CLXXVI. COLORIMETRIC DETERMINATION OF SUBSTANCES CONTAINING THE GROUPING $-CH_2.CO-$ IN URINE EXTRACTS AS AN INDICATION OF ANDROGEN CONTENT

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In the expectation that a measure of endocrine activity in the human patient might be given by the level of excretion in the urine of compounds related to the male hormones, attention has recently been directed to the extraction of urinary constituents possessing androgenic activity and their measurement. As an alternative to biological assay, chemical methods have been applied. Zimmermann [1935] first suggested that steroid sex hormones could be determined quantitatively by the use of *m*-dinitrobenzene, which gives a red colour in presence of alkali with compounds containing an active methylene group. A year later [Zimmermann, 1936] he published a modified method which was applied to pure compounds (androsterone, testosterone, oestrone and equilin), and to urine extracts. Wu & Chou [1937] described a modification of the method, and used it on urine extracts with androsterone as a reference substance. Oesting & Webster [1938; cf. Oesting, 1937] used the Zimmermann technique, and roughly correlated their figures with the comb-growth produced in capons by inunction of the same extracts on the comb.

None of these investigators has considered simultaneously both capon assay and colorimetric assay in terms of pure hormones, and there was thus an obvious gap to be filled before it would be possible to test the colorimetric assay as an indicator of the androgenic activity of urine extract in terms of international units. The correlation of the figures obtained in this way would be expected to give data which, in addition to testing the value of the colorimetric assay on the assumption that excretion of androgenic activity was diagnostically significant (as has been done by Oesting & Webster), would also throw light on the chemical nature of the androgenic substances excreted in the urine, providing clues to the metabolism of steroid hormones, and giving a tangible chemical property which would serve as a guide in the analytical investigation of urine extracts.

As part of a scheme of work on the determination of hormones in blood and urine, undertaken under the auspices of the Hormones Committee of the Medical Research Council, investigations of methods of extraction of androgens from urine and their assay on capons have been in progress at this Institute for some time. These have been extended to a comparison of colorimetric and capon assays, and, on the chemical side in particular, to attempts to improve the sensitivity of the colorimetric method and to investigate its specificity.

The account of experimental work which follows deals in turn with the description of the modified colorimetric method finally adopted, a study of some of the many factors influencing the reaction, an investigation of the behaviour

of various methyleneketones of steroid and other types, and the correlation of colorimetric assay with capon assay for urine extracts from normal and certain abnormal clinical types.

Experimental

Reagents. (1) *Routine method of colorimetry of androsterone*

(*a*) *Alcohol.* Ordinary commercial "absolute" alcohol is used, the only further specification being that it should not have a content of aldehyde exceeding 0·0025%.

(*b*) m-*Dinitrobenzene.* A well-crystallized and fairly pure material (we used B.D.H. "extra pure", M.P. 89–89·5°) is taken and further purified as follows: 20 g. are dissolved in 750 ml. of 95% alcohol warmed to 40° and 100 ml. of 2*N* NaOH are added. After 5 min. the solution is cooled, and 2500 ml. of water are added. The precipitated *m*-dinitrobenzene is collected on a Büchner funnel, washed very thoroughly with water, sucked dry and recrystallized twice in succession from 120 ml. and 80 ml. of absolute alcohol. The material must be well crystallized in almost colourless needles, M.P. 90·5–91°. Admixture of a 1% alcoholic solution with an equal vol. of aqueous 2*N* NaOH should give no colour after an hour. The reagent is a 2% w/v solution of this material in absolute alcohol. It is stored in a brown, stoppered bottle in the dark, and is stable for 10–14 days. In the actual colorimetric measurement (see below) the control solution, without methyleneketone, should give a pale straw colour having a value of $E_g = 0{\cdot}20$–$0{\cdot}21$ in a 1 cm. cell compared with alcohol.

(*c*) *Potassium hydroxide.* The reagent solution is 2·5*N* KOH in absolute alcohol. 9 g. of KOH (B.D.H. "Analar" pellets) are dissolved with mechanical stirring in 50 ml. of absolute alcohol, and the solution filtered through a hardened paper (Whatman No. 50) at the pump. The concentration is checked by titration of 0·5 ml. with 0·1*N* H_2SO_4 (methyl orange indicator) and the solution diluted with alcohol if necessary, to bring it within the limits of 2·48 and 2·52*N*. The solution is stable for 2–5 days if stored in a refrigerator. It must be discarded as soon as the faintest colour is perceptible.

Mode of operation

Test tubes used for the reaction must have been cleaned with nitric and chromic acid mixture. Into one tube, to serve as "blank", are measured out in succession, from 1 ml. pipettes graduated to 0·01 ml., 0·2 ml. of alcohol, 0·2 ml. of *m*-dinitrobenzene solution and 0·2 ml. of KOH solution. The solution of the test substance is measured out into a second tube, and then there is added sufficient alcohol to make the volume up to 0·2 ml., followed by the reagents. The time of adding the KOH is noted. The tubes are well shaken to disperse the dense KOH solution, lightly stoppered, and placed in a water bath kept at $25 \pm 0{\cdot}1°$ by means of a thermo-regulator. The tubes are shielded from all but dull, diffused light by a screen. After an hour, 10 ml. of alcohol are added to each tube, and the contents mixed and transferred to the cells of the colorimeter, which are then closed by microscope coverslips.

Measurement of the colour

Use of the Spekker photoelectric absorptiometer. In this instrument the light absorbed by a coloured solution is measured photoelectrically by a null method. The light transmitted by the test solution falls on a photoelectric cell, the current from which is balanced by current from a second cell with an adjustable iris in front of it. The test solution is then replaced by the blank or control

solution, and balance again obtained by closing in the path of the beam a shutter actuated by a wheel with a logarithmic scale, from which an "absorption coefficient" (E) is read off directly. Using light which has passed through a selective filter, a value of E for a limited range of wave-lengths is obtained. In this work, cells of 1 cm. thickness have been employed, with an Ilford "spectrum green" filter (max. transmission at 5200 Å.). As a routine, readings (denoted by E_g) on one pair of solutions are made in triplicate: in our experiments these differ by not more than 0·01 over the range $E_g = 0{\cdot}1$–$0{\cdot}5$, which is the most suitable for measurement. It is desirable to complete the measurement within 5 min. of the dilution with alcohol. The methyleneketone content of the test substance in terms of androsterone is then determined from the absorptiometer reading (E_g) by reference to a calibration curve constructed from measurements with androsterone. All the recorded measurements, unless otherwise specifically stated, have been made by comparison of the test solution with a control blank containing reagents only.

