Section of Therapeutics and Pharmacology

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The Significance of the Excretion of Sex Hormones in the Urine

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ABSTRACT.—Recent work on the extraction and assay of urinary sex hormones is reviewed. The " sex hormones " known to be excreted in the urine are not identical with the hormones which have been actually isolated from the organs of secretion. Experimentally it is found that when active substances are administered only a very small proportion is excreted in recognizable form, and the excreted form may even be inactive. This general statement applies to œstrogens, androgens, progestin, and gonadotropic hormone. Hormone activity in the urine is, therefore, an uncertain index of hormonal activities in the body. However, with increasing knowledge of the chemistry and metabolism of hormones, methods of urine assay have been devised which give results capable of correlation with known or assumed physiological processes, notably in pregnancy and the normal menstrual cycle, and obvious effects are produced by certain types of tumour.

In the case of male hormone activity the lack of relation between urinary androgens and sexual condition has led to the assumption that the androgens in urine are largely derived from sources other than the gonads, probably from the adrenal glands. Work on this question is described in detail.

RÉSUMÉ.—Revue des travaux récents sur l'extraction et l'estimation des hormones sexuelles de l'urine. Les " hormones sexuelles " qu'on sait être excrétées dans l'urine ne sont pas identiques à celles extraites des organes sécrétoires mêmes. On trouve expérimentalement qu'après l'injection de substances actives qu'une très petite proportion seulement en est excrétée dans l'urine en forme reconnaissable, et que la partie excrétée peut même être inactive. Cette remarque générale s'applique aux oestrogènes, androgènes, à la progestine et à l'hormone gonadotrope. L'activité hormonale de l'urine est donc un index incertain de l'activité hormonale de l'organisme.

Quoique nous puissons nous attendre à une amélioration de cette situation avec l'augmentation de nos connaissances sur la chimie et le métabolisme des hormones et le développement de méthodes d'estimation dans l'urine, la valeur diagnostique des analyses hormonales est en général limitée aux états où l'organisme est inondé par un excès d'hormones, telles que la gestation et certaines tumeurs.

Description détaillée de travaux sur l'excrétion d'hormone masculine, et spécialement d'études par l'estimation biologique et chimique colorimétrique de l'excrétion de substances androgènes par des sujets normaux, des castrés des deux sexes, des eunuchoïdes, des cas de maladie d'Addison et des cas certains ou soupçonnés d'hyperplasie surrénale. L'absence d'interrelation entre les androgènes urinaires et l'état sexuel a mené à la théorie que les androgènes urinaires sont dérivées en grande partie de sources autres que les gonades, et probablement des surrénales. Dans deux cas de tumeur surrénale de grandes quantités d'androgène furent excrétées, et de la dehydroandrosterone fut extraite de l'urine.

ZUSAMMENFASSUNG.—Neuere Arbeiten über Extraktion und quantitative Bestimmung der Geschlechtshormone im Harn werden besprochen. Die " Geschlechtshormone ", deren Ausscheidung im Harn bekannt ist, sind mit den aus den sezernierenden Organen isolierten Hormonen nicht identisch. Experimentell wurde festgestellt, dass nach Einverleibung aktiver Stoffe nur ein sehr kleiner Teil in identifizierbarer Form im Harn ausgeschieden wird und dass die Ausscheidung sogar in Form inaktiver Verbindungen stattfinden kann. Diese allgemeine Angabe gilt für Oestrogene, Androgene, Progestin und das gonadotrope Hormon. Die hormonale Wirksamkeit des Harnes ist somit kein zuverlässiger Massstab für Beurteilung der hormonalen Aktivität des Organismus.

Während mit weiterer Zunahme unsere Kenntnisse der Chemie und des Stoffwechsels der Hormone sowie durch Verbesserung der Harnanalysenmethoden eine Besserung dieser Sachlage zu erwarten steht, ist der diagnostische Wert der Hormonanalyse auf Zustände beschränkt, die mit einer Ueberschwemmung des Körpers mit Hormonen einhergeht, z.B. Schwangerschaft und gewisse Geschwulstarten.

Untersuchungen über die Ausscheidung männlicher Hormone im Harn werden ausführlich beschrieben u.zw. mit besonderer Berücksichtigung von biologischen sowie chemischen und colorimetrischen Untersuchungen über Ausscheidung von androgenen Stoffen bei Normalen, bei Kastraten beider Geschlechter, Eunuchoiden, Fällen von Addisonscher Krankheit und einwandfreien oder fraglichen Fällen von Nebennierenhyperplasie. Die Tatsache, dass keine Beziehung zwischen dem Androgengehalt des Harns und dem sexuellen Zustand besteht, hat zur Annahme geführt, dass die Androgene im Harn nicht aus den Geschlechtsdrüsen stammen, sondern aus anderen Organen, vermutlich den Nebennieren. In zwei Fällen von Nebennierengeschwulst wurden sehr grosse Mengen Androgen ausgeschieden und Dehydroandrosteron wurde aus dem Harn isoliert.

Introduction.—The problem of the significance of the excretion of sex hormones in the urine is far from being settled, and is, moreover, too wide and too complex to be covered in a short review. It is proposed, in the following paper, to examine critically certain general aspects of the subject as they appear to the chemical laboratory worker, and to illustrate these by an account of some of the work which has been carried out on one branch of the subject, urinary androgens, at the National Institute for Medical Research, in co-operation with my colleagues, Mrs. N. H. Callow, Mr. C. W. Emmens, Dr. A. S. Parkes, and a number of clinical collaborators. During the past year this work has formed part of a scheme undertaken by arrangement with the Hormones Committee of the Medical Research Council.

The nature of the sex hormones in urine.—In reviewing the history of work on the gonadal hormones, the impression is often given, generally unintentionally, that the discovery of physiological activity in the urine has been the first step in the direct path to the isolation of the hormone itself. As examples there may be cited the discovery by Aschheim and Zondek (1927) of œstrogenic activity in urine of pregnancy, which led to the extraction and isolation of œstrone and œstriol, or the discovery by Loewe and Voss (1929) of androgenic activity in male urine, leading to the isolation of androsterone. In fact, there is no evidence of the secretion of these particular compounds by the endocrine glands. Pedantically one might say that they were, therefore, not hormones, but compounds with hormone-like activity. It might be said that androsterone and dehydroandrosterone, isolated by Butenandt and his co-workers (Butenandt, 1931 ; Butenandt and Dannenbaum, 1934), so far from being sex hormones, were excretory transformation products which happened to have hormonal activity, and even, in the case of dehydroandrosterone, probably a product derived not from the male gonads but from the adrenal glands.

