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This is to certify that the undersigned have read and recommend to the Committee on Graduate Studies for acceptance, a thesis submitted by Mervyn J. G. Huston, B.Sc. entitled:-

THE EXCRETION OF ESTROGENS AND ANDROGENS IN THE URINE

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THE EXCRETION OF ESTROGENS AND ANDROGENS IN THE URINE

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PREFACE

An essential antecedent for an investigation of any branch of sex function is a clear understanding of the fundamentals of the sex cycle. For this reason a brief resume of the basic phenomena is presented here, as a preface to the limited field of this investigation.

It is now well established that at puberty, certain hormones secreted by the pituitary gland (gonadotropins), stimulate the gonads, initiating and controlling Graafian follicular maturation, ovulation and corpus luteum formation. As a consequence of this stimulation, the ovary elaborates estrogens from the Graafian follicles and progestogens from the corpus luteum. In the male, the interstitial tissue of the testes is stimulated to produce androgens.

This phenomenon occurs cyclically during the period of sexual maturity in the female and the structural and secretory changes in the ovaries are reflected in periodic alterations in the endometrium and myometrium, in the vaginal mucosa and in the mammary glands.

The estrogenic hormone secreted by the ovarian follicles is essential for initiating and controlling the functional activity of the female organism, secreted in sufficient amount it produces the so-called proliferative phase of endometrial development, increases the secretory activity of the uterine serous glands,

enhances the contractility of the uterine muscle and causes proliferation of the mammary duct system. When ovulation occurs at approximately the fourteenth day of the cycle, the follicle ruptures and is converted into a corpus luteum. As this develops it secretes a progestogen. The period of activity of the corpus luteum is about fifteen days and the progestogen secreted during this time converts the vascular proliferated endometrium into a functional secretory type, preparing it for the nidation of the ovum which is now travelling down the Fallopian tube toward the uterus. The progestogen nullifies any further action of the estrogen, producing thus relaxation of the uterine muscle and stimulating acinar development in the mammary gland. Changes paralleling these in the endometrium take place in the vaginal mucosa, and these offer a basis for some diagnostic procedures in evaluating ovarian hormone function (e.g. vaginal smear).

If ovulation is followed by fertilization, the duration of corpus luteum activity is prolonged and a decidua is formed. If fertilization does not occur the corpus luteum degenerates and as a direct consequence, degenerative changes take place in the highly specialized endometrium, resulting in its shedding or menstruation. This is associated with involutional

changes in the mammary gland, and mucosal changes in the vagina. The cycle is then repeated. If fertilization occurs and pregnancy supervenes, at about the end of the third lunar month, the placenta takes on the function of an endocrine gland and secretes large amounts of progestogen. This tends to stabilize pregnancy.

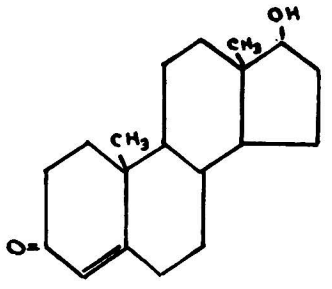
NOMENCLATURE AND CLASSIFICATION OF SEX HORMONES

Androgens:- A collective term for all substances which are able to restore, to some extent, the male genital tract following castration atrophy; or stimulate its growth directly; or induce or maintain, or both, the secondary sex characteristics of the male.

Testosterone

(the true hormone of the testis).

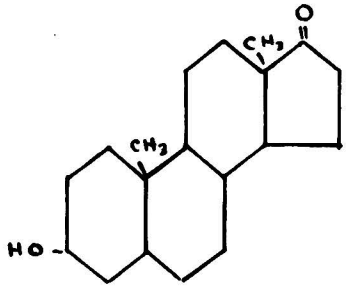
Sources: male urine and synthetically, by degradation of cholesterol.



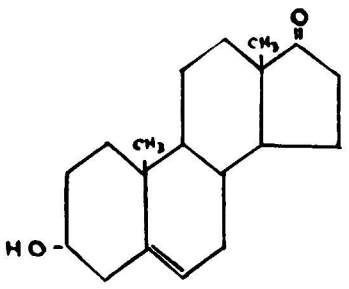
Androsterone

(an excretion product of testosterone).

Sources: male urine and synthetically, by degradation of cholesterol.



Dehydroandrosterone
(probably an intermediate product in the synthesis of testosterone within the body).



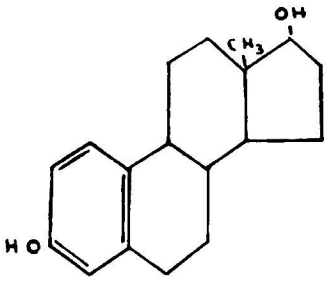
Sources: male urine and synthetically by degradation of cholesterol.

Gynecogen:- A collective term for all substances which are able to restore, to some extent, the female genital tract following castration atrophy; or stimulate its growth directly; or induce or maintain, or both, the secondary sex characteristics of the female.

This group is subdivided into two groups of substances: estrogens and progestogens.

Estrogen:- A collective term for all substances producing an estrous (proliferative) growth in vagina, uterus and mammary glands, and female secondary sex characteristics.

Estradiol
(the true follicular hormone).

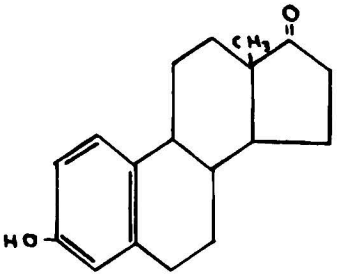


Sources: Ovary and pregnancy urine.

Estrone

(an excretion product of estradiol).

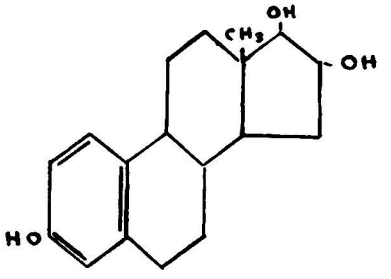
Sources: Urine



Estriol

(an excretion product of estradiol).

Sources: Pregnancy urine and placental tissue.

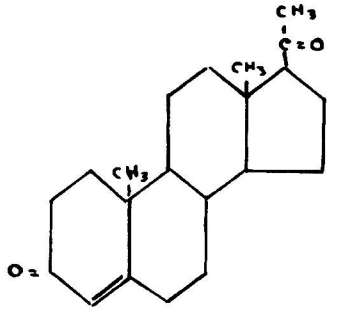


Progestogen:- A collective term for all substances which have the property of producing progestational changes in the female genital tract.

Progesterone

(the corpus luteum hormone).

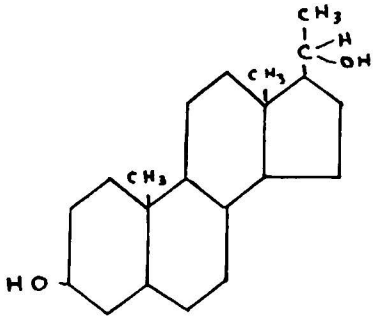
Sources: Corpus luteum and synthetically from stigmasterol.



Pregnanediol

(an inactive excretion product of progesterone).

Sources: Pregnancy urine.



Section I

THE EXCRETION OF ESTROGENS AND ANDROGENS IN THE URINE

Introduction

This investigation presents an examination of some of the methods of extraction, purification and assay of urinary estrogens and androgens. Some of the variables effecting these determinations are investigated with a view to improving and simplifying the processes; and ascertaining more of the chemical characteristics of the substances excreted. A standard procedure for extraction, purification and assay of both groups of hormones has been developed in order to establish a basis for comparing and correlating the findings of various investigators.

Scope

In all cases total estrogens and total androgens are determined, no effort being made to separate them into their constituent parts. Although some information of physiological significance is derived from the laborious differential assay of the different estrogenic and androgenic fractions, (1), the total excretion values are as satisfactory an index for clinical use as the confused picture of sex endocrine metabolism permits.

In the cases of assays on urine from patients, a thorough clinical examination is not included beyond

a statement of their gross pathologic condition. The course of the disease or of treatment where treatment was instituted, is not presented in detail, except insofar as that information is relavent to the findings, since minute variations in the clinical picture of sex endocrine dyscrasiae are of little significance until the gross changes are understood.

Review of Literature

Ever since Ascheim and Zondek discovered estrogenic material in pregnancy urine in 1927 (2), and Loewe and Voss found androgenic material in male urine in 1929 (3), extraction of urinary sex hormones has been the subject of much interest and investigation. Generally speaking, investigations were performed with two purposes:-

A. It was thought that information obtained from urinary hormone assays might be used as an index of hormone economy within the body, and,

B. that an examination of the degraded forms in which hormones are excreted might assist in the development of ideas regarding their metabolism. In both respects the investigations have been successful to a limited degree.

The use of urinary hormone assay as an index of hormone economy is possible only in a very general manner. Siebke has stated (4) that hormone assays on

excreta cannot give an exact idea of general hormone economy. Considerable confirmatory evidence for this view has been recorded (5)(6)(7). Both sexes excrete so-called "male" and "female" hormones in amounts that overlap considerably (7), but on the average, normal women excrete more estrogenic material and less androgenic material than do normal men (8). Different workers are in disagreement as to the significance of the androgen to estrogen excretion ratio, (7)(8)(9)(10). There are wide variations between normal individuals and also wide variations in the daily excretion of the same individual (7)(11)(12).

Excretion values from abnormal subjects of both sexes overlap considerably with each other and with normal, but tend to parallel the degree of hypo- or hyper- function (7). In some pathological conditions extremes of excretion occur. Thus for example, the urine of an individual with adrenal cortical tumour contains huge amounts of androgenic material (13)(14), while that from a case of granulosa cell tumour of the ovary has a very high estrogen content (15)(16). In these conditions urinary hormone assays present certain diagnostic evidence of specific endocrine dysfunction and assist in differentiating between these syndromes and others in which the clinical findings may be quite

similar (14)(17).