Calibration curve. The androsterone used as standard was a purified sample with M.P. 184–185° (corr.). In the beginning of this work measured amounts of an alcoholic solution were evaporated to dryness in a stream of nitrogen on a

Table I. *Calibration of Spekker absorptiometer*

Androsterone (mg.)	0·025	0·05	0·075	0·10	0·15	0·20	0·25	0·30
E_g (*a*)	—	0·205	—	0·395	0·545	0·66	0·74	0·80
E_g (*b*)	0·10	0·19	0·275	0·365	0·50	0·60	—	—

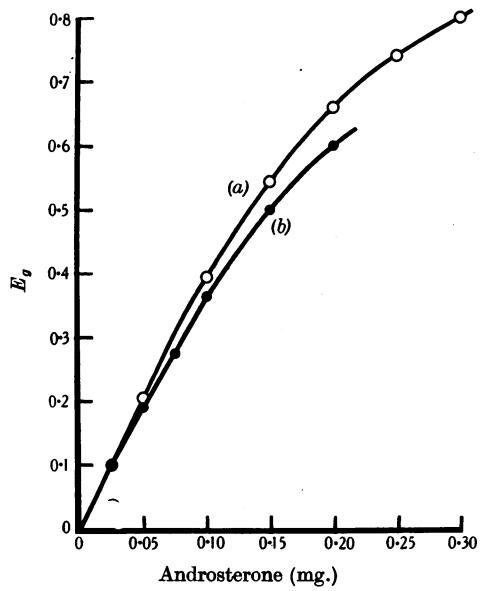

Fig. 1. Calibration curves for the reaction: androsterone + m-$C_6H_4(NO_2)_2$ + KOH (test solution − blank) on Hilger Spekker absorptiometer with Ilford "spectrum green" filters.

water bath, and alcohol and reagents added to the residue. It was soon found that loss occurred owing to the volatility of androsterone, and after this the androsterone was measured out in the form of appropriate amounts (0·1–0·2 ml.) of 0·025–0·1 % solutions in absolute alcohol, made up to a total volume of 0·2 ml.

Using the precautions specified above, it has been possible to reproduce colorimetric values for certain weights of androsterone within comparatively narrow limits, but there is a residue of variation which we have not been able to control. In all probability this is due to impurities in the alcohol and the *m*-dinitrobenzene. In view of the number of factors influencing the reaction, as described in detail below, the agreement is satisfactory. Over a period of seven months, during which the calibration curve has been frequently checked, the maximum variation has been between the limits shown in Table I and curves (*a*) and (*b*) in Fig. I.

At the upper limit suggested for readings, $E_g = 0{\cdot}5$, the difference, in terms of weight of androsterone, is 12%. This difference does not, of course, represent the error in the determination of unknown materials when the position of the calibration curve with a new set of reagents is confirmed in the routine manner by colorimetry of a known amount of androsterone. Thus, ten measurements with 0·1 mg. of androsterone over a period of 12 days with two apparently similar batches of reagents gave a value of 2·0% for the standard error of the absorptiometer reading, corresponding to a standard error of 2·7% in the estimation of the weight of androsterone after reference to the calibration curve.

Use of a visual colorimeter of plunger type. With the co-operation of Mr S. W. Stroud, working in the Courtauld Institute of Biochemistry, Middlesex Hospital, an investigation has been made of the use of a Leitz two-stage colorimeter in the colorimetry of androsterone and urine extracts. With this instrument observations can be made with light which has passed through selective filters. It is only necessary to choose a colour standard. Potassium permanganate solution has an absorption spectrum which is very similar in the green, except for its banded character, to the net absorption spectrum of androsterone and reagents less that of reagents alone. Permanganate solution plus blank can therefore be matched approximately against test solution plus water, and accurate matching is possible in the light transmitted by the Leitz "yellow-green, 531" or "green-yellow, 551" filters: the former, transmitting light nearer the centre of the absorption band, gives higher readings.

The colorimeter is set up with test solution, prepared in the usual way, in the top cup and water in the bottom cup on one side, and, on the other side, blank in the top cup and $N/1000$ $KMnO_4$ in the bottom cup, the last being in a 20 mm. layer. The top plungers, which move together, are adjusted until the beams through the two sides are matched, and the depth of the layer is then read off on the scale.

A calibration curve was constructed with weights of androsterone from 0·05 to 0·225 mg. and the data are given in Table II and plotted in Fig. 2 in the form of weight of androsterone against the reciprocal of the scale reading.

Estimations made in this way on six urine extracts were compared with estimations on the Spekker absorptiometer, and gave the following results in terms of colorimetric equivalent, in mg., of androsterone per day's output, the pairs of figures being from Leitz and Spekker instruments, respectively: (*a*) 7·5, 8·0; (*b*) 7·0, 7·5; (*c*) 15·5, 15·3; (*d*) 15·7, 15·8; (*e*) 1·5, 1·75; (*f*) 24·0, 22·5. The agreement is fairly good. The disadvantage of this technique with the Leitz instrument is the impossibility of making absolute determinations of the absorption in the violet. As explained below, this may be an important matter in the recognition of occasional urine extracts in which the chromogen is abnormal, and the value of E_g alone bears no relation to the maximum of the absorption band.

Table II. *Calibration of Leitz colorimeter*

Androsterone (mg.)	0·05	0·075	0·10	0·125	0·15	0·175	0·20	0·225
Scale reading (531 filter)	38·3	23·4	17·1	14·3	11·9	10·0	8·7	8·4
Scale reading (551 filter)	31·5	19·3	14·6	11·3	9·2	7·6	7·3	6·6

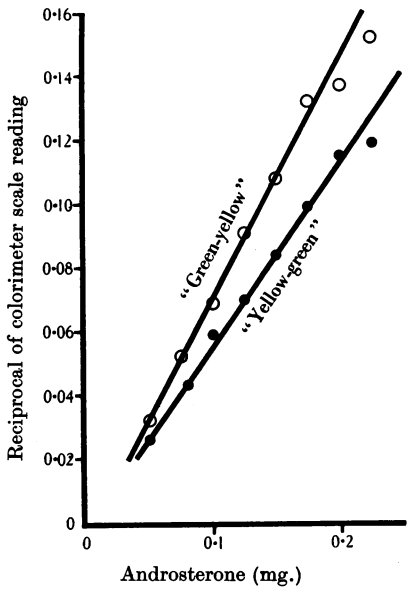

Fig. 2. Calibration curves for the reaction: androsterone + m-$C_6H_4(NO_2)_2$ + KOH (test solution + water/blank + 0·001N $KMnO_4$) on Leitz colorimeter with "green-yellow" and "yellow-green" filters.

Application to urine extracts

Colorimetric measurements have been made on the neutral fractions of urine extracts made by the method described by one of us [Callow, 1936, 1] with minor modifications. The acid and phenolic fractions contain ketonic material, and must always be removed. The neutral fraction is usually taken up in alcohol at a concentration such that 1 ml. contains the equivalent of either 0·1 l. of original urine, or 0·1 day's output, and 0·1 ml. of this solution is taken and diluted with 0·1 ml. of alcohol for colorimetry as described above. The colour of the urine extract itself is not sufficient to interfere with the determination. An unusually highly coloured extract gave a value of $E_g = 0{\cdot}01$ when diluted in the proportion of 0·01 l. urine to 10·6 ml. Moreover, treatment with charcoal generally produces no appreciable change in the colorimetric reading (Oesting [1937] found that androgenic activity was not lost in this way). As in the case of androsterone, evaporation of alcoholic solutions of urine extracts, unless very carefully done, entails loss not only by volatilization but also by creeping, and this method of taking a known amount was abandoned.