Reference to the formulæ in fig. 1 will illustrate the meaning of this distinction between primary hormones and excreted products. Of the natural œstrogenic compounds, α-œstradiol (Formula i), isolated by MacCorquodale, Thayer, and Doisy (1936) from sows' ovaries, and œstriol (Formula ii), isolated by Browne (1933) from Collip's placental extract " Emmenin ", are the hormones definitely known to occur in the body. Although Westerfield and Doisy (1937) have detected ketonic œstrogens in ovarian tissue, œstrone (Formula iii) has been isolated only from urine. Œstriol also occurs in urine, and was actually first isolated from that source. Œstradiols are found in the urine of the pregnant mare (Wintersteiner and Hirschmann, 1937), but the occurrence of œstradiol as an excreted product from the human body is only a supposition made on the grounds that a substance more active than œstrone or œstriol appears to be present. From these three compounds it would seem that the primary hormone, œstradiol, is converted by the body into the other two by oxidation, possibly in the course of acting. The next stage before excretion is that of conversion into water-soluble products by coupling with sulphuric acid or glycuronic

acid, a process which is the normal mechanism of the body for ridding itself of unwanted hydroxy-compounds.

The progestational hormone of the corpus luteum is progesterone (Formula iv), which has been isolated from sows' corpora lutea. The progestational activity of urine, blood, or placenta, is so small that no attempt has been made to isolate active compounds from these sources. Thus Loewe and Voss (1934) extracted only 1 rabbit unit of progestin activity from 20 litres of urine in the last quarter of the menstrual period or in the second month of pregnancy, and Bloch (1936) could not detect progestin in 500 c.c. of blood of pregnant women. Venning and Browne (1936, 1937), have shown that the excreted form of progesterone is the biologically inactive compound pregnanediol (Formula v). Progesterone therefore undergoes complete reduction and complete inactivation before excretion, in contrast to œstradiol. Other pregnane derivatives have been isolated from urine; thus Hartmann and Locher (1934-1935) found that the isomeric *allo*-pregnanediol accompanied pregnanediol, and Marker and his co-workers (1937 *a*, *b*, 1938) have isolated *epi-allo*preg-

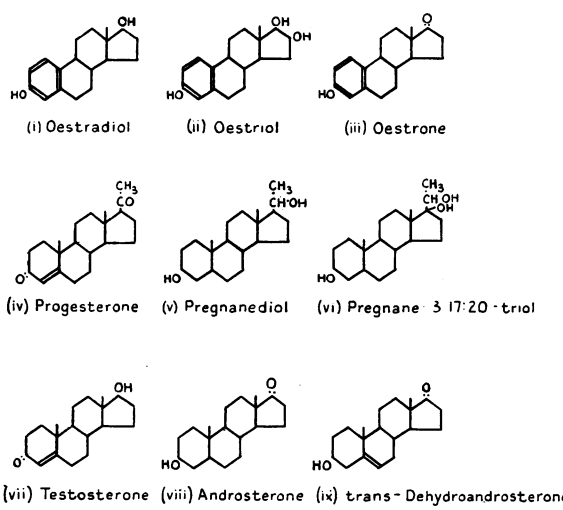

FIG. 1.—Formulæ of hormones and certain excreted products.

nanolone and two partly investigated pregnanetriols from pregnancy urine, whilst Butler and Marrian (1937) isolated pregnane-3 : 17 : 20-triol (Formula vi) from the urine of cases of adrenal hyperplasia. In the last case at least the urinary compound is more probably derived from the adrenal than from the corpus luteum.

Of the many androgenic compounds known, testosterone (Formula vii), isolated by David (1935; cf. David *et al.*, 1935) from bulls' testes, is the only one whose occurrence in the testis is established, though Ogata and Hirano (1934) have reported the isolation of another active, crystalline product from boars' testes. Androsterone (Formula viii) and *trans*-dehydroandrosterone (Formula ix), isolated by Butenandt from male urine, are commonly regarded as the excreted forms of male hormone. They are both active, but less so than testosterone. If we accept the assumption of derivation from testosterone, then the transformation in the body has been a combination of reduction at one position in the molecule with oxidation at another position. On the other hand, Butenandt and his co-workers have also (1937) isolated *epi*-ætiocholanediol, a completely reduced product, from male urine. The author also has isolated *trans*-dehydroandrosterone in very small amount from normal

female urine. The fact that *trans*-dehydroandrosterone is excreted in cases of adrenal tumour suggests the alternative that it is derived from the adrenal glands rather than from the gonads. This is not the occasion to enter into the chemistry of the adrenal glands, but a survey of the compounds isolated from ox adrenals by Reichstein and his school, by Kendall *et al.*, and by Wintersteiner and Pfiffner gives an idea at once of the complexity of the field and of the close relation of the adrenal family of compounds to the sex hormones. There are compounds with the side-chain in position 17 which have the same carbon skeleton as progesterone, and also compounds with the simple androstane skeleton. One of these latter compounds is itself androgenic, and a degradation product also has activity. We have here in these four groups a large family of chemically allied compounds produced in the ovary, the placenta, the testis, and the adrenal gland. Androstane derivatives are known to have multiple hormone activities (cf. Deanesly and Parkes, 1937), and if one further considers the possibilities of side-reactions by which related compounds may be produced as by-products, it will not appear surprising that œstrogenic and progestin activity is found in the testis, androgenic activity in the ovary, and œstrogenic, androgenic, and progestin activity in the adrenal (Zondek, 1934 *a*; Courrier, 1934; Dorfman, Gallagher, and Koch, 1935; Callow and Parkes, 1936; Parkes, 1937 *a*; Lipschütz, 1937; Seeman, 1937). If you consider how the estimate of the importance of a compound is conditioned by the ease with which it can be isolated, and the possible occurrence of unsuspected slightly active or inactive by-products, you will appreciate some of the difficulties and uncertainties of constructing a complete scheme of sex hormone metabolism and attaching precise significance to urinary sex hormones.