The excretion values for estrogens are a little more satisfactory than are those for androgens since more is known of their production, distribution, utilization and destruction. In females the excreted amount varies during the menstrual cycle, with two peaks of excretion: one approximately at the time of ovulation and one just prior to disintegration of the corpus luteum. The excretion drops to zero very shortly preceding menstruation (18)(19)(See also chart #1). Excretion during pregnancy rises steadily as pregnancy advances (20)(See chart #2).

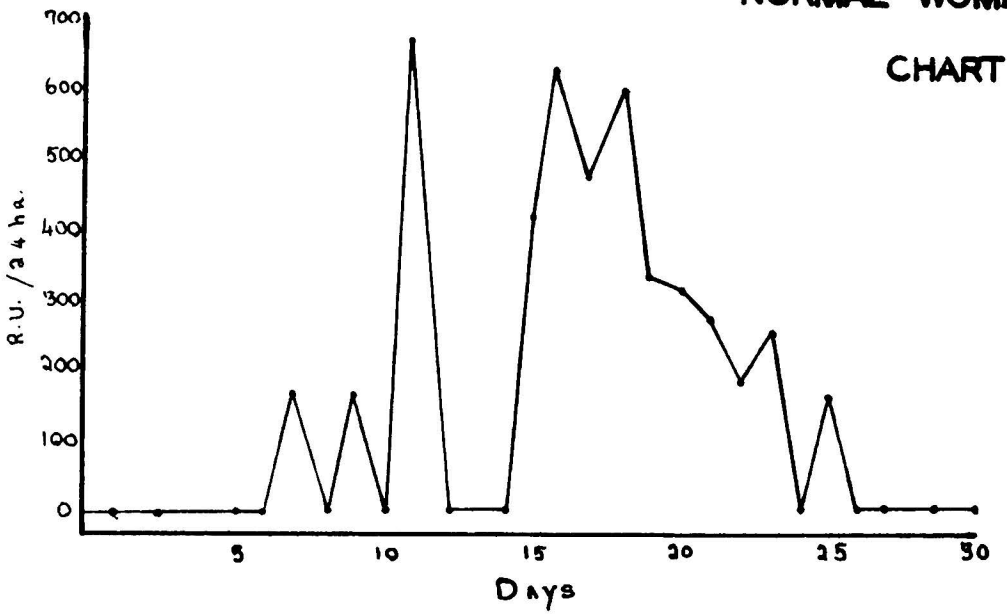
In certain disorders of gynecic function significant changes from normal in estrogen excretion occur, so that urinary assay in these cases offers a useful diagnostic aid. Cases of virilism have normal or lowered excretion (14). In granulose tumours of the ovaries the excretion is maintained at a high level (16). Male hypogonads excrete a larger amount of estrogenic substances than do normal males, (7).

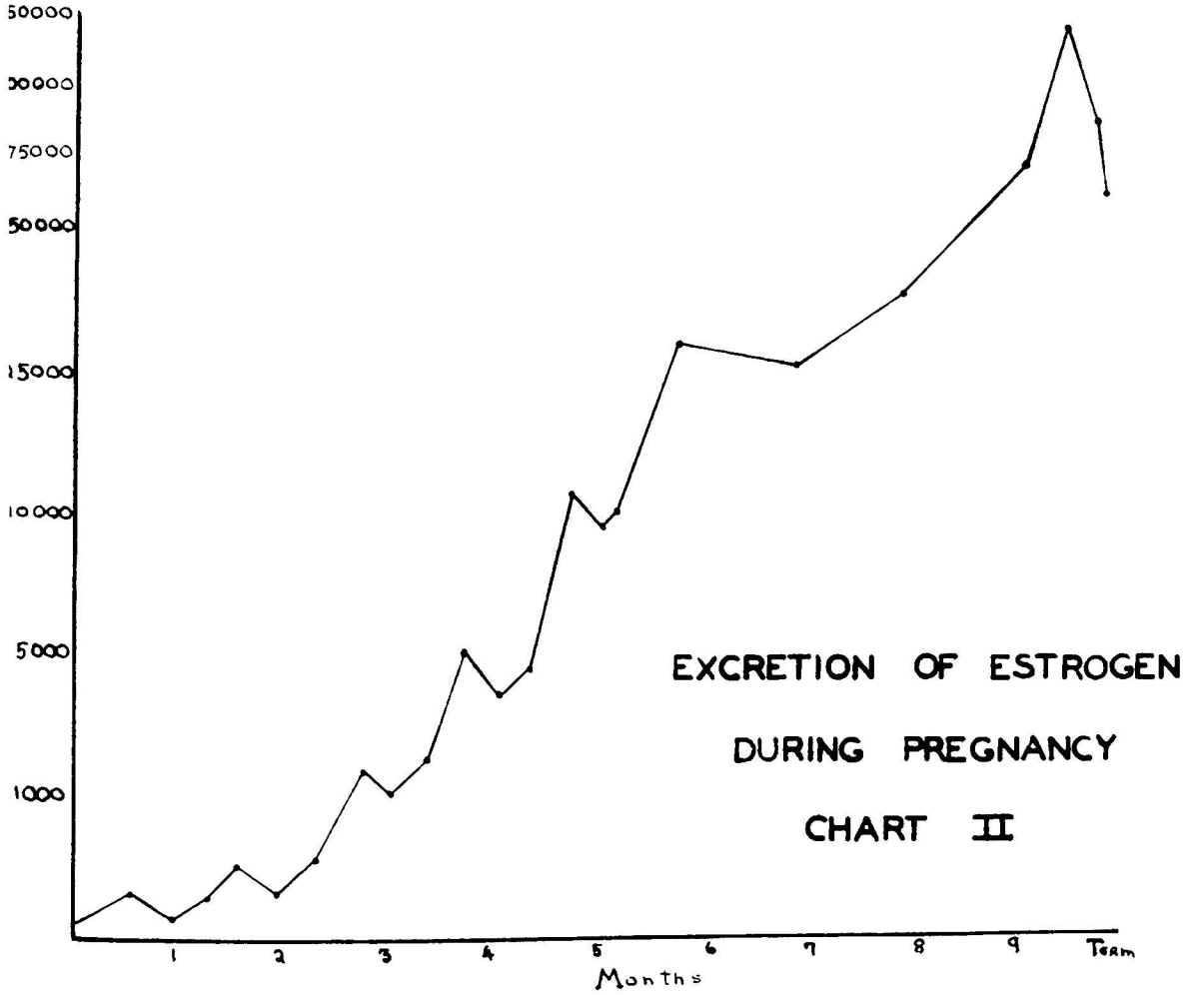
Of significance with regard to the unreliability of urinary excretion as an index of hormone economy is the observation that a very low percentage of administered hormone can be recovered from the urine. Kemp and Pedersen - Bjergaard (21) report about 6%. Other

EXCRETION OF ESTROGEN

NORMAL WOMEN

CHART I





EXCRETION OF ESTROGEN

DURING PREGNANCY

CHART II

workers approximate this figure (22)(23)(24).

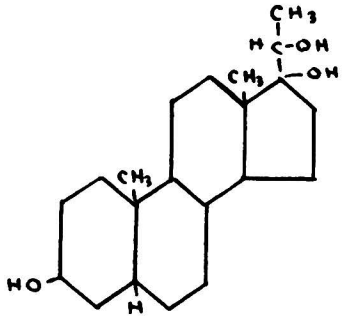
Several important advances have been made in an effort to clarify the metabolism of the sex hormones. These have taken the direction of examining the urine for metabolites of the various sex hormones.

It has been established that testosterone, the active male hormone is excreted as partly inactivated androsterone and dehydroisandrosterone (10). However it is not known what part liver and kidney perform in the conjugation and inactivation of the circulating hormones (25). Testosterone is produced in the testes, yet testes are not essential for androgen excretion--witness the excretion by women and castrates (7)(5)(26)(27).

Similarly it has been established that estradiol is produced by the ovary and is excreted as the less active estrone and estriol (19). However castrate females and normal males also excrete estrogenic substance (28)(29) so that the ovaries cannot be the only source of estrogenic material. As is the case with androgens, the association of estrogens with liver and kidney function is not fully understood (30)(31).

The anterior pituitary produces through its tropic hormones a profound effect on the adrenal cortex and the gonads. All the complexities of this association

The similarity of these compounds to a substance pregnanetriol 3,17,20 isolated from the urine of women with adreno genital syndrome by Butler and Marrian (35) is apparent.



Section II

ESTROGENS

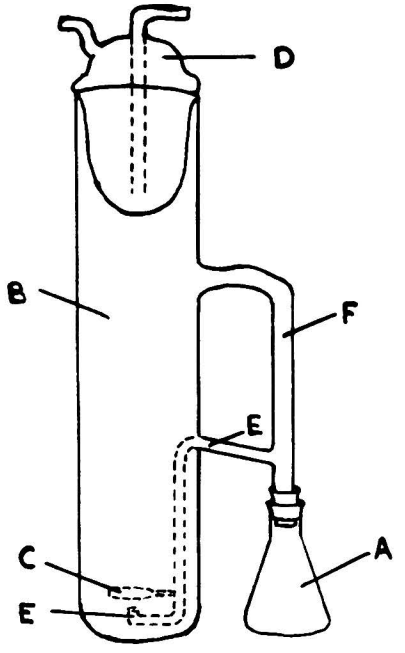
Estrogens are excreted in part as free substances and in part combined with glycuronic acid in variable ratio (19). Assay on untreated urine does not yield significant results as the combined fraction is biologically inactive. Hydrolysis to liberate the estrogens from combination has therefore become an integral stage in all methods of extraction. Procedures described vary greatly in the duration of hydrolysis and in the pH of the acid - urine mixture. Most investigators agree that a relatively drastic hydrolysis is essential for optimum efficiency (23)(41). A few (42)(43) suggest that over severe hydrolysis has a destructive effect on the estrogens, yet the values reported by adherents of both extremes seem to agree fairly well in similar instances.

A great variety of extraction apparatus and solvents have been employed. Benzene, ether, $CHCl_3$, and CCl_4 have been used. The length of time that extraction is necessary varies with the efficiency of the apparatus.