The absorptiometer reading is converted, by reference to the calibration curve, into an equivalent in weight of androsterone, which is then expressed as mg. of "sterone" per litre or per day's output of urine. We have coined the term "sterone", to avoid giving the wrong impression that this value represents the biological and not merely the chromogenic equivalent of androsterone.

(2) *Factors influencing the reaction with androsterone*

General. In preliminary trials it was immediately found that, with unpurified *m*-dinitrobenzene, the technique of Wu & Chou [1937] gave a higher colorimetric reading than that of Zimmermann [1936] with the same amount of androsterone; the former method was therefore chosen as the starting-point for investigation of the factors influencing the sensitivity. Certain of these had already been studied by Zimmermann (Z.) and by Wu & Chou (W.C.), namely: (1) the solvent (Z., W.C.), (2) concentration of alkali (Z., W.C.), (3) concentration of *m*-dinitrobenzene (Z., W.C.), (4) time of measurement after mixing (Z.), (5) temperature of development (Z., W.C.), (6) illumination (Z.). With so many variables concerned (and this list is not exhaustive) it was to be expected that further systematic work would lead to an increase in the sensitivity of the reaction. Moreover, by choosing conditions under which the effect of variation in any factor was changing most slowly, the reproducibility of the readings might be increased.

Solvent: purity and amount. Small amounts of aldehyde in the alcohol cause increased absorption in both the test and the blank solutions, but more in the latter, so that, as a result of over-compensation, the value of test minus blank decreases with increase in the aldehyde. The figures in Table III were obtained using our routine technique with 0·1 mg. of androsterone. The absorptiometer with the "spectrum yellow-green" filter was used for the determination of aldehyde in samples of alcohol by means of the colour produced with Schiff's reagent. In separate experiments the same alcohol, of known aldehyde content, was used for dissolving the androsterone, the *m*-dinitrobenzene and the potassium hydroxide.

Table III. *Influence of acetaldehyde on* E_g *with 0·1 mg. androsterone*

% MeCHO	Test minus blank	Blank minus EtOH	Test minus EtOH (calc.)
0·0005	0·395	0·11	0·505
0·0017	0·37	0·17	0·54
0·0025	0·35	0·195	0·545
0·005	0·31	0·27	0·58

The effect of altering the total amount of solvent in the reaction mixture was tested as follows: portions of 0·25 mg. of androsterone were dissolved in 0·15, 0·22 and 0·26 ml. of alcohol, and to each were added 0·2 ml. of 2 % *m*-dinitrobenzene solution and 0·19 ml. of 2*N* alc. KOH. Controls were made up to the same total volumes, viz. 0·54, 0·61 and 0·65 ml. The values of E_g after dilution at the end of 1 hr. were respectively: 0·65, 0·62 and 0·57. The effect of using 95 % alcohol instead of absolute alcohol as a diluent after incubation was to reduce the value of E_g for 0·1 mg. of androsterone from 0·365 to 0·35. With the earlier technique the difference was greater. The specifications in the routine method of amount of alcohol, and of content of aldehyde and water, were adopted as giving a sensitive reaction whilst setting limits attainable in practice.

Purity and concentration of m-*dinitrobenzene.* Ordinary *m*-dinitrobenzene gives a red colour with potassium hydroxide. This has been attributed to the presence of dinitrothiophene [Meyer & Stadler, 1884] which is detectable in a quantity of 0·1 μg. [Stadler, 1885] by this reaction. Nitration of "thiophene-free" benzene gave a product which still gave a colour with alkali, and we therefore followed the method of Willgerodt [1892] for removal of chromogenic material, details of which are given above. This method must be adhered to

strictly, for overheating, or prolongation of the alkali treatment, gives poorly crystallized products of low M.P. which are useless for colorimetry. One such sample, after repeated crystallization from alcohol, then ethyl acetate, yielded yellow crystals, sparingly soluble in alcohol, M.P. 148° (soft at 145°), identified as 3:3′-dinitroazoxybenzene (lit. M.P. 143°, 146·5°). (Found (micro-analysis by Dr G. Weiler): C, 50·36; H, 3·08; N, 19·9. Calc. for $C_{12}H_8N_4O_5$: C, 50·0; H, 2·8; N, 19·45%.)

The effect of impurity in the *m*-dinitrobenzene on the value of E_g is shown by the figures in Table IV, dealing with the variation of E_g with amount of KOH when different samples of *m*-dinitrobenzene were used, the "purified" samples having passed through the alkali process.

The effect of the amount of *m*-dinitrobenzene used was investigated at two stages of the development of the final method. We first confirmed Wu & Chou's

Table IV. *Variation of* E_g *with amount of KOH*

Wt. of androsterone (mg.)	0·25	0·25	0·10	0·10	0·05
m-Dinitrobenzene ...	Unpurified	Purified	Unpurified	Purified	Purified
Vol. of 2*N* alc. KOH (ml.)	E_g				
0·09	0·32	—	—	—	—
0·12	0·47	0·365	—	—	0·12
0·15	0·54	—	—	0·28	—
0·16	—	0·53	—	—	—
0·19	0·56	0·63	—	—	0·165
0·20	—	—	0·32	0·34	—
0·25	0·51	0·66	0·34	0·365	0·20
0·275	—	—	0·345	—	—
0·30	—	0·61	—	0·34	0·20

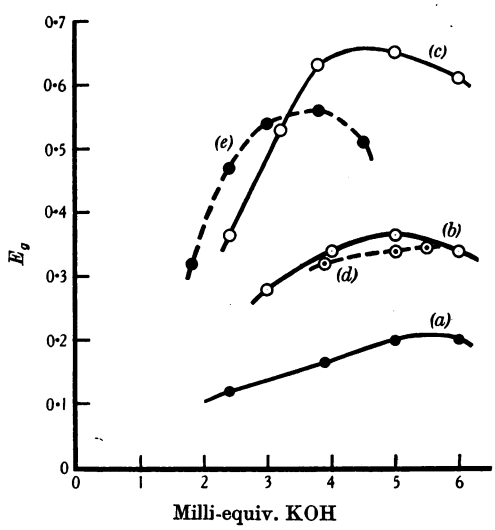

Fig. 3. Variation of E_g with amount of KOH. Curves (*a*), (*b*), (*c*): purified *m*-$C_6H_4(NO_2)_2$; 0·05, 0·1 and 0·25 mg. androsterone. Curves (*d*), (*e*): unpurified *m*-$C_6H_4(NO_2)_2$; 0·1 and 0·25 mg. androsterone.

observations that low values were obtained when small amounts of *m*-dinitrobenzene were used. In the final form of the reaction, 0·1 mg. of androsterone in 0·1 ml. of alcohol with 0·2 ml. of 2·5*N* KOH and varying amounts of 2%

m-dinitrobenzene, viz. 0·14, 0·18, 0·20, 0·22 and 0·24 ml., made up to a total volume of 0·6 ml. with alcohol, gave the following values of E_g: 0·365, 0·36, 0·365, 0·375, 0·36. There was thus no systematic variation outside the limits of experimental error over this range of concentrations.