Hormone metabolism.—The number of independent variables which must be considered is very large. The processes which may go on in the body include absorption from the alimentary tract or from depots of oil solution injected intramuscularly; formation (whether by synthesis from simpler compounds or by degradation from sterols); more or less complete degradation of the formed hormone by reduction and oxidation, to some extent in the course of utilization; and esterification. The organs which may be concerned include not only the gonads and the accessory organs, but the adrenals, the liver, and the kidneys. Well may Siebke (1937), one of the veteran workers in this field, say:

> "We are absolutely convinced that the hormone assays carried out by us on excreta cannot give an exact idea of the general hormonal economy of the body. Too many unknown factors are acting: we do not know how much hormone is provided to the body by the food, how much is formed in the body itself, how much there is in circulation, and in what chemical form the hormone produces an effect on the appropriate organs; we do not know how much is stored, used up, and inactivated in one way or another; we fix on one figure alone, the fraction which is rejected. Even this figure we do not get by methods which are beyond criticism or absolutely exact; for, as far as concerns our own results, they deal only with determination of hormone which can be obtained by benzene extraction, but we do not know how many forms of hormone—perhaps soluble only in water—have escaped us in this process of preparation."

Survival of administered hormones.—One obvious test of the significance of the observed urinary excretion can be made, namely, the recovery from the urine of administered hormone. Luchsinger and Voss (1929) and Zondek (1934 *b* and *c*) made experiments of this kind. More detailed work was done by Robson, MacGregor, Illingworth and Steere (1934), and also by Kemp and Pedersen-Bjergaard (1933). The last workers investigated both fæces and urine in normal subjects, before and after oral administration of œstrin, and found that about 6% of the activity could be recovered from the urine. In a later study (1937), the administration of œstrin to men or castrate women was found to lead to the excretion of from 3–12% of it in the urine within the next two days, the proportion excreted being somewhat

greater after oral than after parenteral administration. Their method of extraction was, however, open to criticism, since they employed a mild hydrolysis which has not, in our experience, the effect of releasing all œstrin from its water-soluble combined form. Mazer and Israel (1936) also investigated the excretion of œstrin after administration, with the object of finding the " substitution dose of œstrogen, gauged by the minimal quantity necessary to maintain a premenstrual level of the principle in the blood and urine of castrated women " in order to judge amounts necessary for therapy. They may have been successful in this, but unfortunately for the question under discussion, they give figures only for the " active " œstrogen, i.e. the free œstrin extractable without hydrolysis. So far the result of work on human subjects has been not so much to provide evidence as to how hormones behave on passing through the body as to condemn methods of extraction. Only 3–12% of the administered hormone is extracted from the urine, but the percentage of the administered hormone which might be extracted is still unknown. Westerfield and Doisy (1937) have carried out experiments on the destruction and transformation of œstrogens in the monkey. They used a hydrolysis method (which is not described in detail) before extraction and found that only 1·5–5·3% of injected œstrogen could be recovered, even from an ovariectomized-hysterectomized monkey. The special interest of their work is the demonstration that œstrone and the non-ketonic œstrins are interconvertible in the body. Pincus and Zahl (1937) have investigated œstrin metabolism in the rabbit by more efficient methods which, they claim, lead to the recovery of up to 98% of the hormone under certain conditions. It is to be hoped that these methods can and will be applied to human urines. Until recently, the whole question of œstrin excretion seems to have been dominated by the unfortunate terms " active " and " inactive " forms of the hormone, with the implied assumption that the ratio of these is determined by the reproductive organs and not by some other organ such as the liver, one of whose supposed functions it is to esterify any excess of phenolic compound in the circulation, and thereby " inactivate " it solely in virtue of an acquired solubility in water and consequent rapid excretion.

Turning now to progestin, the state of affairs appears somewhat more satisfactory. It has already been mentioned that progestin activity is found in only minute amount in urine. There is, however, an inactive but chemically tangible transformation product, pregnanediol. Pregnanediol was first isolated from human pregnancy urine by Marrian (1929) (cf. Dingemanse *et al.*, 1930, and Butenandt, 1930). Later Odell and Marrian (1936) and Venning and Browne (1936) isolated pregnanediol glycuronide, and worked out analytical methods by which it could be recovered from urine (Venning, 1937 ; cf. Beall, 1937). Venning and Browne (1937) then demonstrated that it occurred in normal urine in the second half of the menstrual cycle, coincidently with the presence of the corpus luteum, and further, that injection of progesterone into human patients was followed by excretion of pregnanediol glycuronide, which could be recovered in an amount equivalent to from 12–46% of the progesterone injected. Here, therefore, we have a method of hormone assay which seems likely to be on a sounder basis than the œstrin assays.

No data have been published hitherto on the recovery of male hormone after administration to human subjects. We have ourselves obtained some results bearing on this subject in the course of investigations designed for another purpose. Under the auspices of the Therapeutic Trials Committee of the Medical Research Council a series of tests was carried out by several clinicians on the effect of androgens on prostatic hypertrophy. In an attempt to obtain as much information as possible, urine from the patients was assayed for androgen content before, during, and after treatment, extracts being assayed on capons by Dr. A. W. Greenwood at the Institute of Animal Genetics, Edinburgh. In the first stage of the trials, the material given was testosterone, in doses of 5 mgm. injected two or three times a week.