The method of extraction we have adopted is that of Leiboff (24) for several reasons:

1. It embodies an efficient severe hydrolysis.
2. The length of extraction is relatively short.
3. A non inflammable solvent, $CHCl_3$, is used.

4. A small amount of the extracting solvent is used.
5. The extraction is continuous with a constant renewal of extracting solvent and withdrawal of extracted estrogens.



Apparatus

B is the extraction chamber. E is the overflow tube extending to the bottom of container B. Letter C is a glass plate to protect the opening of E. Letter F is the tube for transferring vapor from A to the extraction cylinder. A is an Erlenmeyer flask for receiving the overflow. D is a condenser.

Method

A 500 c.c. aliquot of a 24 hr. urine is acidified with 50 c.c. of conc. HCl and evaporated to one third its volume by boiling, and then cooled. This is the hydrolysis procedure.

$CHCl_3$ is placed in B to a level 2 inches higher than C.

The hydrolyzed urine is poured into B through a long stemmed funnel. The urine will form a layer above the chloroform.

More chloroform is slowly poured into B until chloroform is seen to pass through tube E into flask A. Enough chloroform is added so that there is about 25 c.c. in A.

Condenser D is set in place and the water turned on.

Heat of such intensity is applied to extractor and flask that the chloroform in bath boils gently and steadily.

When the $CHCl_3$ in B boils it rises partly up into the urine and drops back again. When the temperature of the urine is elevated to that of boiling $CHCl_3$, the $CHCl_3$ will bubble vigorously through the urine and vaporize and be liquified by the condenser. An emulsion forms between the layer of $CHCl_3$ and urine. The flame must be regulated to prevent the emulsion from entering tube E.

While this is proceeding the $CHCl_3$ in the flask A is vigorously heated and evaporates through the arm F and is liquified by the condenser. The increased volume of $CHCl_3$ in the extractor causes the $CHCl_3$ to rise in E and spill over into the flask.

Within 2 hours all the $CHCl_3$ soluble material in the urine has been extracted and concentrated in flask A.

The flask is then disconnected and 5 c.c. of peanut or sesame oil added to the $CHCl_3$ extract and the $CHCl_3$

removed in vacuo.

The chloroform-free extract is now ready for assay.

We modified the procedure in that we extracted for 3 hours and added the $CHCl_3$ fraction from B to that from A. This was to insure total extraction and incorporation of all extracted material.

We employed sesame oil throughout.

Method of Assay

Although a number of other methods have been tried (45)(46), the most satisfactory method of assay for estrogenic substance is the rat vaginal smear method of Allen and Doisey (47).

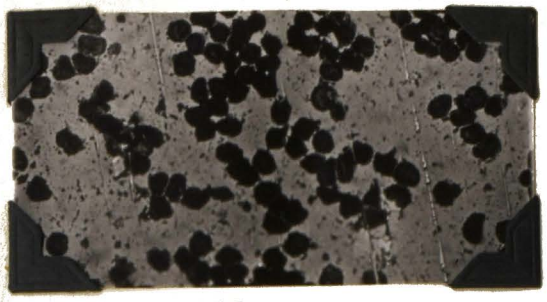
Young adult female rats are spayed and smears taken one week later. The rats are not used for assay until all are definitely negative. Any that do not give a negative slide after several days are discarded.

We use the following criteria for the classification of vaginal smears:-

I. Preponderence of leucocytes associated with a few compact and squamous cells. Fig. I.

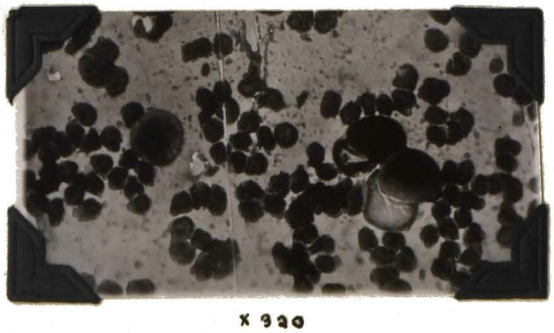
II. Predominance of compact cells with a few squamous cells and leucocytes. Fig. II.

II+. Compact cells only in large numbers of a characteristic appearance with the stained part drawn in, leaving a white ring around the cell or part of



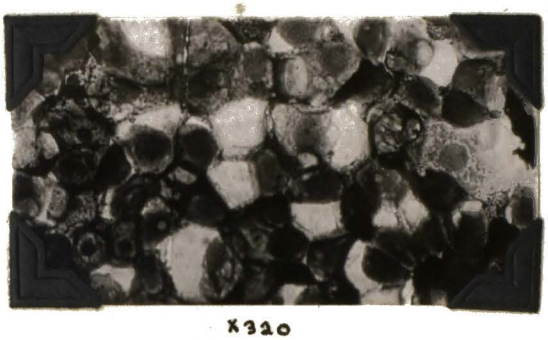
X 920

FIG I



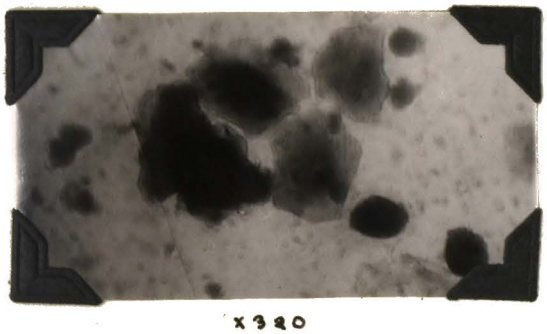
X 920

FIG II



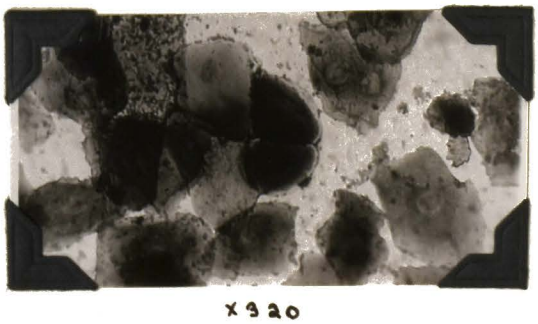
X 920

FIG III



X 920

FIG IV



X 920

FIG V

it. Fig. III.

III. Predominance of squamous cells with a few leucocytes and compact cells. Fig. IV.

IV. Squamous cells only. Fig. V.

I and II are interpreted as negative.

II+, III and IV are interpreted as positive.

The category of II+ was included after an examination of a large number of smears. It was found that this type of smear was often seen preceding or following a slide of complete cornification. It was therefore considered sound to assume that if a slide of this type was seen the rat could be considered positive, as it must be either passing into or out of estrus.

Experiments were done to determine the time of maximum response after injection. It was found satisfactory to take smears on the second, third and fourth days after injection. See Table I.

The material is administered in one injection subcutaneously under the loose skin of the flank. Ten rats are employed for each assay wherever the size of the dose permits. As an initial dose 0.2 c.c. of the 5 c.c. of extract is given. If there is no response with this, a larger dose is employed. If all the rats are positive, a smaller dose is given. The dose is varied until some of the rats are positive and some negative.

Table I

Rat Lot #1
Cage #25
Extract #1
Dose 0.2 c.c.

Feb./40	20	21	23	24	25	27	28
1	I		II	IV	IV	II	II
2	I		I	IV	IV	II	I
3	I		II+	IV	IV	II	I
4	II		I	IV	IV	II+	I
5	I		II	IV	II+	I	I
6	I		II	IV	IV	IV	II
7	I		II+	IV	IV	I	I
8	I		II	IV	IV	I	I
9	I		II	IV	IV	II	I
10	II		II	IV	IV	II	I

Rat Lot #5
Cage #C
Extract #21
Dose 0.1 c.c.

Feb./40	19	20	P.M. 21	A.M. 22	P.M. 22	A.M. 23	P.M. 23
1	I		II	II+	II+	IV	IV
2	I		III	II+	II+	IV	IV
3	I		II	III	II+	II+	II+
4	I		II	II+	II+	II+	II+
5	I		II	III	II+	II+	II+
6	I		II	II+	II+	IV	IV
7	I		II	II	III	II+	II+
8	I		II	II+	III	II+	II+
9	I		I	II+	II+	II+	II+
10	I		II	II+	II+	II+	II+

Table I - cont'd.

Rat Lot #7
Cage #A
Extract #20
Dose 0.1 c.c.

Feb.40	19	20	P.M. 21	A.M. 22	P.M. 22	A.M. 23	P.M. 23
1	I		II	II+	III	IV	IV
2	I		I	II+	III	IV	IV
3	I		II	II+	II+	IV	II+
4	I		I	II+	II+	IV	IV
5	II		II	II+	II+	IV	IV
6	II		I	II+	II+	II+	II+
7	I		II	II+	IV	IV	II+
8	II		II	II+	III	II+	II+
9	I		II	II+	IV	II+	II+
10	I		I	II+	III	II+	II+

Rat Lot #4
Cage #D
Extract #44
Dose 0.05 c.c.

Feb./40	24	24	26	27	28
1	II		II+	III	II+
2	II		II+	IV	II+
3	I		II	I	I
4	I		II	II	I
5	I		I	I	I
6	I		II	III	II
7	I		III	II	I
8	II		II	II+	II
9	I		I	III	II
10	I		III	I	I

Where there is no response with a dose up to 0.4 c.c. the result is reported as "negative with a dose of 0.4 c.c.", as this would be indicative of an excretion less than 1γ. However in some instances where, by examination of other urines from the same patient, a low value is expected, a larger initial dose is given and injections up to 1 c.c. used. In these larger doses fewer rats than ten must necessarily be injected. In cases where the extract is very potent, it is necessary to dilute an aliquot of the extract with more sesame oil.

As a standard, crystalline estradiol benzoate in sesame oil was used. Groups of rats were injected with various concentrations of estradiol benzoate to determine the smallest quantity of material which would produce the maximum response. This is considered to be the dose which is just sufficient to bring all of a group of rats into oestrus. Nine out of ten rats came into full oestrus with a dose of 0.0824γ of estradiol benzoate. As this figure represents a potency higher than is usually reported for this substance (48), and nine out of ten rats were positive, it was considered expedient to use 0.1γ as a basis for comparison throughout. This figure has advantages with regard to ease of calculation and convertibility (I.U. = 0.1γ).