Effect of amount of potassium hydroxide. Series of tests were carried out in which androsterone was dissolved in $(0{\cdot}41-x)$ ml. of alcohol, 0·2 ml. of 2% alcoholic *m*-dinitrobenzene added, and then x ml. of $2N$ alcoholic KOH (x being varied from 0·09 to 0·3 ml.). Table IV gives the values of E_g for three series, one with an unpurified sample of *m*-dinitrobenzene, and these are shown graphically in Fig. 3.

The figure demonstrates particularly clearly the effect of purification of the *m*-dinitrobenzene. The proportion of KOH at which the absorption is maximal varies not only with the quality of the *m*-dinitrobenzene, but also with the amount of androsterone. The choice of 5 milli-equiv. of KOH in the standard method seems a satisfactory compromise, but the amount is evidently critical.

Effect of temperature and time of development. In general agreement with Wu & Chou, it was found that the absorption of the mixture diluted after 1 hr. increased by about 3% with a rise of 1° in the temperature of development.

With the technique modelled on that of Wu & Chou, it was found that with variation of the time of development the value of E_g rose to a maximum at about 1 hr., as shown by the series of figures in Table V, and there is no appreciable difference between 60 and 65 min.

Table V. *Variation of* E_g *with time of development of reaction mixture*

Time of development (min.)	0·05 mg. androsterone	0·10 mg. androsterone (*a*)	0·10 mg. androsterone (*b*)	0·10 mg. androsterone (*c*)	0·25 mg. androsterone (early technique)
30	0·17	0·315	—	—	0·45
45	0·20	0·35	—	—	0·51
50	—	—	0·355	0·35	—
60	0·20	0·39	0·375	0·37	0·61
65	—	—	0·375	—	—
70	—	—	0·37	0·36	—
75	0·19	0·39	—	—	0·60
90	—	0·365	—	—	—
105	0·18	0·34	—	—	—
120	0·16	0·35	—	—	—

Effect of light. When the reaction mixture was developed in bright sunlight the value of E_g for 0·25 mg. of androsterone was 0·31 as compared with $E_g=0{\cdot}61$ with development in dull, diffused daylight. On the other hand, further diminution of the illumination had no effect; the value $E_g=0{\cdot}60$ was obtained after development in the dark. (Early technique.)

Stability of colour after dilution. The colour fades slowly after dilution of the reaction mixture, and the fading appears to be accelerated by exposure of the solution to light or air. In diffuse daylight and with closed absorptiometer cells the values of E_g for 0·1 mg. of androsterone were 0·37 immediately after dilution, unchanged after 25 min., and 0·325 after 4 hr.

Similarly with 0·25 mg. of androsterone, when the solutions were poured back into test tubes and replaced in the thermostat in between measurements, the values of E_g immediately after dilution, and after 30 and 60 min., were 0·61, 0·57 and 0·52 respectively.

Remaining sources of error. A potential source of variation is water in the alcohol or in the potassium hydroxide. This factor has not been investigated.

The hidden sources of error responsible for the residue of uncontrolled variation probably include this, the quality of the *m*-dinitrobenzene and manipulative errors. The presence of small amounts of cholesterol has no effect. The presence of digitonin reduces the colour: 0·1 mg. of dehydroandrosterone and 0·5 mg. of digitonin gave a value of E_g corresponding to 0·08 mg. No errors due to alteration of the filters or of the photoelectric cells have been detected in control measurements of the absorption of an "orange" glass filter made over a period of a year.

(3) *Specificity—the reaction with other compounds*

Spectral colorimetry. Zimmermann [1936], using a Pulfrich photometer, plotted curves which were a rough indication of the absorption spectra of the reaction mixtures of androsterone, oestrone, testosterone and creatinine, using

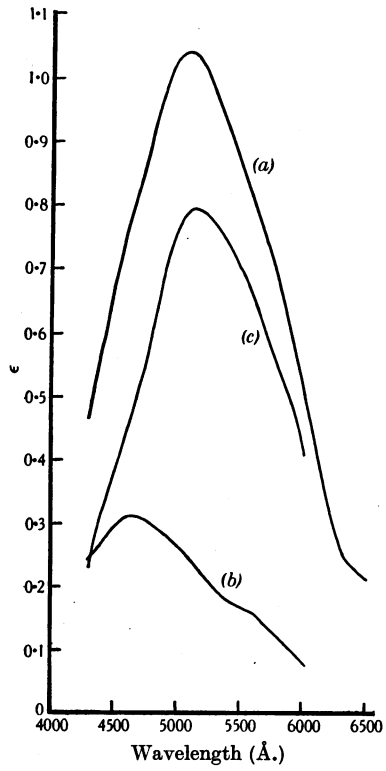

Fig. 4.

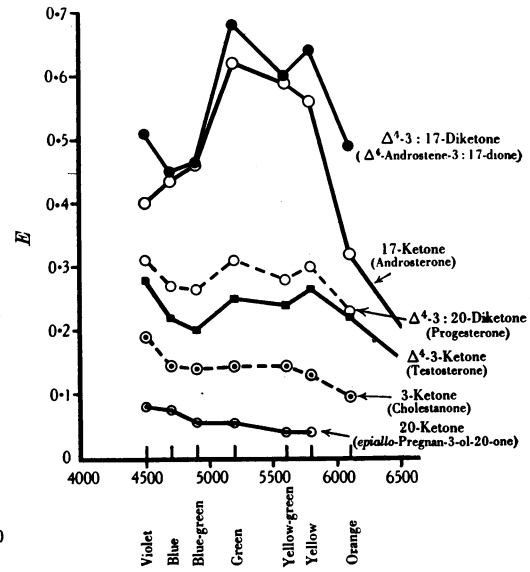

Fig. 5.

Fig. 4. Absorption spectra of (*a*) reaction mixture, 0·1 mg. of androsterone + *m*-$C_6H_4(NO_2)_2$ + KOH, and (*b*) of reagents alone, each against EtOH, with (*c*) calculated curve for test solution − blank. Hilger-Nutting spectrophotometer; 2 cm. cells; photometry of photographs at intervals of (*a*) $\epsilon = 0{\cdot}05$, and (*b*) $\epsilon = 0{\cdot}02$.