In this series (Table I) the differences in urinary androgen were small, and the figures must not be given too much importance, but it was certainly surprising to find that in some of the cases administration of testosterone appeared to produce an actual decrease in androgen excretion, apparently followed by a recovery after treatment

TABLE I.—ANDROGEN EXCRETION BY PATIENTS RECEIVING TESTOSTERONE

Patient*		Androgen content of urine (International units per day ; 7-day sample) Before	During	After
Ca.	C	17	11	14
Da.	A	17	16·5	—
Far.	C	10	9	—
Faw.	B	17	22	—
Ja.	B	—	16	24
Pa.	C	10	12	—
Pr.	A	13	? 9	9
Wi.	A	10	10	23

* A, patients of Dr. J. Carver ; B, patients of Mr. V. W. Dix ; C, patients of Dr. Raymond Greene.

had ceased. Tentatively, one explanation which suggested itself was that a depressant effect on androgen excretion was produced by way of the pituitary. Later cases were given larger doses of testosterone propionate instead of the free hormone. Table II gives data on cases treated by Dr. Raymond Greene. Here again the

TABLE II.—ANDROGEN EXCRETION BY PATIENTS RECEIVING TESTOSTERONE PROPIONATE

Patient	Androgen content of urine (International units per day ; 7-day sample) Before	During	After	Dosage of testosterone propionate when sample was taken
Wee.	13	? 9	—	10 mgm./wk.
R.	11	14	—	20 mgm./wk.
T.	20	21	30	20 mgm./wk.
Ca.	14	15	—	40 mgm./wk.
Wes.	? 9	11	? 8	40 mgm./wk.
Be.	? 11	13	? 7	50 mgm./wk.
Co.	17	46	—	50 mgm./wk.
Bo.	15	54	—	100 mgm./wk.

differences are small. With doses of 20 or 40 mgm. per week there are small increases, and only when 50 or 100 mgm. per week were given was there an unambiguous increase in the amount of androgen extractable from the urine, amounting to about 6% of the injected dose as a maximum in one case. Table III shows two results obtained recently with eunuchs, one treated by Dr. G. L. Foss, and the other under treatment

TABLE III.—ANDROGEN EXCRETION BY EUNUCHS RECEIVING TESTOSTERONE PROPIONATE

Patient	Date	Note of treatment		Androgen excretion: Capon assay i.u./day	Colorimetric assay mgm. "sterone"/day
S.	31.10.37 to 2.11.37	20 mgm. and 50 mgm. on 29 and 30.10.37	Free :	<4	2·1
			Combined :	160	30·7
,,	31.12.37 to 2. 1.38	After 5 weeks' rest from treatment	Total :	34	8·4
C.	1.12.37 to 6.12.37	No treatment	Total :	9	3·1
,,	29.12.37 to 2. 1.38	50 mgm./day	Total :	81	11·6

by Dr. Levy Simpson. The first case, details of which have been published by Dr. Foss (1937), had a long history of intensive treatment with testosterone propionate over a hundred days ; calculated on the basis of the amount given just before the collection of urine, in the next two days some 16% of the administered activity was recovered by extraction from the urine. Seven weeks later, and five weeks after cessation of treatment, the urinary androgen extracted had fallen to 34 international units per day. It is not certain whether this was his basal level, since the prolonged effect obtained by injection of testosterone propionate in oil is most

pronounced. The second case, though receiving a higher dose, shows a still lower percentage recovery. The conclusion from these results is that, in general, the destruction and inactivation of male hormone in the body after absorption is so intense, and the yield of administered hormone recovered in the urine is so small, that the present method of urinary androgen assay is unlikely to give results which have any great significance in respect to the career of male hormone in the body.

Finally, before leaving the subject of hormone metabolism, reference must be made to the gonadotropic hormone. Zondek (1935) briefly reported that in human subjects injected with prolan, about 10% of the amount administered was excreted in the urine. Friedman and Weinstein (1937) have recently investigated the recovery of gonadotropic hormone from the urine of normal males receiving extract of pregnancy urine. Small doses produced no detectable increase in the gonadotropic activity of the urine, and only repeated injection produced an increase which was estimated at not more than 20% of the injected activity.

On the whole, therefore, the amount of the four types of sex hormonal activity which can be recovered in recognizable form in the urine after administration is so small, that it seems permissible to argue, on this evidence alone, that we cannot obtain in this way an accurate picture of sex hormone activity in the body in normal subjects, or in those suffering from minor hormonal disturbances.

The possibility of applying assays of sex hormones to the diagnosis of the commoner menstrual disorders and of pathological conditions in non-pregnant women is of considerable interest. For this purpose it is obviously necessary to know the state during the normal menstrual cycle, and a review of our knowledge of normal conditions throws a critical light on the dependability of hormone assay methods and on ability to detect abnormalities by excretion beyond the limits of normal variability. Space does not allow of a detailed review here, but reference to papers published during the last eleven years on œstrin excretion alone demonstrates the slowness with which agreement is being reached merely on the type of normal variation in relative amounts in the course of the menstrual cycle, whilst estimates of the absolute levels of normal excretion are widely different, although they show some signs, in recent investigations, of tending to a maximum.

Lack of sexual differentiation in hormone excretion.—Yet another angle from which hormone assays may be regarded critically is provided by the facts of excretion by one sex of hormones named after the other sex. In the earlier period of sex hormone research the simple theory was held by many that substances with male and female sex hormone activity were entirely characteristic of male and female organisms respectively, and there was a tendency to assume that these substances, apparently responsible, not only for obvious tasks in the reproductive system and for the production and maintenance of secondary sexual characteristics, but even for psychological phenomena, were segregated in the two sexes. The existence of hormones properly belonging to one sex in the organs or excreta of the other sex was announced with surprise and even resisted. For instance, Frank and Goldberger (1928) accepted with some reluctance the fact that œstrin occurred in male blood, and later (Frank, Goldberger, and McGee, 1928) speculated hopefully that the occurrence of œstrin in male blood " might offer a physical basis for homosexuality ". Again, Laqueur *et al.* (1927), in reporting that they found œstrin in normal male urine, considered it necessary to say explicitly that they found it in urine from individuals " of whose ' manliness ' there can be no doubt ". The excretion of œstrone by the stallion (Zondek, 1934 *a* ; Deulofeu and Ferrari, 1934 *a* and *b* ; Häussler, 1934) is now so well known that it has become a " classical example ". Without going further into the question, it may be mentioned that the administration of testosterone has been shown to increase œstrin excretion in men (Steinach and Kun, 1937 *a* and *b*). This observation, which seems to be confirmed by some figures of our own on eunuchs, suggests interesting possibilities in hormone metabolism.

Excretion of androgens.—The most obvious example of this lack of differentiation of the excretions of the two sexes is provided by the excretion of androgens. This has been the subject of investigation at the National Institute for Medical Research, and it is proposed to take this opportunity to report briefly on some of our results. Two other groups of workers, in Amsterdam (Dingemanse, Borchardt and Laqueur, 1937 *a* and *b*), and in Chicago (Gallagher, Peterson, Dorfman, Kenyon, and Koch, 1937), have been busy on the same subject, and our results are to some extent complementary and confirmatory.