In order to test the method of extraction, varying amounts of aqueous estrone solutions were added to samples of urine, extracted and assayed. No augmentation was found. Some of the crystalline estrone in oil was therefore injected but again no response was elicited. It was concluded that the material (which had been stored for a considerable length of time) had lost potency.

Experiments of a like nature were then carried out using estradiol benzoate, with reasonable recovery after extraction.

500 c.c. H_2O + 5 c.c. Est. Benzoate solution - 10/10 rats + with 0.4 c.c.

500 c.c. male urine + 5 c.c. Est. Benzoate solution = 8/10 rats + with 0.4 c.c.

It will be noted a lower potency was recovered when the estradiol benzoate was added to male urine and extracted than when it was added to water. This suggests the possibility of estrus inhibiting substance in male urine.

The $CHCl_3$ extracts prepared from different urines varied considerably in the amount and color of solid material. This is material soluble in $CHCl_3$ but not in oil. Pregnancy urines tend to have more extraneous material than the others examined. The amount bears no relationship to potency.

Variable amounts of toxic substance are extracted along with estrogenic substance from urine. Whether or not the toxicity is due to the solid material mentioned above is not known. It may be in part, but the amount of dark brown or reddish material does not always parallel toxicity. Some of the extracts examined were fatal to rats in doses of 0.1 c.c., while others showed no toxic effects up to 1.0 c.c.

One sample contained a very large amount of a white crystalline material in the $CHCl_3$ extract. This material was isolated and identified as hippuric acid. When this extract was injected it was found to be quite toxic to the rats. Several other extracts had smaller amounts of this substance. Hippuric acid, may be one of the toxic substances extracted by $CHCl_3$; but there may also be others which we have not been able to isolate.

After injection, particularly of a large dose, the rats are often lethargic and eat and drink very little, but usually recover after a short time.

As well as the Leiboff method of extraction for estrogenic substance we carried out some experiments using the method of Callow (10)(flow sheet #1). This method is primarily designed for the extraction of androgens, but the first stage of extraction removes estrogens as well. These may be separated from the

Flow Sheet I


1 litre urine + 150 c.c. conc. HCl + 125 c.c. CCl_4. Refluxed 2 hours
(1)
(2) Aqueous Residue + 125 c.c. CCl_4. Refluxed 2 hrs.
(7) CCl_4 + (4) + (6) CCl_4 removed
(4) CCl_4 Added to (7)
(8) Residue in benzene
Washed twice with 20 c.c. sat'd. $NaHCO_3$
(6) CCl_4 Added to (7)
(10) Benzene wash 5x with 25 c.c. 2N NaOH
(9) Washings
Acidified with HCl
Extracted twice with 20 c.c. Ether
(14) Benzene wash with H_2O till neutral
(13) washings added to (15)
(12) Aqueous Discarded
(11) Ether Added to (23)
(16) Benzene
(15) Washings + (13)
(17) Benzene removed
Acidified with HCl
(18) Residue in ether
Extracted 3 x with 50 c.c. Ether
(22) Aqueous Discarded
(23) Ether + (11)
Washed twice with 20 c.c. H_2O
(24) H_2O Discarded
(25) Ether
Dried with Na_2SO_4 (26)
5 c.c. oil added (27)
Ether removed in vacuo (28)
Residue in 10 c.c. absolute EtOH
21)

androgen fraction as indicated in the flow sheet #1.

It was hoped that by some modification of this method a satisfactory assay for both estrogens and androgens might be carried out on a single sample. Callow does not claim a total yield of estrogens by this method. The mild hydrolysis does not liberate all the combined estrogenic material; and the introduction of a stronger hydrolysis would cause destruction of the androgens.

We carried out several experiments on the aqueous residue after the CCl_4 fractions had been removed. An aliquot of this residue was subjected to the Leiboff extraction and assayed. In all cases the amount extracted was too small to elicit response in the highest doses administered.

It was hoped that by adding the value for estrogen of this fraction to that obtained by the Callow procedure that this total would represent the total estrogen content as determined by the Leiboff method. Theoretically this is correct but in view of the lengthy assay using two methods and the fact that the extract from the urine residue is very weak and requires very large doses with variable and frequently toxic effects, the method was found to be impractical.

A table of the results obtained by assay on various

conditions of endocrine disturbance is appended. Where two methods of assay on the same urine were carried out, the results are apposed for comparison.

It will be noted that the results obtained by the method after Callow are very much lower than those obtained by the Leiboff method (TableI). Only about 14% of the potency is thus determined. This method can be considered then of no practical value.

The cases of threatened abortion receiving progesterone show a great variety of excretory values - from an amount too small to be determined (less than 1γ) to 160γ.

It has been reported (19) that the excretion of estrogens rises steadily as normal pregnancy advances (Chart II). The low values obtained in the cases of threatened abortion receiving progesterone may be of significance. The threatened abortion may be a result of an estrogen deficiency as well as of progesterone which was augmented by injection. On the other hand the low excretion of estrogen may be a result of the injection of lutocylin, as it may have an inhibiting effect on the anterior pituitary and depress the gonadotropic hormone, with consequent diminution of estrogen secretion. If progesterone administration inhibits the production of necessary estrogen, then the low values observed in these patients who were unable

Table II

Number of Extract	Date	Name	Diagnosis	Treatment	Leiboff Estradiol Benzoate		Callow Estradiol Benzoate	
					γ/24 hr.	Rat Units 1 R.U.= 0.75γ	γ/24 hr.	Rat Units 1 R.U.= 0.75γ
15	1940 Jan. 9	Mrs. R.	Threatened abortion	Lutocylin (progesterone)			1.40	10.50
17	" 10	"	"	"	7.14	53.55	4.85	36.37
19	" 12	"	"	"			2.60	19.50
16	" 9	Mrs. M.	"	"	<1.00	<7.50		
18	" 10	"	"	"			<1.00	<7.50
20	" 13	Mrs. C.	"	"	74.80	561.00	7.48	56.10
21	" 15	"	"	"	160.00	1200.00	21.00	157.50
23	" 25	Mrs. W.	"	"	1.48	11.10		
28	Feb. 19	Mrs. L.	"	"	<1.00	<7.50		
38	March 20	Mrs. J.F.	"	"	17.78	133.35		
41	" 21	" "	"	"	42.56	319.20		
43	" 22	" "	"	"	45.53	341.47		
44	" 23	" "	"	"	20.09	150.67		
50	" 28	" "	"	"	43.40	325.00		
52	" 29	" "	"	"	44.15	331.12		
61	April 27	" "	"	"	9.76	73.20		
63	May 1	" "	"	"	5.20	39.00		
64	" 1	Mrs. L.C.	"	" 5 mg./day	0.24	1.80		
65	" 13	" "	"	"	<1.00	<7.50		
66	" 14	" "	"	"	0.20	1.50		
67	" 15	" "	"	"	0.67	5.02		
54	April 1	Mrs. I.N.	" also diabetes	" Lutocylin	4.97	37.27		
40	March 21	Mrs. D.	Habitual abortion	"	<1.00	<7.50		
45	" 23	"	"	"	<1.00	<7.50		
8	1939 Nov. 21	Miss L.J.	Amenorrhoea	- - - - -	<1.00	<7.50		
25	1940 Jan. 30	Miss S.	"	- - - - -	<1.00	<7.50		
14	1939 Dec. 9	Miss J.H.	"	Estradiol diprop twice a week from Oct. 27	3.62	27.15		
6	Oct. 31	Miss V.	Virilism	- - - - -	1.45	10.87		
60	1940 April 27	Mrs. R.B.	Menorrhagia	- - - - -	4.50	33.75		
29	Feb. 29	Miss B.	"	- - - - -	1.05	7.87		
33	March 12	"	"	Perandren	1.22	9.15		
22	Jan. 15	Mr. G.D.	Eunuchoidism	- - - - -	<1.00	<7.50	0.56	4.20
24	" 29	Mr. N.W.	"	- - - - -	<1.00	<7.50	0.57	4.27
27	Feb. 1	" "	"	Test. Prop. 90 mg. since Jan. 29	<1.00	<7.50	<0.50	<3.70
26	" 1	Mr. M.	Cushing's disease	- - - - -			0.60	4.50
7	1939 Nov. 1	Mr. H.	Normal	- - - - -	<1.00	<7.50		

to carry to term even with adequate progesterone, are of significance. The effect of estrogens in priming tissue for progestational action is well known, so it is possible that a certain amount of estrogen is necessary for a satisfactory tissue response from administered progesterone in threatened abortion.

Only one (Mrs. I.N.) of the cases of threatened or habitual abortion was successful in carrying to term.

Of the three cases of amenorrhoea, two were extremely low while the third, who had been receiving injections of estradiol dipropionate was considerably higher although still in the low normal range.

The two cases of menorrhagia had low normal figures. This would indicate that the menorrhagia was not due to excessive estrogen secretion.

The case of virilism had an excretion value in the low normal extreme.

The cases of eunuchoidism and the case of Cushing's syndrome had very low excretion values.

Section III

ANDROGENS

Androgens like estrogens are excreted partly in the combined state in which they are inactive (7). However a much milder hydrolysis than is required for estrogens must be employed, to avoid destruction of freed androgen.

The literature describes a great variety of extractors using various solvents. We adopted the method of extraction and purification described by Callow (10), as it has several advantages:-

1. It requires no special apparatus.
2. A non inflammable solvent, CCl_4 is used.
3. It embodies a mild hydrolysis.
4. The extracted androgens are removed from the action of the acid.
5. The procedure is of relatively short duration - 6 hours.

One disadvantage of this method is that it is not continuous. Three extractions with fresh solvent are necessary.

The steps in this procedure are described in Flow Sheet #1.

A few preliminary experiments were done with the charcoal purification in absolute alcohol solution but this did not give as clear a solution as that obtained

when purification was done in ether solution (step #19 - flow sheet #1).

