of the castrated rats, and on the excretion of 17-ketosteroids in the 48-hour urine of the rats. In all cases, the responses of the rats in terms of increases in weights of accessory sex glands were higher with the presumed equivalent steroids than with the mixtures of extracted androgens for each "local drugs" (Table XCIX and C). However the percentage difference between the two series of androgens was low enough to show the similarity in their response.

The pattern of responses was also similar in both cases with mixture of androgen extracts from A_4 and its presumed equivalent steroid giving the highest response while again the mixtures of androgens from A_3 and its presumed equivalent pure steroid giving the lowest response. The pattern of 17-ketosteroid excretion also followed those of the accessory sex glands.

Results of this experiment, combined with those of experiment V, indicate clearly that not only are androgens present in the extracts but that the responses obtained so far were due to the presense of these androgens in the various extracts. Experiment IX further confirmed the above conclusion when the effects

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of the mixtures of the androgens extracted from each "local drugs" was compared with those of the mixtures of their presumed equivalent pure androgens on the capon's comb. The responses obtained are shown in Tables XXXIII and XXXIV. The only difference as we have noticed all along was the large responses always associated with extracts from A_3 when administered to capons and the low responses obtained when administered to castrated rats.

Having established in experiment VII that other substances are present in the whole crude drug we again decided to compare the effects of the whole crude drug with the mixture of androgens in each of the local drug on the weights of the accessory sex glands of the castrated rats as well as in the excretion of 17-keto-steroids in the 48-hour urine. The details of the experiment has been reported in Experiment A. The responses obtained are recorded in Tables XXXV to XXXVII.

The responses of the accessory sex glands and the amount of 17-ketosteroids excreted were very much higher with each of the whole crude drug when compared with mixtures of androgens extracted from each of the local drugs. These results suggest and confirm the observations of early workers (Deanesly and Parkes (1936), Koranchevsky et al (1936) that the presence of impurities

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might augument the actions of androgens in the whole crude extract. It is also possible that not all the androgens have been extracted from the local drugs.

In experiment XI, which was similar to the last experiment except that capons were used instead of castrated rats, the increase in comb size in capons injected with the whole crude drug were also higher than with those injected with mixtures of androgens extracted from each of the local drugs.

As stated earlier, the mechanism by which the impurities alter the action of androgens are not known. But a possibility is their activating action on the enzymes that catalyse the androgen dependent processes in the body. Also, since the index we have used (increase in weight of accessory gland and comb size) depend on protein build up in these organs it is possible, that the amino acid and protein in the whole crude drug are easily utilised by the body in the formation of protein in the target tissues. It is also known that acetates are precursors of amino acids. The presence of fatty acids and lipids in the crude extract, might have an effect on the formation of useful amino acids which might then be used for the growth of the target organs.

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It is worthwhile to mention at this point that all the various experiments carried out so far have pointed to the fact that there are definitely male steroid hormones in the "drug" designated as A_3. This local drug is a concoction of five different types of roots. It is not possible at this stage to say exactly which one of them is really responsible for the production of the male steroid hormone. But it is clear that androgens are present in the drug.

Worthy of note also is the continuous difference in the response of castrated rats and capons to both the whole crude drug designated A_3 and its purified extracts administered either singly or as a mixture. While the response in the rat is usually very low, it is high in the capons. What is responsible for this is not clear yet, but there is a very strong possibility of species differences in the metabolism of these androgens extracted from plant roots. Further experiments will be performed to clarify this point.

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SUMMARY

Four local virilising medicinal preparations made from animal and plant parts have been analysed. Bioassay and chemical methods were used for their qualitative analysis on castrated rats and capons. The various androgens in the four preparations were separated and purified by thin-layer chromatography. They were identified by both colourimetric and infrared spectrophotometric methods, and estimated by the Zimmermann reaction.

By injecting the extracted androgens either singly or as a mixture into castrated rats and capons and comparing their responses with their equivalent pure androgens further pointers as to their identities were obtained.

It is assumed that the presence of impurities in the whole crude preparation might be responsible for the high responses produced by this preparation when compared with mixtures of the extracted androgen from each of the "drugs".

It was shown that androgens were present in a concoction of plant roots and that there is a possibility of species differences in the metabolism of these androgens from plant roots by castrated rats and capons.

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CONTRIBUTION TO KNOWLEDGE

1. Androgens capable of stimulating the male sex organs are present in four local virilising preparations. They have been isolated and purified. It is tentatively suggested that they might be identified as Androstenedione, 5β-Androstan, 3, 17, dione, Dehydro-iso-androsterone, and testosterone ester.
2. Urinary 17-ketosteroid excretion in rats is shown to be minimal.
3. Compounds with Androgenic properties are present in a concoction of plant roots which are capable of stimulating the male sex organs.

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ABBREVIATIONS.

1. Quotation of References

In the text, the following system of reference abbreviations is used.

(i) For a single author:- the name of the author and year of publication.

(ii) For two authors:- the names of the two authors and year of publication.

(iii) For three authors:- the name of the author first mentioned followed by et al and year of publication.

The list of reference is in alphabetical order of first authors.

2. System of Journal abbreviation

The system of journal abbreviation used in the list of references in this thesis, is that adopted in the World List of Scientific Periodicals London 4th edition; Butterworth scientific publications, (1963 - 1965 and supplements).

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