Androgenic material in urine occurs in a so-called "inactive" form which is soluble in water and not extractable by benzene or other fat-solvents (cf. Adler, 1934). Sufficiently vigorous hydrolysis by acid liberates the steroid hormones from the water-soluble compounds, and one of the first steps has been to discover conditions under which the optimum yield is obtained. Both Dingemanse *et al.* and Gallagher *et al.* (loc. cit.) have investigated this point more or less systematically, and our own work has dealt with it in considerable detail. Once it had been established that the first factor necessary was a sufficient amount of mineral acid, series of experiments on samples from pooled urine have been carried out in which the time of hydrolysis at the boiling point was varied. Without hydrolysis at the boiling point the yield of androgen is negligible. Within the first half-hour the yield obtainable rises to a maximum, and then it falls, evidently owing to destruction. Peterson, Gallagher, and Koch (1937) (cf. Gallagher, Koch, and Dorfman, 1935) chose fifteen minutes, while we have adopted from half an hour to one hour as the optimum period. In other respects the method described elsewhere (Callow, 1936 *a*) is followed. However, judged by results, the Chicago method, the rather different Amsterdam technique, and our own method, give figures for normal urine which are of the same order of magnitude—a singular triumph in hormone assay. We have given attention to one matter which has not been considered by other workers, namely the effect of conditions of storage. After allowing male urine to stand at room temperature (10–15° C.) without preservative for a week, the loss of androgenic activity in one experiment amounted to 36%, and other more striking examples of loss of activity have been obtained. This is an important point in the collection of urines to be assayed for diagnostic purposes.

The assay of the neutral fraction of urine extracts for androgenic activity, referred to here, was carried out by Mr. C. W. Emmens using the method of injecting capons and measuring the comb growth produced, according to the technique described by him in detail elsewhere (Emmens, 1938). Comparison with androsterone, the international standard of male hormone activity, is made by means of a dose-response curve constructed from the stock of capons receiving standard doses of androsterone; at least one control group of five capons being injected with androsterone over the period during which assays of urine extracts are being made.

Recently (Callow, Callow, and Emmens, 1937) we have investigated the colour reaction with *m*-dinitrobenzene, first proposed by Zimmermann (1935) for male hormones and later applied to the estimation of hormones in urine extracts by Zimmermann (1936), by Wu and Chou (1937), and by Oesting (1937). We have considerably increased the sensitivity of this reaction by modifying it, and an amount of extract equivalent to 10 ml. of a normal urine is sufficient to give an easily measurable colour; we have been able, by analysis of the colour, to increase the specificity of the test, and by correlation with capon assay we have been able to raise it to the status of a good index of androgenic activity.

Examination of the approximate absorption spectra of the colour reactions given by the steroid sex hormones and related compounds demonstrates that, in interaction with *m*-dinitrobenzene and potassium hydroxide, compounds of one particular class give a strong absorption band in the green, namely the 17-keto-compounds, among which are androsterone and dehydroandrosterone, the known androgenic constituents

of urine. Androstadien-17-one and androstan-17-one, for specimens of which we are indebted to Dr. R. L. Warren of the Royal Cancer Hospital, give similar curves. As a convenience, results are expressed in terms of milligrammes of " sterone " per litre of urine or per day's output of urine, the name " sterone " being a non-committal term for a 17-keto-compound like androsterone or dehydroandrosterone without specifying exactly what it is. In fig. 2 the values of this colorimetric assay are plotted against values from capon assay for a very varied collection of urines. Normal male or female urines are shown by the solid or half-moon points respectively. The line has been fitted by my colleague, Mr. Emmens, and has the equation $y = 6{\cdot}4x - 16{\cdot}7$, where y = content of international units by capon assay, and x = " sterone "

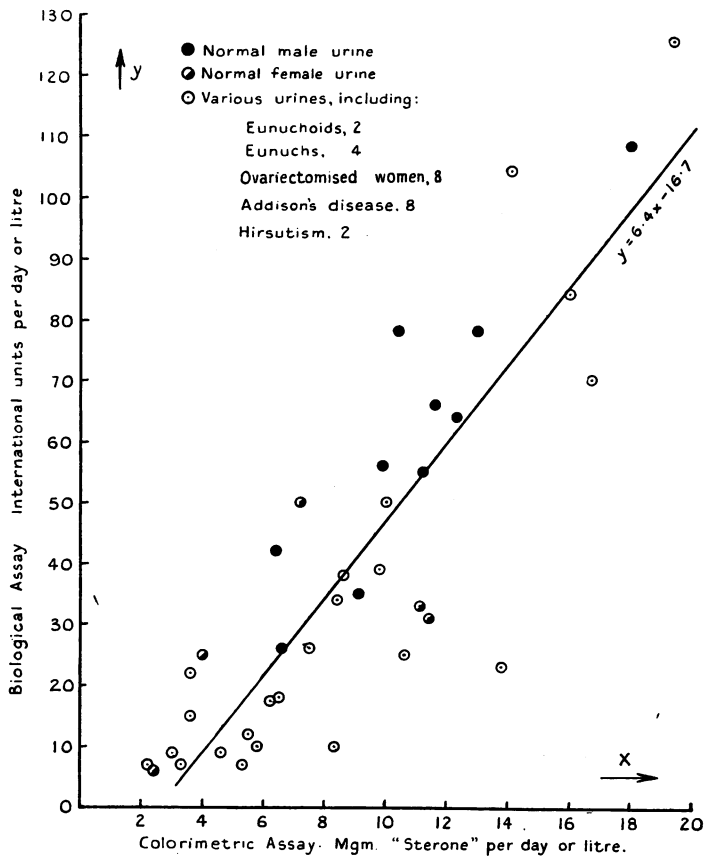

FIG. 2.—Relation between capon assay and colorimetric assay for urine extracts from various sources.

content measured colorimetrically. Were the only androgenic and chromogenic constituent of the urine extract pure androsterone, the equation would be $y = 10x$, and were it pure dehydroandrosterone it would be $y = 3x$. The position of the experimental line suggests that there is a biologically inactive, but chromogenic, impurity in the extracts, and that, apart from this, the androgenic material is a mixture of substances chemically and biologically resembling androsterone and dehydroandrosterone. Neither the biological nor the chemical tests are sufficiently specific for a more definite statement to be made ; obviously the occurrence of related

androgenic and chromogenic compounds cannot be excluded. It seems probable, from some recent preliminary tests, that chromogenic but biologically inactive substances may be removed by extraction with benzene before hydrolysis, and that the extract then obtained after hydrolysis may give a yet closer relation between colorimetric and capon assay. A detailed account of this investigation will be published with Mrs N. H. Callow and Mr. C. W. Emmens.