Consolazio and Talbott (58) report a 25% higher result using CCl_4 as the solvent for purification and decolorization.

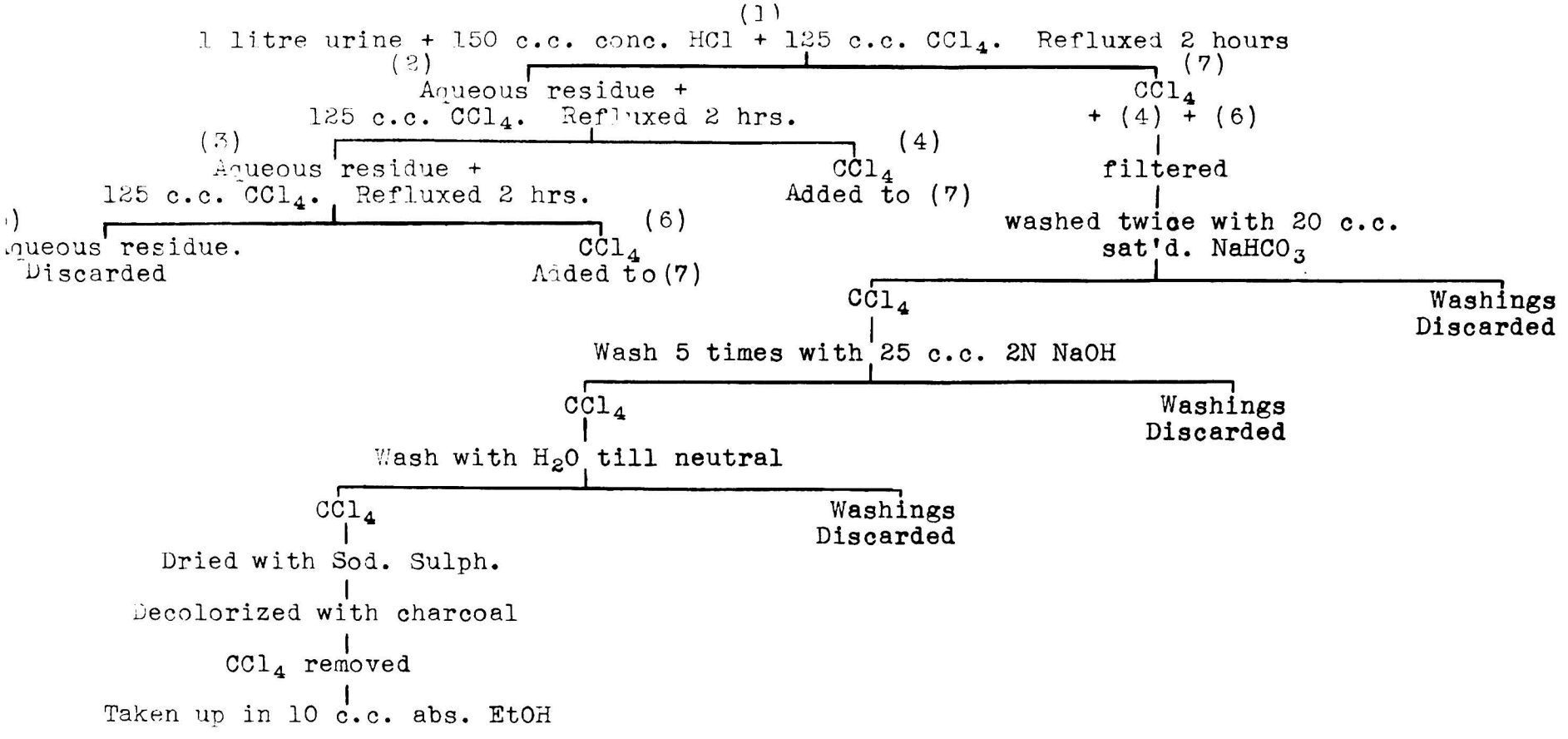
This claim was investigated and an increase of 26.2% over the previous method found.

Number	Solvent for Purification	c.c.	C.U.	Mg./24 hr. excretion
90 A	CCl_4	0.1	4.5	10.21
90 B	Benzene and ether	0.1	3.8	8.09

This procedure, as well as yielding higher results, shortens the purification considerably by eliminating the two steps using benzene and ether. This method was adopted (flow sheet #2).

The method of assay we employed is a modified Zimmerman (49) reaction with meta-di-nitro-benzene as the color reagent. This reaction is not specific for urinary androgens. However it has been shown that the intensity of color production is parallel to biologic activity (50)(51). The advantages of the colorimetric assay are great with respect to ease of operation, sensitivity, high reproductibility and low error.

Flow Sheet II



We tried some preliminary experiments on the method of Oesting (51) using crystalline androsterone. We did not find satisfactory color production by this method as color intensity did not consistently represent known androgenic content.

We then tried the method of Friedgood (54) with satisfactory results. This method was used throughout.

1. Reagents

2% m-dinitrobenzene* in absolute EtOH.

15% aqueous KOH.

2. Apparatus

The Hellige - Oesting Hormone Comparator consists of a revolving disc containing different colored glass plates, surmounting a colorimeter of two tubes. The blank tube is placed at the right and the unknown at the left. The disc is revolved until a color is found which changes the color of the blank tube to a color comparable to the unknown. The strength of the unknown is then read directly off the disc in color units (1 color unit is equivalent to 10γ androsterone).

3. Procedure

Aliquot portions of the alcoholic hormone solution are measured into test tubes and the alcohol evaporated off carefully over a water bath at 70 - 75°C. After

* Purified by a method proposed by Callow, Callow and Emmens(53)

cooling to room temperature, 0.15 c.c. of 95% EtOH, 0.2 c.c. of 2% m-dinitrobenzene in absolute EtOH and 0.2 c.c. of 15% aqueous KOH are added to each tube. The contents are mixed thoroughly. At the same time a control tube containing similar amounts of the two reagents and alcohol is prepared. The tubes are placed in the dark in a water bath at precisely 25°C for 75 minutes, with occasional shaking. The contents of the tube are then diluted to 7 c.c. with 95% EtOH. After being thoroughly mixed they are compared immediately in the Oesting colorimeter.

There are a number of variables affecting color production that must be standardized for consistent results. These variables are:- percentage of m-dinitrobenzene, dilution and alkalinity of reaction mixture, duration, light and temperature. A number of techniques have been reported using different standards of control of these variables (51)(52)(53)(54)(55).

4. Experimental

A series of tests was run to determine the effect on color production of fresh and old solutions of m-dinitrobenzene and KOH. The results are recorded in Table III.

As the reagent m-dinitrobenzene solution ages the depth of the red color increases in both the unknown

Table III

#	c.c. of Ext.	Color Units	m-dinitro-benzene	KOH	Results
1	0.05	3.1	fresh	fresh	Clear solution - good color comparison.
1	0.07	3.8	"	"	
2	0.05	3.1	"	one month old	Clear solution - good color comparison.
2	0.07	4.0	"	"	
3	0.05	3.1	"	five months old	Blank and test both very cloudy. Depth of color about same as fresh.
3	0.07	3.4	"	"	
4	0.05	3.8	one month old	fresh	Much deeper red in all solutions including blank. Poor color comparison due to appearance of brown pigments.
4	0.07	4.0	"	"	
5	0.05	3.1	"	one month old	Much deeper red in all solutions. Brown pigments present making comparison difficult.
5	0.07	± 4.0	"	"	
6	0.05	3.1	five months old	fresh	Very bright red in all solutions. Very poor color comparison.
6	0.07	± 4.0	"	"	
7	0.05	± 5.0	"	five months old	Dark brown solutions. Impossible to make any color comparison.
7	0.07	± 5.0	"	"	

and the blank. At a month comparison is unsatisfactory due to the appearance of brown pigments; at five months impossible.

The KOH solution gives satisfactory results up to a month. At five months the solution is very cloudy.

The m-dinitrobenzene solution should be made up fresh every two weeks; the KOH solution every month.

We first standardized the discs with crystalline androsterone. It was found that the color production is proportional to the amount of hormone present. The most effective range of the colorimeter is above 1.4 C.U. The lower limits are variable, as the extent of color change is not great per unit of hormone. The point at which the maximum color change per unit of hormone is observed is between 2.8 and 3.4 C.U. Therefore this range is the most effective and is used throughout wherever possible. Colors that fall between that of two color discs are given a value half way between the two discs.

The results obtained in standardizing the color discs are shown in Table IV and Chart III.

Table IV

Known Conc.	C.U.	C.U.	C.U.	C.U.	C.U.	C.U.	C.U.	C.U.	C.U.
2γ	0.2	0.2	0.2-	0.2	0.2	0.2	0.2	0.2	0.2
3γ	0.2-	0.3	0.2	0.2+					
4γ	0.2	0.3	0.3	0.3	0.3	0.3	0.3		
6γ	0.4-	0.4	0.3+	0.4+	0.4	0.4			
8γ	0.8	0.8	0.4	0.4	0.8	0.8	0.8		
9γ	1.1	0.8-							
12γ	0.8+	0.8-							
14γ	1.4	1.1	1.1+	1.1+	1.1+	1.4	1.4		
15γ	1.4	1.1							
18γ	1.7	1.7							
20γ	2.4	2.4	2.4	2.4	2.0	2.4	2.4		
24γ	2.8	2.8							
28γ	3.1	2.8	3.1	3.1	3.1	3.1	3.1	3.1	
30γ	3.1	3.1	3.1						
34γ	3.4	3.1	3.4+	3.4	3.4	3.4-	3.4-	3.4	
36γ	4.0	4.0							
40γ	3.5	4.5	4.0	3.8	4.0	4.0	4.5	4.5	4.5
42γ	4.5	4.5							
48γ	5.0	5.0+							
50γ	4.5	5.0+	5.0	5.0	5.0	5.0+	5.0+	5.0+	5.0