Fig. 5. "Absorptiometric spectra" with absorptiometer and Ilford "spectrum" filters of the reactions of typical steroid ketones + *m*-$C_6H_4(NO_2)_2$ + KOH.

measurements of the absorption within a limited range of wavelengths by inserting Zeiss "S" filters. We have used a similar method on a number of substances with the Spekker absorptiometer and Ilford "spectrum" filters. The

maxima of transmission of these filters, as given by the makers, occur at the following wave-lengths: violet, 4300 Å.; blue, 4700 Å.; blue-green, 4900 Å.; green, 5200 Å.; yellow-green, 5500 Å.; orange, 6000 Å. The range of transmission is of the order of 500–600 Å., except with the violet and orange filters, which are less selective. Measurements through these filters are designated below by the symbols E_v, E_b etc.

In the case of the reaction with androsterone, an accurate photographic measurement of the absorption spectrum has been made with a Hilger-Nutting spectrophotometer. Fig. 4 shows the absorption spectra of the reaction mixture obtained under standard conditions, in specially purified alcohol, with 0·1 mg. of androsterone (curve *a*) and of the blank, with reagents alone (curve *b*), comparison being made in each case with alcohol. The androsterone reaction shows a broad absorption band extending over the centre of the visible region, with a maximum at 5010 Å., whilst the reagents alone give low general absorption with a maximum at 4650 Å. Curve (*c*) shows the calculated difference between test and blank solutions. Reference to Fig. 5 shows that absorptiometric measurements with selective filters actually give a fairly faithful version of the net absorption spectrum after subtraction of the blank solution. The Ilford "spectrum-green" filter was selected for measurement of the absorption on the empirical ground that it gave the highest readings. The net absorption spectrum shows that any selective filter having maximum transmission somewhere between 5000 and 5400 Å. would be suitable for the purpose.

The reaction has been carried out generally under standard conditions, but in some cases using an earlier technique, with a number of methyleneketones, and the "absorptiometric spectra" have been measured with the Spekker absorptiometer and Ilford "spectrum" filters with the object of seeing whether compounds of different classes could be distinguished from one another. We are indebted to Messrs Ciba Ltd. for most of the androstane derivatives we have examined. The data obtained are in Table VI.

Influence of the position of the keto-group on the intensity and spectral characteristics of the colour. The first step in investigating the degree of specificity of the reaction with *m*-dinitrobenzene and alkali was to find whether it varied with the position of the keto-group in steroid compounds. Zimmermann [1936] had already shown that the approximate absorption spectrum given by testosterone was of a type different from that given by androsterone or oestrone, and Kaziro & Shimada [1937], using the Zimmermann technique, reported that the colour reaction was "positive" with 3-ketocholanic acid and "negative" with 6-, 7- and 12-ketocholanic acids.

The data we have obtained on various 3-, 6-, 17- and 20-ketones, given in detail in Table VI, show that the production of an intense, broad absorption band with a maximum in the green, is, among the steroid compounds, characteristic of the 17-ketones. In none of the groups of compounds is the type or intensity of the absorption much affected by distant substituents, and the spectra plotted in Fig. 5 may be taken as representative of their classes.

Among the *17-ketones*, we have investigated dehydroandrosterone in some detail with respect to factors influencing the intensity of the reaction on the same lines as androsterone. The effects of altering the concentration of the reagents and the time of development were the same as with androsterone. Dehydroandrosterone and oestrone gave calibration curves which followed closely that of androsterone. Androstan-17-one and $\Delta^{3:5}$-androstadien-17-one were very kindly given to us by Dr F. L. Warren of the Royal Cancer Hospital, and we take this opportunity of thanking him.

Table VI. *"Absorptiometric spectra" of steroid and other methyleneketones and of neutral fractions of urine extracts with* m-*dinitrobenzene and KOH*

Class and substance	Wt. (mg.)	Time of development	Absorptiometer readings (test – blank) and wavelength examined: E_v (max. 4500 Å.)	E_b (max. 4700 Å.)	E_{b-g} (max. 4900 Å.)	E_g (max. 5200 Å.)	E_{y-g} (max. 5600 Å.)	E_y (max. 5800 Å.)	E_0 (max. 6100 Å.)
17-Ketones:									
Androsterone	0·1	1 hr.	0·21	0·26	0·305	0·385	0·35	0·31	0·18
	0·2	1 hr.	0·40	0·435	0·46	0·62	0·59	0·56	0·32
Dehydroandrosterone	0·2	1 hr.	0·30	0·34	0·42	0·58	0·475	0·44	0·265
Androstan-17-one	0·1	1 hr.	0·19	0·23	—	0·37	—	—	—
	0·2	1 hr.	0·365	0·41	0·47	0·62	0·555	0·53	0·135
$\Delta^{3:5}$-Androstadien-17-one	0·1	1 hr.	0·20	0·245	—	0·38	—	—	—
	0·2	1 hr.	0·43	0·45	0·48	0·67	0·61	0·61	0·355
Oestrone	0·1	1 hr.	—	—	—	0·42	—	—	—
	0·2	1 hr.	0·39	0·45	0·46	0·66	0·60	0·585	0·345
3-Ketones:									
Androstan-17-ol-3-one	0·2	20 min.	0·26	0·245	0·265	0·31	0·33	0·32	0·23
	0·2	1 hr.	0·225	0·18	0·17	0·21	0·19	0·19	0·135
Cholestanone	0·2	2 min.	0·18	0·195	0·205	0·26	0·285	0·265	0·175
	0·2	5 min.	0·26	0·23	0·24	0·295	0·33	0·325	—
	0·2	10 min.	0·235	0·21	0·22	0·28	0·31	0·31	0·22
	0·2	20 min.	0·20	0·175	0·175	0·23	0·25	0·265	0·185
	0·2	40 min.	0·16	0·115	—	0·15	0·145	0·15	—
	0·2	1 hr.	0·19	0·145	0·14	0·145	0·145	0·13	0·095
Δ^4-3-Ketones:									
Testosterone	0·1	1 hr.	0·18	0·15	—	0·155	0·15	0·165	—
	0·2	1 hr.	0·28	0·22	0·20	0·25	0·24	0·265	0·22
Cholestenone	0·1	10 min.	0·04	0·02	—	0·03	—	—	—
	0·1	20 min.	0·08	0·05	—	0·06	—	—	—
	0·1	30 min.	0·085	0·08	—	0·075	—	—	—
	0·1	1 hr.	—	—	—	0·135	—	—	—
	0·2	1 hr.	0·27	0·22	0·20	0·24	0·23	0·245	0·20