The feature of practical interest which appears in this diagram is that, in spite of its being made up from several classes of urines, the sources of which are sharply differentiated physiologically, it is impossible to distinguish segregation of any particular class into a group. One reason for this may be the multiplicity of factors influencing the androgen excretion of individuals. Dingemanse *et al.* have shown that there is a variation of urinary androgen with age. Before puberty and in senility androgen is present, but in small amount; the largest amount is present in the sexually active adult. Chou and Wu (1937) report that the male hormone excretion of Chinese is higher in the summer than in the winter, but as their results are based on colorimetric assays unchecked by capon assay, they require confirmation. It may be that constituents derived from the food interfere with the estimation. Dingemanse *et al.* and Gallagher *et al.* found large differences between individuals, and, further, wide variations in the daily excretion by the same individual. Our own examinations of subjects believed to be free from sexual dysfunction entirely confirm the existence of a large variation in the excretion of individuals from time to time. Thus, seven consecutive collections, each over three days, of urine from a male, aged 36, diagnosis hæmatemesis, showed daily levels, in mgm. of "sterone" per day, of 6·2, 6·9, 10·0, 9·0, 9·1, 7·2, 10·6. A second male patient, aged 38, diagnosis melæna, in six consecutive collections, each over six days, gave the figures 3·5, 8·3, 10·8, 12·3, 9·8, 8·6. The collection of these urines was kindly arranged for us at the Middlesex Hospital, by Professor E. C. Dodds. Women show equally high variability. Although Dingemanse *et al.* found in a single case that the highest excretion occurred during the post-menstrual period, Gallagher *et al.* did not confirm the existence of cyclic variation. Our own results indicate a tendency in the direction of higher values at the beginning of the cycle, but we have not yet sufficient data to be able to say whether this behaviour is general.

Bearing in mind the uncertainties which are introduced by these wide limits of variability in the normal subject, we may consider again the possibility of distinguishing between subjects grouped according to certain obvious physiological abnormalities. Tables IV and V show the distributions with respect to androgen excretion, expressed either in international units (capon assay) or mgm. of "sterone" (colorimetric assay) of patients classified according to their physiological state. These are preliminary data obtained in investigations which, when completed, my colleagues hope to publish in detail.

TABLE IV.—DISTRIBUTION OF LEVELS OF ANDROGEN EXCRETION IN THE URINE OF VARIOUS TYPES. BIOLOGICAL ASSAY.

Androgen excretion (international units per day or per litre)	< 10	10—19	20—29	30—39	40—49	50—59	60—69	70—79	80—89	90—99	100—109	110—119	> 119
Normal females (5)	1	—	1	2	—	1	—	—	—	—	—	—	
Normal males (6)	—	—	1	—	1	—	1	2	—	—	1	—	
Eunuchoids (3)	—	—	—	1	—	—	—	—	1	—	1	—	
Eunuchs (5)	1	2	1	1	—	—	—	—	—	—	—	—	
Ovariectomized women (12)	4	5	1	—	1	—	—	—	—	—	—	1	
Addison's disease (6)	2 (♀)	1 (♂)	2 (♀) 1 (♂)	—	—	—	—	—	—	—	—	—	
Hirsute women (13) ? adrenal hyperplasia)	2	1	—	1	2	—	2	1	1	—	—	1 [+ 2 cases; adrenal tumour. 150-2,500 i.u./day and 220 i.u./day]	2

TABLE V.—DISTRIBUTION OF LEVELS OF ANDROGEN EXCRETION IN THE URINE OF VARIOUS TYPES. COLORIMETRIC ASSAY

"Sterone" excretion (mgm. per day or per litre)	< 2·2—3·9	4—5·9	6—7·9	8—9·9	10—11·9	12—13·9	14—15·9	16—17·9	18—19·9	
Normal females (7)	2	2	2	1	—	—	—	—	—	
Normal males (10)	—	—	3	3	1	2	—	1	—	
Eunuchoids (3)	—	1	—	—	—	1	—	1	—	
Eunuchs (4)	1	—	—	2	—	1	—	—	—	
Ovariectomized women (14)	1	4	5	1	2	—	—	—	1	
Addison's disease (6)	2 (♂) 1 (♀)	1 (♀)	2 (♀)	—	—	—	—	—	—	
Hirsute women (12) (? adrenal hyperplasia)	1	1	2	1	1	—	1	1	—	[+ 2 cases; 27 and 33; + 2 cases adrenal tumour, 150 and 175 mgm./day]

Normal women (in the top line) vary between limits of excretion of 5 and 60 international units of androgen per day. Normal men (in the section immediately below) vary between the limits of 20 and 110 international units of androgen per day. As a group, the women have a lower level of excretion than the men, but the limits overlap, and it is impossible to distinguish one sex from the other from examination of the urine by these methods. There is a school of thought which considers that such a difference between the sexes ought to exist; even if the quantity is the same, some qualitative difference should exist. At present, no characteristic difference can be discerned, although, admittedly, our methods of chemical examination are not highly selective. It is still possible, however, that with increasing specificity, analysis may show some differentiation. Referring to the other sections of the diagram, it can be seen to what a small extent androgen excretion is dependent on the sexual condition.

Eunuchoids [two of these are patients under the observation of Dr. Crooke, and particulars of one of them, S.A., have been published (Crooke, 1937)], in which testicular function appears to be completely suppressed, would appear to have an androgen excretion which is, if anything, above the average for normal men. Eunuchs, of which there are four examples, show a low level of androgen excretion, but not necessarily outside normal limits.