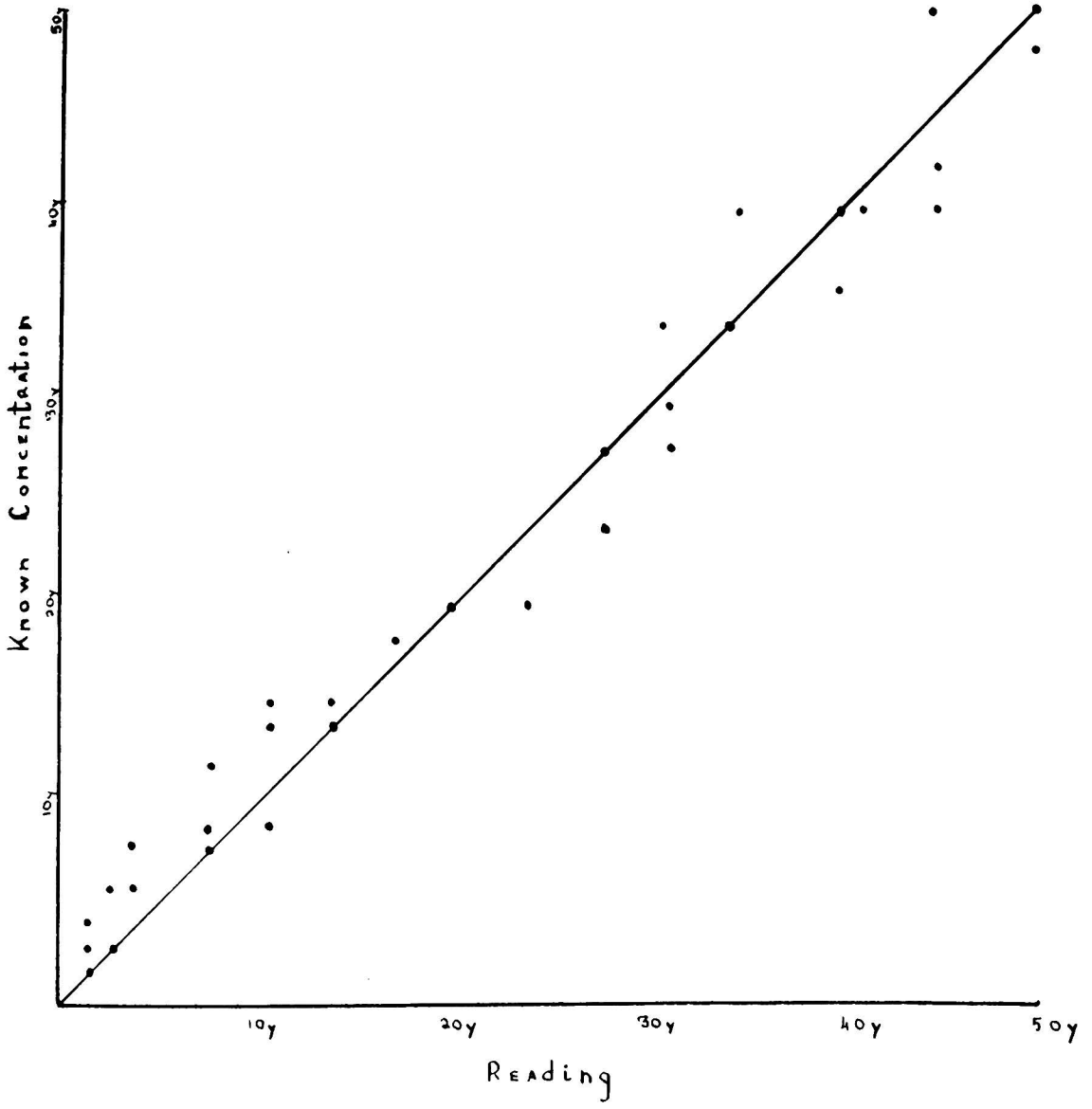


CHART III

Some other crystalline hormones were assayed for color production.

α β diethyl stilboestrol produced no color.

Progesterone produced a brownish color which was not comparable in the colorimeter.

Testosterone was much weaker per unit weight than androsterone and the color produced was not a good match in the colorimeter.

Estrone gave a satisfactory color of a potency practically identical with androsterone weight for weight.

Dehydroandrosterone gave a good color comparison of slightly higher (10%) intensity than androsterone weight for weight.

For a comparison of the color produced by these compounds and androsterone see Table V.

Urines from normal and abnormal individuals of both sexes were extracted and estimated by the method outlined. In many of the tests brownish pigment appeared that masked the red of the reaction and made a comparison difficult. This is particularly noticable in extracts of weak potency where large amounts of the alcoholic solution were used.

Table VI presents the results obtained in normal urine.

Table V

	Testosterone		Estrone	Dehydroandrosterone			Androsterone
Conc.	C.U.	C.U.	C.U.	C.U.	C.U.	C.U.	C.U.
2γ	-	0.2-	0.3	-	0.2	0.2-	0.2
4γ	0.2-	0.2-	0.4-	0.3	0.3	0.3	0.3
8γ	0.2-	0.2-	0.8-	0.8	0.8-	0.8	0.8
14γ	0.2-	0.2	1.4-	2.0	2.0-	2.0-	1.4
20γ	0.2	0.2+	2.0	3.1	3.1	2.8	2.4
28γ	0.6	0.4	3.1	3.4	3.4	3.4	3.1
34γ	0.8	0.8	3.4-	4.0-	3.7	4.0-	3.4
40γ	1.4	1.1	4.0	5.0	5.0	5.0-	4.5
50γ	2.0	2.0	5.0+	5.0+	5.0+	5.0+	5.0

: : : : : : : : :

Table VI

Normal

#	Date 1940	Name	Sex	Color of CCl_4 Ext.	Males Mg./24 hrs. as Androsterone	Females Mg./24 hrs. as Androsterone
68	Nov. 4	M.H.	♂	Bright cherry red	12.00	
70	" 5	"	"	" " "	12.00	
73	" 6	"	"	" " "	13.20	
76	" 7	"	"	" " "	8.77	
79	" 8	"	"	Deep " "	8.17	
80	" 9	"	"	Bright " "	8.55	
83	" 10	"	"	" " "	9.09	
69	" 5	J.N.	"	Light orangey red	12.00	
72	" 6	"	"	Orangey yellow	14.00	
75	" 7	"	"	Light orangey red	14.43	
77	" 8	"	"	Light cherry red	12.85	
81	" 9	"	"	Orangey rose	13.07	
82	" 10	"	"	" "	13.22	
71	" 5	F.S.	"	Pale orangey rose	13.60	
85	" 12	"	"	Pinky rose	12.40	
86	" 13	"	"	Orangey red	7.60	
78	" 8	M.D.	"	Orangey rose	15.03	
74	" 7	M.H.	♀	Pinky orange		8.70
90	Dec. 22	M.M.	"	Pale rose		10.21
				AVERAGE	11.76	9.45

The average for normal males is 11.76 mg./24 hrs. This is slightly higher than that reported by Drips and Osterberg (8) who report an average of 9.3 mg./24 hrs.

The average for normal females is 9.45 mg./24 hrs. This is also slightly higher than that reported by Drips and Osterberg who report an average of 7.4 mg./24 hrs.

It will be noted that some of the normal male excretion figures fall below the average normal female excretion. This overlapping between sexes of excreted androgen values has been noted by other workers (5)(8)(7)(10).

The color of the CCl_4 extracts varied from a deep cherry red to a pale orangey yellow. The depth of color bears no relationship to androgenic potency as can be seen from Table V.

In order to show daily fluctuation in the same individual more clearly, the results obtained in the assay of urine from subject M.H. and J.N. are represented in Chart IV.

The subject M.H. varies during the week from a high of 13.20 mg./24 hrs. to a low of 8.17 mg./24 hrs.; with an average of 10.25 mg./24 hrs.

The subject J.N. varies from 14.43 mg./24 hrs. to 12.00 mg./24 hrs. with an average of 13.25 mg./24 hrs.

These values are higher than reported by Koch (7)