3:17-Diketone:									
Androstane-3:17-dione	0·1	1 hr.	—	—	0·33	0·41	0·37	—	—
	0·2	1 hr.	0·53	0·47	0·52	0·68	0·62	0·62	0·41
Δ^4-3:17-Diketone:									
Δ^4-Androstene-3:17-dione	0·1	1 hr.	—	—	—	0·42	—	—	—
	0·2	1 hr.	0·51	0·45	0·465	0·68	0·60	0·64	0·49
20-Ketones:									
epiallo-Pregnan-3-ol-20-one	0·1	1 hr.	—	—	—	0·045	—	—	—
	0·2	1 hr.	0·085	0·075	0·055	0·055	—	—	—
3:20-Diketone:									
Progesterone	0·1	1 hr.	0·20	0·19	—	0·19	—	—	—
	0·2	1 hr.	0·31	0·27	0·265	0·31	0·28	0·30	0·23
6-Ketones:									
Cholestan-6-one	0·2	1 hr.	—	—	—	0·01	—	—	—
Cholestane-3:6-dione	0·2	1 hr.	0·14	0·105	·09	0·11	0·10	0·115	0·09
Other compounds:									
*cyclo*Pentanone	0·058	5 min.	0·35	0·445	0·53	0·70	0·62	0·62	0·46
	0·058	10 min.	0·32	0·35	0·405	0·57	0·53	0·49	0·35
	0·058	20 min.	0·335	0·34	0·35	0·47	0·43	0·395	0·30
	0·058	30 min.	0·33	0·29	0·31	0·44	0·40	0·385	0·29
	0·058	1 hr.	0·38	0·32	0·30	0·40	0·36	0·33	0·25
*cyclo*Hexanone	0·067	1 hr.	0·35	0·22	0·175	0·19	0·14	0·12	0·18
Acetophenone	0·08	1 hr.	0·42	0·46	0·52	0·66	0·50	0·33	0·15
Acetone	0·04	1 hr.	0·295	0·29	0·27	0·29	0·22	0·20	0·15
Urine extracts (bulk collections)									
Female FU. 57.B	0·02 l.	1 hr.	0·18	0·19	0·205	0·27	0·24	0·21	0·135
FU. 62.B	0·01 l.	1 hr.	0·21	0·21	0·245	0·29	0·28	0·24	0·15
FU. 76.B	0·01 l.	1 hr.	0·30	0·30	0·33	0·43	0·405	0·36	0·225
Male MU. 38.B	0·01 l.	1 hr.	0·26	0·26	0·29	0·38	0·335	0·295	—
MU. 39.B	0·01 l.	1 hr.	0·23	0·225	0·265	0·32	0·30	0·26	0·165
MU. 75.B	0·01 l.	1 hr.	0·225	0·21	0·265	0·37	0·30	0·26	0·15

Saturated 3-ketones show a characteristic rapidity in development of the colour, which then fades, and after 1 hr. of development only a very low, general absorption is shown. The behaviour of cholestanone was investigated in detail, and the most intense colour, characterized by a band in the yellow and yellow-green, is given after only 5 min. development. This behaviour seems to be common to compounds having the group —CH_2—CO—CH_2—. *cyclo*Pentanone, for a specimen of which we are indebted to Prof. R. P. Linstead, gives a band with maximum in the green, which is developed rapidly.

Δ^4-*3-ketones*, in contrast to the saturated 3-ketones, require a long time of development, and, in the case of cholestenone, some detailed figures for which are given in Table VI, it seems that development of the maximum colour is not complete after 1 hr. All the Δ^4-3-ketones show a characteristic peak in the yellow, in addition to one in the green, and this is visible even when the powerfully chromogenic 17-keto-group is also present, as in Δ^4-androstene-3:17-dione (cf. Fig. 5).

The *20-keto-group* gives a very low, but measurable general absorption. In combination with a Δ^4-3-keto-group it causes a small general rise in the absorption.

The *non-steroid* compounds were measured in amounts which were the molar equivalents of 0·2 mg. of androsterone. Acetone shows the rapid development of colour characteristic of dimethyleneketones. Acetophenone is noteworthy for the resemblance of the curve to that of 17-ketosteroids, except for considerably lower absorption in the yellow and yellow-green. This resemblance, in spite of a considerable difference in structure, demonstrates with what caution absorption spectra of this colour reaction should be accepted as indicative of structure without confirmatory evidence.

Absorption spectra of the colour reaction with urine extracts. The absorptiometer measurements obtained with extracts from bulk collections of male and female urines are given in Table VI and plotted in Fig. 6 where they are compared with the absorptiometric spectrum of 0·1 mg. of androsterone. It is evident that there is no characteristic difference between extracts from either male or female urine and a typical 17-ketosteroid beyond the minor one of having a value for E_v which is relatively higher and of the same order as E_b. However, in certain abnormal cases there are major differences in the absorption in the shorter wavelengths. We have, in practice, made measurements of E_v and E_b as a routine, in addition to E_g. Certain urines yield extracts which give at $E_g = 0{\cdot}37$ values of E_v or E_b or both which are of the same order as E_g ($E_v/E_g > 0{\cdot}8$). It is clear that these contain chromogenic compounds not present in appreciable amount in normal urine, and, since the value of E_g is liable to be affected by absorption in the adjacent regions of the spectrum, there must be some doubt whether such extracts can fairly be compared with others on a scale of values of E_g. The ratio E_v/E_g tends to be high when the chromogen content is low, and is probably then an indication of the presence of a substance giving rise to general absorption. There is also qualitative evidence, from the rate of development of colour with urine extracts, that substances like saturated 3-ketosteroids are present in small amount. Although in certain cases an overestimate of the biological activity was made when the value of E_v/E_g was high, in other cases, in which, presumably, the urine component giving absorption in the violet did not affect E_g, the relation was normal, and no general rule can be laid down except to regard with suspicion an extract with a high value of E_v/E_g. In the following treatment of the correlation of colorimetry and biological assay no selection of urine extracts in respect of values of E_v/E_g has been made. In two

cases the abnormal colour was traced to contamination resulting from extraction of vulcanite stoppers by toluene used to preserve the urine. Naturally, such contamination must be avoided in collecting specimens intended for colorimetric assay.

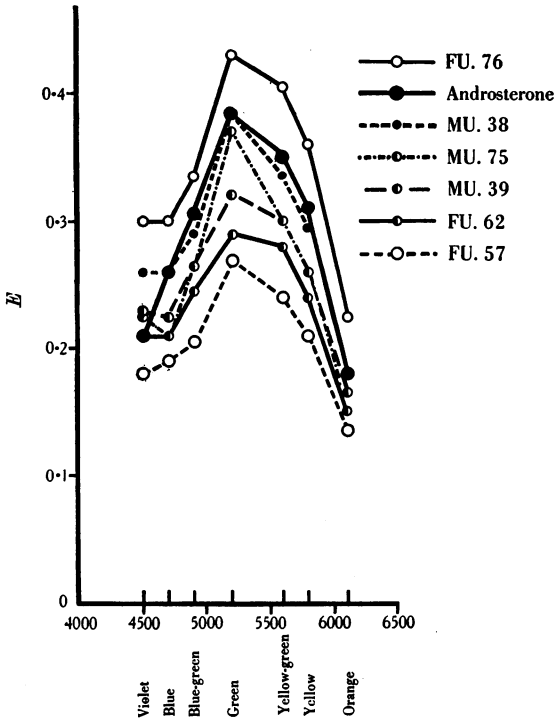

Fig. 6. "Absorptiometric spectra" of the reaction of extracts of men's (MU.) and women's (FU.) urine + m-$C_6H_4(NO_2)_2$ + KOH compared with 0·1 mg. of androsterone. (FU. 57 equiv. 0·02 l.; other urine extracts equiv. 0·01 l.)