These cases remove the uncertainty as to whether the castrated male excreted significant amounts of male hormone. Chou and Wu (1937) reported definite androgenic activity in both capon assay and colorimetric assay in the urine of a eunuch aged 68, but McCullagh and Renshaw (1934) had previously failed to find androgen activity in the urine of eleven eunuchs; Koch (1936) reported low positive results, but later Kenyon *et al.* (1937), using an efficient method of extraction, found only traces in two cases, amounting to 1 i.u./litre in one case and 3·5 i.u. in another. It might be conjectured that possibly decomposition had occurred in the course of making the large collections to which they refer. After some unfortunate experiences, we consider it most important that urine should be extracted as fresh as possible, even if a preservative has been added. Two of our five cases, one under Dr. S. Levy Simpson and the other under Dr. P. M. F. Bishop, show values well within normal limits without having previously received any hormone therapy, although the colorimetric assay figure (13·8 mgm./day) in the latter case is anomalous. A third is, perhaps, suspect on account of his male hormone treatment. The medical history of the remaining two is less well established.

Ovariectomized women (mostly patients under the observation of Dr. Levy Simpson) form a group, with one exception, definitely below the average of normal women but still overlapping the normal limits. Consideration of these three groups seems definitely to exclude gonadal function as a major factor in determining androgen excretion. Turning, for reasons I have already mentioned briefly, to the adrenal as a possible site of origin of urinary androgens, we obtained, through the agency of

Dr. Crooke and Dr. Levy Simpson, urine from a number of cases of Addison's disease. On the whole, the low level of androgen in most of these cases gives support to the hypothesis suggested. Unfortunately, complete adrenalectomy is not a possible operation in the human, and the degree of adrenal cortical deficiency in these patients is difficult to assess.

When, however, we turn from deficiency to excess, and consider cases of suspected adrenal hyperplasia included in the remaining class of subject, cases of hirsutism, we come to a series of observations which lead on to the part of this investigation which seems most likely to bear fruit of more than academic interest. Simpson, de Fremery, and Macbeth (1936) examined a series of cases of adrenogenital syndrome and found in the urine of four out of seven cases a significant excess of androgen, though in only one case was this very large. Since then, Slot (1936) has recorded a case of virilism, due to adrenal tumour, with an excretion of 2,200 i.u. androgen per litre of urine. Kenyon *et al.* (1937) (cf. Cahill *et al.*, 1936) have recorded an excretion of 480 international units per day in a case of carcinoma of the adrenal cortex.

With the co-operation of several clinicians, we have investigated the androgen excretion in a series of cases which showed signs, in varying degrees, of virilism, hirsutism being the common symptom. It will be seen that the range over which the figures for androgen excretion spread is a wide one, stretching from 7 i.u. per day for an aged woman up to values which are outside the limits of the diagram. Considering the moderate figures, our results are entirely in accord with those of Kenyon *et al.*: that as a rule normal amounts of androgenic material are excreted, but that a moderate excess of androgens is occasionally found. One of these cases, with normal excretion, has been operated on recently, and an apparently normal adrenal was found and removed. In another case with moderately high androgen (about twice normal) an enlarged adrenal has been diagnosed by X-ray examination. Dr. Crooke considers that in his series of patients there is some correlation between high androgen excretion and degree of menstrual disturbance. On the whole, however, it is difficult to find any line of demarcation or to suppose that hirsutism is due only to a hypersecretion of an androgen which can be recognized in the urine. The dividing line comes at a much higher level, and in an entirely separate category come two cases of adrenal tumour. In one case, from the London Hospital, the patient was a girl aged 7 years, with a large adrenal tumour which, removed at autopsy, weighed 5 kg. At one stage, the urine contained 3,500 i.u. of androgen per litre. A few weeks later a specimen had, by capon assay, 500 i.u. per litre and, by colorimetric assay, 170 mgm. of " sterone " per litre. 70% of the androgenic activity could be accounted for by dehydroandrosterone, which was isolated in pure crystalline form (Callow, 1936 *b*). Recently, the urine from a second case, a 19-year-old girl from whom an adrenal tumour was removed by operation, has been examined. This showed an androgen excretion of 320 i.u./litre by capon assay, and a colorimetric assay of 140 mgm. of " sterone " per litre. Dehydroandrosterone was again readily isolated from the urine in a yield of about 60 mgm. per litre. Adrenal tumour can, therefore, be readily and quickly diagnosed with the aid of androgen assay, either colorimetric or biological, of the urine.

A contrast to these cases was provided by a case of Cushing's syndrome in an adult woman, studied clinically by Dr. Crooke. This case showed hirsutism of male type, but we were unable to detect any hormonal abnormality in the urine, and the androgen excretion, in particular, was within normal limits and actually lower than the normal average. At autopsy the adrenals were of normal size.

The andrenogenital syndrome is comparatively rare in men. Burrows, Cook, Roe and Warren (1937) found in the urine of a man with an adrenal tumour a very high œstrogen content, with a moderately high androgen content. They isolated from the urine the compound androstadien-17-one, closely related to dehydroandrosterone.

The occurrence of high œstrin excretion in cases of adrenal tumour has been reported by Frank (1934, 1937), Graef *et al.* (1936), but the enormous amounts reported by these writers do not seem to be a constant occurrence (cf. Slot, 1936). Of our own cases, subject to the reservation that œstrin extractions have not always been efficient, it can be said that the child certainly excreted excessive œstrin for her age, but did not give an extraordinary value, and in Dr. Levy Simpson's case there is no indication of an obviously excessive amount.

The fact that only in cases of adrenal tumour is an enormous excess of androgen found, and that the principal active compound is dehydroandrosterone, leads to the assumption that the dehydroandrosterone found normally is an excretory product derived from the adrenals, and even suggests that urinary androgens are derived entirely from this source. I doubt whether this latter general conclusion is justified, for it seems possible that the adrenals and the gonads are, to some extent, complementary to each other in androgenic activity. The question of the source of androgenic substances in the urine has recently been reviewed by Parkes (1937 *b*). The complex question of the relation between the adrenals and the gonads is outside the scope of this paper.