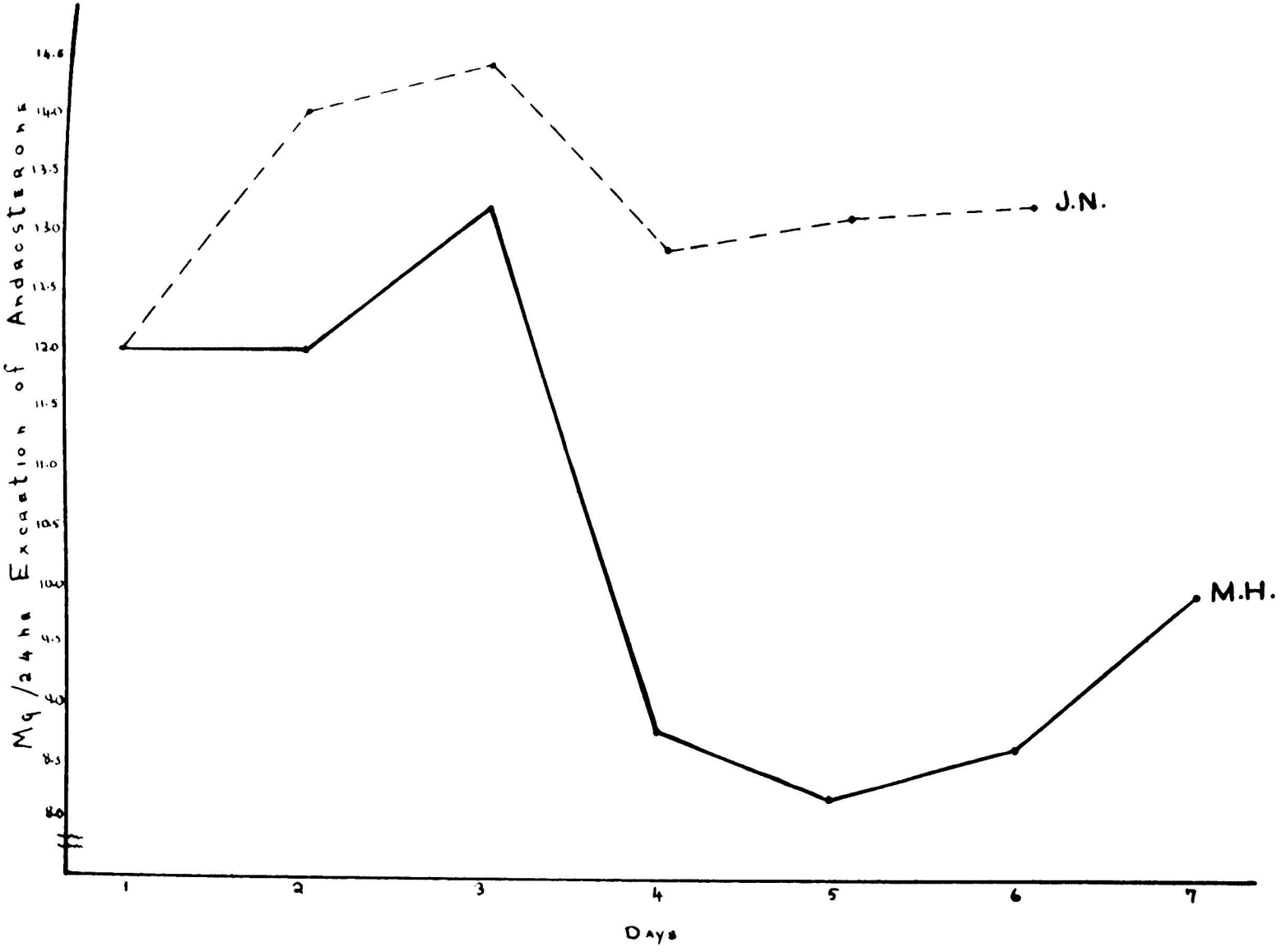

Mg/24 hs Excretion of Andrastenone
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
J.N.
M.H.
1
2
3
4
5
6
7
Days

CHART IV

for variation in normal individuals (20 - 69 I. U./day) but approximate limits for males as reported by a number of workers (5)(7)(8)(10).

With these normals as a basis for comparison an examination of the results obtained from the urine of abnormal subjects can be made. The excretion values in the abnormal cases are recorded in Table VII.

The cases of threatened abortion receiving lutocylin by injection have a subnormal excretion of androgens. Three cases of hypogonadism, one case of Cushing's Syndrome and one castrate examined also have a lower than normal rate of excretion. The case of eunuchoidism had an excretion figure within normal limits. After receiving 10 mg. of testosterone propionate three times a week for 9 days, the excretion figure was raised from 11.69 to 13.16 mg./24 hrs.

In some cases assays were done on the same urine for both estrogenic and androgenic substance. The results in these instances are contained in Table VIII.

The Androgen/Estrogen ratio in the cases of threatened abortion varies over a wide range and is on the average very low. The cases of eunuchoidism show a ratio much higher but again considerable variation is found.

Table VIII

#	Date 1940	Name	Sex	Condition	Treatment	Androsterone Mg./24 hrs.	Estradiol Benzoate γ/24 hrs.	An./Est. Ratio
15	Jan. 9	M.R.	♀	Threatened abortion	Lutocylin	1.11	1.40	0.79
17	" 10	"	"	"	"	0.65	7.14	0.09
19	" 12	"	"	"	"	0.92	2.60	0.36
18	" 10	M.M.	"	"	"	0.39	< 0.50	> 0.76
20	" 13	M.C.	"	"	"	0.997	74.80	0.01
21	" 15	"	"	"	"	1.50	160.00	0.009
22	" 15	G.D.	♂	Eunuchoidism		4.69	0.56	8.38
24	" 29	N.W.	"	"		11.69	0.57	20.51
27	Feb. 7	"	"	"		13.16	< 0.50	> 26.04

: : : : : : : : : :

Thirteen normal urines were left at room temperature for several days and then extracted for androgens. The CCl_4 in all cases was withdrawn from the urine. Of these CCl_4 extracts, three were assayed immediately; the other ten were left at room temperature in the light for seven months and then assayed. The results are presented in Table IX. Values obtained from normal, freshly extracted, freshly assayed urine are included for comparison. (Taken from Table VI).

It will be noted that the average of the values obtained from urine that has stood 4 - 5 days and assayed immediately (0.70 mg.) is very much lower than that obtained when fresh urine is assayed immediately (11.76 mg.). This indicates that urines should be extracted fresh for optimum efficiency. Many workers are in agreement with this observation (43).

On the other hand the urines which were left 4 - 5 days and extracted and left in CCl_4 for seven months show an average figure (12.94 mg.) approximating that of freshly extracted urine immediately assayed, (11.76 mg.). The one female urine so treated is higher (19.76 mg.) than the freshly extracted and assayed female urine we examined (9.45 mg.); or male urine (11.76 mg.).

The effect of urine standing before extraction may be to combine some of the color producing substance

Table IX

Urine extracted:	Fresh		After 4-5 days		After 4-5 days	
Assayed:	Immediately		Immediately		After seven months	
	Mg./24 hrs. Androsterone		Mg./24 hrs. Androsterone		Mg./24 hrs. Androsterone	
	♂	♀	♂	♀	♂	♀
	12.00	8.70	0.60		6.82	19.76
	12.00	10.21	0.27		12.22	
	13.20		1.23		14.99	
	8.77				15.34	
	8.17				11.90	
	8.55				10.80	
	9.09				27.45	
	12.00				12.58	
	14.00				4.39	
	14.43					
	12.85					
	13.07					
	13.22					
	13.60					
	12.40					
	7.60					
	15.03					
Average	11.76	9.45	0.70		12.94	19.76

in a form in which it exhibits less color value. This material may be extracted by the CCl_4 and on standing before assay, be reconverted to its original color producing form; or it may be converted to another compound having similar or higher color value.

Similar unexplained increases in the ketosteroid content of urine extracts when assayed by Zimmermann reaction after storage of these substances in 95% alcohol, have been noted by Friedgood and Berman, (59).

It was decided to investigate the possibility of time and temperature increasing the color producing power of fresh extracts. A large amount of urine was extracted fresh and aliquots of the CCl_4 fraction, in various stages of purification were left at different temperatures for varying lengths of time.

Up to two months aliquots left, at room temperature, at 50°C and at 65°C showed no change from an aliquot assayed immediately after extraction. Table X.

Two aliquots were refluxed on a water bath for 48 hours and 206 hours respectively. In neither was there any effect on the assay. Table X.

These results show the stability to heat and time of freshly extracted androgens in CCl_4.

Three androgen extracts in absolute EtOH were retested after standing seven months at room temperature.

Two had lost 83.9% and 90.3% respectively; and one had gained 31.25%. Table X.

Experiments were done on androgenic extracts in absolute EtOH left at room temperature in daylight, and at 50°C and at 65°C in ovens. Up to three months no change in color potency was found. Table X.

Urinary androgens in absolute EtOH must be stable for three months at temperatures up to 65°C.

Table X

#	Colorimetric Assay Initial c.c.	C.V.	Solvent	Treatment	Colorimetric Assay after treatment indicated c.c.	C.V.	Result
89	0.05	3.1	CCl_4	Refluxed 48 hrs.	0.05	3.1	No change
93	0.05	3.1	CCl_4	" 206 hrs.	0.05	3.1	"
91	0.05	3.1	CCl_4	Left at room temp. 1 month	0.05	3.1	"
114	0.10	3.1	CCl_4	" " " " 2 "	0.10	3.1	"
115	0.10	3.1	CCl_4	" " 50°C for 2 "	0.10	3.1	"
116	0.10	3.1	CCl_4	" " 65°C " 2 "	0.10	3.1	"
48	0.04	3.1	EtOH	" " room temp. 3 "	0.04	3.1	"
53	0.05	3.1	EtOH	" " " " 3 "	0.05	3.1	"
24	0.05	3.1	EtOH	" " " " 7 "	0.20	2.4	Lost 83.9%
27	0.04	2.8	EtOH	" " " " 7 "	0.50	3.4	" 90.3%
26	0.025	0.8	EtOH	" " " " 7 "	0.10	4.5	Increased 31.2%
82	0.05	3.1	EtOH	" " 50°C for 1 "	0.05	3.1	No change
88	0.05	3.1	EtOH	" " 65°C " 1 "	0.05	3.1	"

Section 4

EFFECT OF ULTRA VIOLET LIGHT AND CHLORINE GAS ON ANDROGENS

Our experiments with heat and time did not throw any light on the mechanism for the apparent increase in color value of an extract from 4 - 5 day old urine, after it has stood seven months. Therefore it was decided to investigate the effect of ultra violet light on androgen solutions.

Preliminary irradiations were performed with the solution 2.5 feet from the light source. At this distance little effect was noted (Table XI). At 10.5 inches a marked and consistent effect was elicited (Table XI). This distance (10.5 inches) from source of light to solution was therefore used throughout.

Table XI

#	Androgen	Distance from source	Solvent	Value pre-irradiation	Time Exposed	Value post-irradiation	Loss %
68	urinary	2.5 feet	EtOH	12000 γ	15 min.	12000 γ	0
88	"	"	CCl_4	3000 γ	"	2400 γ	20.00
74	"	10.5 inches	EtOH	3000 γ	45 min.	1900 γ	36.60
99	"	"	CCl_4	3000 γ	10 min.	1133 γ	62.23

The solutions of androgen irradiated were of four types:-

1. Crystalline androsterone in absolute EtOH.
2. Crystalline androsterone in CCl_4.
3. Urinary androgen in absolute EtOH.
4. Urinary androgen in CCl_4.

The values obtained are included in Table XII and are presented graphically in Chart V.

Discussion

1. Crystalline androsterone in absolute EtOH on exposure to ultra violet light shows an initial rapid destruction followed by a more gradual loss.

2. Crystalline androsterone in CCl_4 is destroyed very rapidly by ultra violet light. Thirty seconds exposure in CCl_4 causes as much destruction as thirty minutes in absolute EtOH.