(4) *Correlation of colorimetry and biological assay*

Method of biological assay. The urine extracts in oily solution have been assayed for androgenic activity by a capon comb-growth method, described in detail elsewhere [Emmens, 1938]. A dose/response curve was constructed by giving injections of international standard androsterone, and checked regularly by at least one control group of 5 capons. Androgenic activity is expressed as being equivalent to so many international units of androsterone per litre (1 I.U. = 100 μg.), and the estimates based on groups of five birds. In four instances an extract has been assayed by the direct inunction of an oily solution on the comb, and compared with androsterone similarly administered. These four estimates fall well within the range of the others, there being therefore no reason to exclude them from the calculations, despite the difference of technique. Their influence on any conclusions drawn from the data is in any case negligible.

Urines investigated. In the course of the scheme of research of which this investigation forms a part, a variety of urines has been extracted and examined, including bulk collections from groups of normal men or women, a small number from men and women in hospital with no obvious sexual dysfunction, and urine

from eunuchs, eunuchoids, ovariectomized women, cases of Addison's disease and hirsutism in females. For the purpose of considering whether colorimetric and biological assays could be correlated, all these have been lumped together; only cases in which hormone-producing tumours have been proved to be present have been excluded. It may therefore be considered that the hypothesis of correlatability is put to a severe test. The possibility of distinguishing between these classes of patients will not be discussed here.

Statistical treatment of the results

Fig. 7 is a scatter-diagram showing the correlation between the colorimetric and biological assay figures for 59 urine extracts, which are given in Table VII. These were obtained from collections from the following: normal men, 4 groups

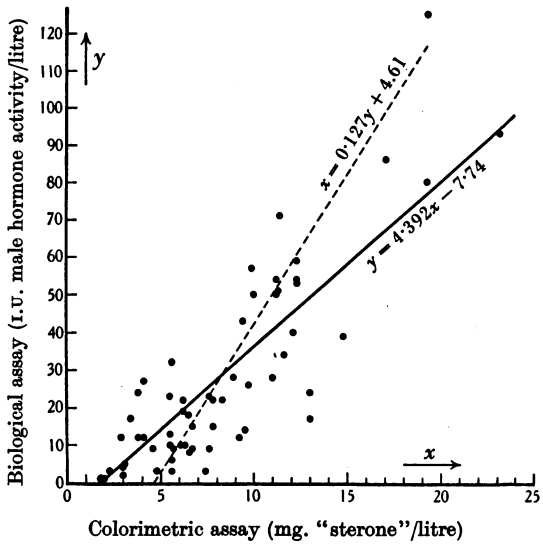

Fig. 7. Correlation of capon comb-growth assay and colorimetric assay of 59 urine extracts.

and 7 individuals; normal women, 4 groups and 6 individuals; eunuchs, 8 (9 samples); ovariectomized women, 8; eunuchoids, 2; hirsute women, 8; cases of Addison's disease, 7 (6 samples from 4 women; 3 men); carcinoma of cervix, 2. To provide comparable figures for calculation, all quantities have been expressed as per litre, though the quantities per day are the significant ones for individual patients and have been measured in most cases. A correlation coefficient has been calculated by grouping the data with units equal to 10 I.U. and 2 mg. "sterone", this coefficient being 0·745. The two regression lines, $y=4{\cdot}392x-7{\cdot}74$ and $x=0{\cdot}127y+4{\cdot}61$ express, respectively, the regression of content of international units by capon assay (y) on "sterone" content (x), and that of "sterone" content on international unit content. The appearance of a better "fit" given by the first of these lines, and the choice of it as a basis for discussion of the meaning of the results depends on the relative errors of the colorimetric and biological tests.

It is known that the error of the capon comb-growth test may be large, but a satisfactory estimate of the average value of this error for a large number of tests is almost impossible to obtain since it depends on both the age of the

Table VII. *Colorimetric and biological assays of urine extracts*

Type and ref. No.	mg. "sterone"/l.	I.U./l.	Type and ref. No.	mg. "sterone"/l.	I.U./l.
(*i*) 1	1·8	1	(*f*) 16	7·6	9
(*j*) 11	2·0	1*	(*d*) 21	7·6	23
(*i*) 3	2·3	3	(*b*) 84	7·8	15
(*j*) 6	2·9	12	(*e*) 72	7·8	22
(*e*) 64	3·0	2*	(*c*) FU. 62	8·3	22
(*e*) 77	3·0	4	(*a*) MU. 75	8·9	28
(*f*) 14	3·1	5	(*f*) 15	9·2	12
(*j*) 8	3·4	17	(*b*) 31	9·4	43
(*c*) FU. 57	3·8	12	(*h*) 25	9·5	14
(*d*) 51	3·8	24	(*d*) 31	9·7	26
(*b*) 61	4·1	27	(*a*) 44	9·9	57
(*e*) 68	4·2	12	(*f*) 10	10·0	50
(*i*) 7	4·6	9	(*c*) FU. 76	11·0	28
(*h*) 21	4·8	3	(*k*) 50	11·2	50
(*h*) 27	5·5	10*	(*a*) 65	11·2	54
(*f*) 9	5·5	13	(*b*) 2	11·3	51
(*i*) 9	5·5	23	(*d*) 11	11·4	71
(*e*) 98	5·6	3	(*a*) MU. 38	11·6	34
(*e*) 86	5·6	6	(*h*) 26	12·1	40
(*b*) 11	5·6	32	(*b*) 21	12·3	53
(*e*) 104	5·7	9	(*g*) 40	12·3	54
(*d*) 81	6·1	10	(*g*) 33	12·3	59
(*f*) 12	6·2	19	(*h*) 31	13·0	17
(*i*) 4	6·2	22	(*k*) 51	13·0	24
(*c*) 38	6·3	10	(*d*) 121	14·8	39
(*f*) 11	6·5	18	(*h*) 38	17·1	86
(*e*) 65	6·55	8	(*h*) 34	19·3	80*
(*i*) 5	6·7	9	(*f*) 13	19·4	125
(*b*) 81	6·7	15	(*h*) 24	23·2	93
(*e*) 73	7·4	3			

(*a*) Bulk collection, normal men.
(*b*) Normal men.
(*c*) Bulk collection, normal women.
(*d*) Normal women.
(*e*) Eunuchs.
(*f*) Ovariectomized women.
(*g*) Eunuchoids.
(*h*) Hirsute women.
(*i*) Addison's disease, women.
(*j*) Addison's disease, men.
(*k*) Carcinoma of cervix.