There appears to be a definite relation between the pituitary and androgen excretion, whether derived from the adrenal or the gonads. Davidson (1937) found that some development of the atrophic prostate and seminal vesicles of castrated rats could be brought about by the administration of adrenotropic extracts of the pituitary. We have been able, by the courtesy of Dr. W. D. Henderson, of the National Hospital, Queen Square, to examine the urine from a case of infantilism due to pressure on the pituitary in a youth aged 22. Before operation the urine extract had a low colorimetric titre, and the capon assay showed the presence of a trace only of androgen. A month after operation these values had notably increased, and still later, at least as regards colorimetric assay, the androgen excretion appears to be normal, not only in amount, but also in its chemical character ; the colour reaction had previously been rather anomalous.

In conclusion, the present state of the question of the significance of the excretion of sex hormones in the urine may fairly be summed up by saying that in cases where relatively tremendous amounts of hormone are produced and excreted, hormone assay is an extremely valuable aid to diagnosis. In quite another category, with our present methods, and even perhaps with improved methods, are the mildly abnormal cases. I think that the assumed significance of urinary sex hormone estimation in such cases must be justified empirically. If we know definitely that in a certain physiological state a given sex hormone is produced in large amount, and we find a corresponding product in the urine, then a connexion is established, and it will be possible to extend to other circumstances an argument based on analogy. Argument in the other direction, from observed excretion to a process in the body, is unsafe in the present state of our knowledge. Here, I think, it is appropriate to quote R. T. Frank and his colleagues (1934) who, summing up nine years' work on hormone assays, say that " such studies, though they serve as valuable aids, cannot replace clinical acumen and experience ".

It is to be hoped that such improvements in methods of extraction and assay may result from the work of chemists and biochemists that some material advance may be made from the present position, and more certain aid be given to the clinician.

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Discussion.—Dr. LEVY SIMPSON said that the investigations of Dr. Callow and his colleagues were, he believed, of fundamental importance, and should ultimately throw a good deal of light on the biological mechanism in endocrinopathies. At the moment, the significance of the data was not always clear, but the apparent confusion in some directions should serve as a stimulus for further study.

In 1933 (*Proc. Roy. Soc. Med.*, 1934, **27**, 383), he began investigations, in collaboration with de Fremery and Macbeth, on the " male " comb-growth, seminal vesicle, and prostate-stimulating hormone, in the urine of virile women. The detailed results were subsequently published (*Endocrinology*, 1936, **20**, 363), and, in summary, showed that patients with the adrenogenital syndrome, or the Cushing's syndrome, might—but did not invariably—excrete an excess of " male " hormone. The lack of consistency was perhaps difficult to appreciate if excretion was a measure of secretion, but this latter was probably not the case. The innate responsiveness or refractoriness of the tissues was also a factor. Since the pituitary did not secrete comb-growth hormone, and since one of the worst cases of adrenal hirsutism had followed bilateral ovariectomy, it seemed probable that the adrenal gland was responsible for the secretion of the excess of comb-growth hormone. Subsequent experience with adrenal tumours, and the disappearance of pathological hormones after their removal, tended to confirm this view, as also did the work on castrated males and females recorded by Dr. Callow.

Clinically, the presence of a gross excess (e.g. more than 300 capon units per day) of comb-growth hormone in the urine of a virile woman was of diagnostic significance, and meant that every effort, including laparotomy, must be employed to track down an adrenal tumour. Unfortunately, adrenal hyperplasia was occasionally associated with values up to 300 units per diem, but more usually the excretion was no greater than 150 units. Gross enlargement of the clitoris favoured the diagnosis of an adrenal tumour, but this was not invariable; and such enlargement might be absent even when an adrenal tumour was present, together with an excess of comb-growth hormone in the urine.

In a male with an adrenal tumour and feminization, including enlargement of the breasts and atrophy of the penis, with impotence, a large amount of œstrogenic hormone was found; this disappeared after operation, but reappeared when metastases occurred. It was, in fact, the first evidence that secondary deposits were occurring. Why should an adrenal tumour in the male appear to give rise to an excess of œstrogenic hormone, and, in the female, to an excess of the comb-growth hormone? At his request, Miss Joan Ross had kindly compared two such tumours, and found that, although the histological appearances were similar, that of the female contained certain characteristically staining discrete granules, which were not present in that of the male. She also observed that similar granules appeared in the inner layers of the adrenal cortex in normal males after puberty, but did not appear in normal females until the age of 50. There might, therefore, be a histological basis for these biological differences.

In a series of ovariectomized women, investigated with Dr. Callow and colleagues, it had not yet been possible to correlate the clinical types with the biological findings. Apparently in the case both of ovariectomy, and of castration in the male, the changes that resulted were dependent upon the initial endocrine constitution of the individual, and considerable differences were met with. Some of the women excreted amounts of comb-growth hormone above normal, but it did not follow that these women showed virilism or hirsutism, to an appreciable degree. It would appear that here, too, the response of the tissues to circulating hormones was an important factor determining the clinical manifestations.

Dr. A. S. PARKES: The original work described by Dr. Callow has been carried out as part of a plan of investigation into the whole subject of the practical possibility and clinical value of what is usually called " hormone analysis ". In the course of this work it has become evident that the technical difficulties of obtaining sound results are very great, and that the significance of the results is not yet obvious. However, as will have been gathered from Dr. Callow's admirable survey, considerable progress is being made, even though much of it

is negative in character, and it is to be hoped that conclusions of practical value will ultimately emerge.

Dr. Callow has emphasized that many of the substances commonly termed hormones are more likely to be of the nature of by-products in the metabolism of other substances. I think, however, that it is difficult to draw any sharp line of distinction between hormones and waste products. There is evidence that the substances we now know as hormones were originally waste products for which animals, in the course of evolution, have acquired a use. Some of the substances which function as hormones in mammals occur in lower vertebrates in conditions in which they can, presumably, be of no value. Thus, the lactogenic hormone, probably responsible in mammals for the secretion of milk, is to be found in the pituitary gland of birds, reptiles, amphibians, and fish. We have heard of the extraordinary mixture of œstrogenic and androgenic substances in both sexes of mammals, and notably in the urine of men and women. If evolution proceeds along the line which it appears to have taken so far, and mammals learn to use these anomalous substances, it may perhaps be prophesied that evolution will be in the direction of general intersexuality.