3. Urinary androgen in absolute EtOH is not destroyed as rapidly as crystalline androsterone in the same solvent. This indicates that urinary androgen is more stable to ultra violet light than crystalline androsterone.

4. Urinary androgen in CCl_4 is destroyed much more rapidly than in absolute EtOH. It is not destroyed nearly as rapidly as crystalline androsterone in CCl_4. This evidence indicates greater stability of urinary androgen to ultra violet light than crystalline

Table XII

Time of Irradiation	Loss % Crystalline Androsterone in Absolute EtOH	Loss % Crystalline Androsterone in CCl_4	Loss % Urinary Androgens in Absolute EtOH	Loss % Urinary Androgens in CCl_4
0.5 min.		53		
3.0 "		97		
5.0 "		96		
10.0 "		97		62
15.0 "	44			
30.0 "	53			97
45.0 "	65		37	
60.0 "	73			98
75.0 "	82		50	
90.0 "	86		60	
105.0 "	90			
120.0 "			67	99

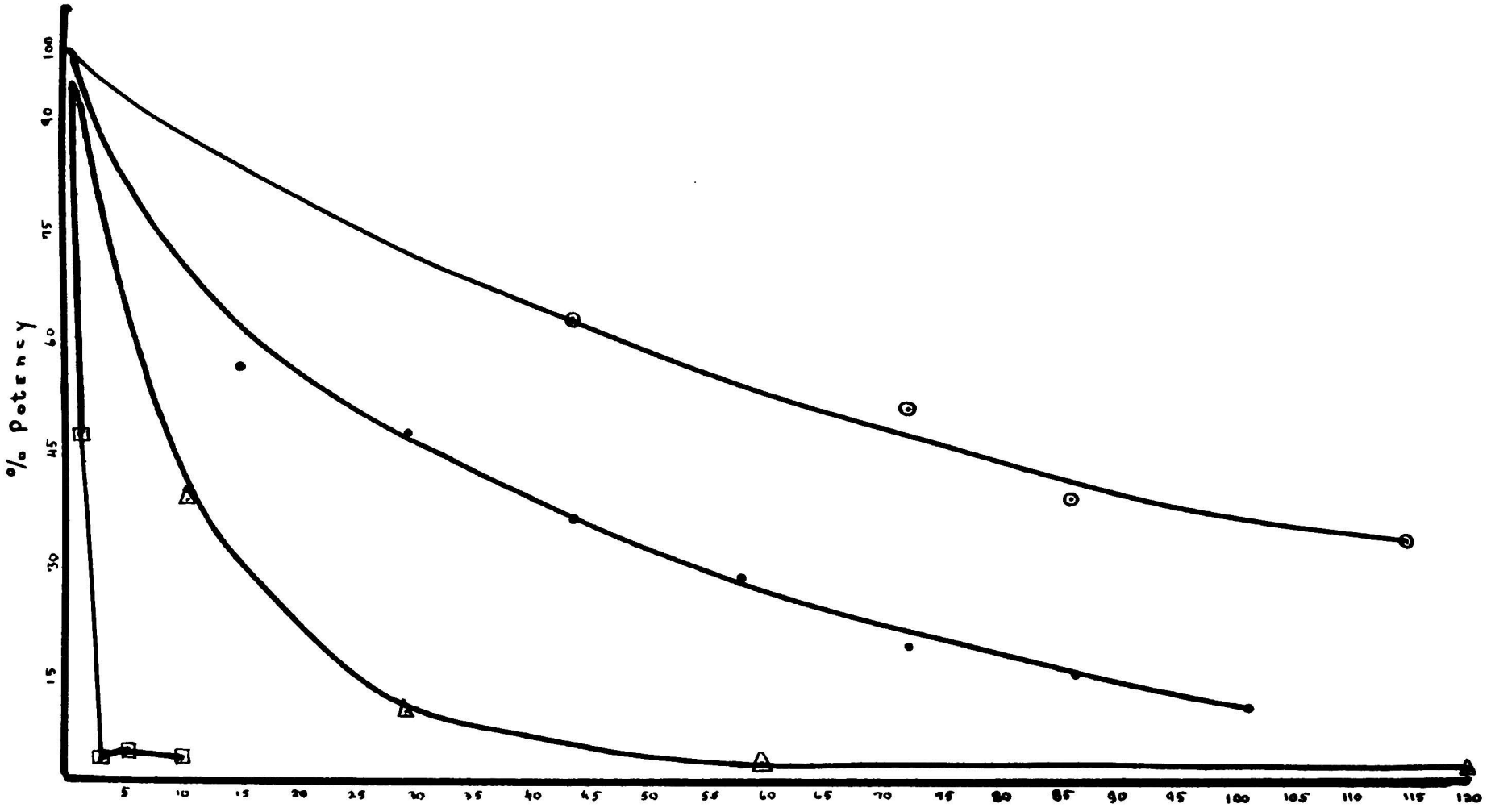

% Potency
100
90
75
60
45
30
15
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
Minutes of Irradiation
⊙ Urinary Androgen in EtOH
• Xtalline Androsterone in EtOH
Δ Urinary Androgen in CCl_4
⊡ Xtalline Androsterone in CCl_4

CHART V

androsterone.

When Group 4 is compared to Group 3, and Group 2 is compared to Group 1, it is evident that there is a very much more rapid destruction of androgen in CCl_4 than in EtOH. As alcohol offers no resistance to ultra violet light, the destruction curve obtained when androgens were irradiated in this solvent represents the maximum destruction per unit of time brought about by ultra violet. It would seem therefore that the greater destruction in CCl_4 must be due to a chemical reaction initiated by ultra violet light.

Lyons and Dickinson (56) showed that CCl_4 is decomposed by ultra violet into phosgene and chlorine. The reaction is represented by the equation:-

$$2CCl_4 + O_2 \longrightarrow 2COCl_2 + 2Cl_2$$

It was thought that one or both of the products of this decomposition (phosgene and chlorine) might be reacting with the androgen. To test this suggestion, dry chlorine gas was bubbled through solutions of crystalline androsterone.

The results are contained in Table XIII.

It will be seen that contrary to expectation chlorine gas has an activating effect on the color production of androsterone as measured by the Zimmerman reaction.

The effect of phosgene has yet to be investigated.

Table XIII

#	Solvent	Time	Value pre-chlorination	Value post-chlorination	Increase %
112	Abs. EtOH	5 min.	428	1100	157.01
119	" "	10 "	428	800	86.91
113	" "	10 "	none	none	no change
118	CCl_4	5 "	480	666	38.75

Our experiments with the effect of chlorine gas on androgens open certain avenues for further investigation:-

1. Effect of other halogens on the reaction.
2. Effect of chlorine and other halogens on some of the closely related ketosteroids.
3. Does enhanced color production as measured by Zimmermann reaction indicate also enhanced biologic activity?
4. Does chlorine effect indicate the possibility of chloride effect, as suggested by Butenandt?(57).

EFFECT OF ULTRA VIOLET LIGHT ON THE PURPLE COLOR OF URINARY ANDROGENS IN CCl_4.

The purple color of urinary androgens in CCl_4 is converted to orange on 5 minutes exposure to ultra violet light, and to yellow in eleven minutes. The yellow color is due to a flocculent precipitate. When this is removed by filtration, the filtrate is water clear.

It was also noted that the solutions of urinary androgens left at room temperature in daylight for three months changed in color. The solutions that had been washed with $NaHCO_3$ and NaOH prior to standing, were colorless; those that had been left in the impure state had changed from purple to a pale rose. Aliquots left in the ice box and ovens, in the dark, had not changed in color. It can therefore be considered that the bleaching is due to daylight.

As previously mentioned, during irradiation of CCl_4 solutions of urinary androgens a light brown flocculent precipitate forms. It is water soluble and gives a positive test for chloride. This precipitate appears even in solutions which have been purified and decolorized before irradiation. Solutions of crystalline androsterone which have been irradiated show no such precipitate. The source and exact nature of this precipitate are as yet undetermined.

Summary

1. A survey has been made of the literature.

2. A standard method for extraction, purification and assay of urinary estrogens and androgens is suggested.

   (a) Estrogens. The method for extraction and purification of estrogens is based on that of Leiboff. The rat smear bioassay of Allen and Doisey is employed, with an improved system of smear classification.

   (b) Androgens. The method for extraction and purification of androgens is based on that of Callow as modified by Consolazio and Talbott. Androgen activity is measured in an Oesting colorimeter using the Zimmerman reaction as adopted by Freidgood.

3. Tables of excretory values of estrogens and androgens in normal and abnormal individuals are included.

   (a) Estrogens. Cases of threatened abortion receiving progesterone varied from < 1.0 to 160.0γ estradiol benzoate/24 hrs.
   Amenorrhoea < 1.0 to 3.62γ estradiol benz./24 hrs.
   Menorrhagia 1.05 to 4.50γ " " "
   Eunuchoidism and Cushing's disease 1.0γ estradiol benz./24 hrs.

(b) Androgens. Normal males 7.60 to 15.03 mg./24 hrs. as androsterone.

Normal females 8.70 to 10.21 mg./24 hrs. as androsterone.

Threatened abortion receiving progesterone 0.39 to 1.50 mg./24 hrs. as androsterone.

Hypogonad males 0.25 to 13.16 mg./24 hrs. as androsterone.

Cushing's syndrome 2.98 to 3.20 mg./24 hrs. as androsterone.

These values are in fair agreement with other reported values where figures are available.

4. The effect of time, heat, ultra violet light and chlorine gas on solutions of androgens has been investigated.

(a) Time. Androgen solutions are stable up to three months. After seven months anomalous results were obtained.

(b) Heat. No change in activity was found after refluxing androgen solutions in CCl_4 for 206 hours; or after leaving them in an oven at 65°C for three months.

(c) Ultra violet light. Androgens are destroyed by ultra violet light much more rapidly in CCl_4 than in EtOH. Urinary androgens are more

stable to ultra violet light than is crystalline androsterone.

(d) Chlorine. The potency of androgen solutions as measured by Zimmermann reaction is enhanced by chlorine gas.

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