* Capon assay by inunction.

birds and the amount of growth stimulated [Emmens, 1938]. However, 20 control groups receiving androsterone for a period during which capon variability was on the high side, and during which approximately one half of the estimates used here were made, yield a correlation of 0·862 between comb-growth and the amount of androsterone injected. The correlation for the whole period is certainly not less than this, and probably as high as 0·9. The z test [Fisher, 1932] reveals that a correlation of 0·745 derived from 59 pairs (the number of extracts assayed) is not significantly different from one of 0·862 derived from 20 pairs. One can conclude therefore that the correlation between biological and colorimetric assays is not much less than that between biological assay and the true amount of androgenic material present, even when the latter is pure androsterone.

The correlation between values of E_g and the amount of androsterone present cannot be accurately expressed by a correlation coefficient, since the relationship is not linear. Fig. 1 demonstrates that the colorimetric determination of androsterone is very accurate, although the accuracy with which urine extracts have been assayed is appreciably less than that indicated in Fig. 1 and the calculation on p. 1315 suggests. There remains no doubt, however, that the greater part of the departure of the correlation between colorimetric and biological assays from unity is due to the errors of biological assay. The small contribution of the errors of colorimetric assay to this departure probably does

not account completely for the remainder, since a perfect fit of the biological and colorimetric estimates could be expected only on uniform material. Thus, if it were assumed that androsterone and dehydroandrosterone were the active substances, differences from sample to sample in the ratio of these two compounds would reduce the correlation of the two estimates, unless balanced by appropriate variations in the content of biologically inactive chromogens. In view of the fact that a given colorimetric reading may be obtained from a series of mixtures of different active and inactive chromogens, each giving a different result when tested biologically, the degree of correlation found is high.

Chemical evidence

Certain preliminary work has been carried out on the analysis of urine extracts into their constituents with a view to the complete identification of the latter; results have been obtained which, subject to the reservations necessitated by the inherent error of capon assay, and the rather unquantitative character of the methods, indicate the association of biological activity with the chromogenic, ketonic fraction. Separation of the ketonic fraction as water-soluble compounds by treatment with Girard's reagent T (trimethylammonium acethydrazide chloride) [Girard & Sandulesco, 1936] gives results such as the following:

450 mg. of extract MU. 75[1] equivalent to 10·25 l. of urine, assayed at 8·9 mg. of "sterone" per litre by colorimetry, or 28 international units of androgenic activity per litre by capon comb growth, yielded, after extraction with methanol and two treatments with Girard's reagent, (*a*) a high-ketone fraction, wt. 86 mg., "sterone" content 6·8 mg. per litre, androgen activity 45 I.U. per litre, (*b*) two low-ketone fractions, one, weight 12 mg., "sterone" 0·19 mg. per litre, and the other, weight 225 mg., with a "sterone" content of 0·37 mg. per litre (but with a high value of E_v), and androgen activity less than 2 I.U. per litre. This shows clearly that the androgenic compounds are confined to the ketonic fraction.

Discussion

The use of "male hormone assay" of urine extracts by biological methods as a method of diagnosis of the physiological condition of a patient entails the assumption, originally based, perhaps, on a presumed specificity of male hormone activity, that the circulating hormones, although they may be destroyed or transformed in the course of excretion, will yet give a product whose activity will be proportional to the physiological activity of the original compounds present in the human body. An incidental assumption is made in supposing that the usual (and only practical) method of assessing the biological activity by the comb-growth of capons gives a measure of the activity in the human body. The factual support for the main assumption is slender, owing to the immense difficulties of chemical analysis of extracts of tissue, blood and excreta. The only compounds with marked androgenic activity so far isolated from the organism are testosterone from the testis, and Δ^4-androstene-3:11:17-trione from adrenal cortical tissue. The only active compounds which have been isolated from normal urine are androsterone and *trans*dehydroandrosterone, or Δ^4-androsten-3-*trans*-ol-17-one, and the amounts identified account for only a fraction of the biological activity. Urines from adrenal tumour cases have

[1] We are indebted to Messrs Boots Pure Drug Co. for this extract made at Nottingham from a 250 l. bulk collection of men's urine. Other data from this extract have been used in Tables VI and VII.

yielded $\Delta^{5:7}$-androstadien-17-one [Burrows *et al.* 1937], and *trans*dehydroandrosterone [Callow, 1936, 2]; in the last case the amount of the compound isolated accounted for 70% of the biological activity. A related, inactive compound, *epi*aetiocholanediol, has been isolated in very small amount from normal urine extract [Butenandt *et al.* 1937]. The genetic relationship between secretion and excretion is thus far from clear with respect to chemical structure. The investigation of the relationship with respect to biological activity is as yet hardly begun, though from the work of Kochakian [1937] and from work carried out in this laboratory in collaboration with clinicians, a preliminary account of which has been given in a lecture by one of us [Callow, 1938], it is already evident that the recovery of androgenic activity from urine extracts is only of the order of 5%, as a maximum, of that administered by injection. Here again, the scale of comparison is comb growth in the capon, although it is known that the relative activities of androgens, which vary even in capons according to the mode of administration, are different in laboratory mammals, and, presumably, in man. Further, the biological assay is of a mixture of substances, and, of two known constituents of this mixture, one has about three times the activity of the other.

It seems, therefore, that an examination of urine extracts by a chemical test, to which the known active urine constituents and closely related steroids respond, will entail no more dangerous assumptions than examination by biological assay.

The first result of this work is that of increasing the accuracy and reproducibility of a method of determining 17-ketosteroids. It has then been shown that a greater degree of specificity can be introduced into this determination by rough measurements of the absorption spectra. A claim for complete specificity would be untenable unless a more exact examination of a far wider range of compounds had been made. The hypothesis that the method gives a measure of the 17-ketosteroids in urine extracts is justified and supported by three considerations. First, the method of obtaining the extracts is such that neutral, non-volatile compounds of high molecular weight are separated. Secondly, after chemical fractionation of the extract, the chromogenic activity is concentrated in the ketonic fraction. Thirdly, the absorption spectra of the coloured compounds formed by this material with *m*-dinitrobenzene in alkali closely resemble those of the compounds formed by pure 17-ketosteroids.

The further hypothesis that a determination of 17-ketosteroids made in this way gives a measure of the androgenic activity is suggested in the first place by the knowledge that two of the compounds to which urine extracts owe their androgenic activity are actually 17-ketosteroids. Secondly, there is the inherent likelihood that 17-ketosteroids will all possess androgenic activity. Confirmation is afforded by the degree of correlation between colorimetric and capon assays. When it is considered that the urine extracts are certainly a complex mixture of compounds with both androgenic and chromogenic properties, and that the first step in obtaining them is a vigorous acid hydrolysis involving both liberation and destruction, which may be selective, under conditions difficult to control, and, further, that an absorption spectrum is a physical property particularly sensitive to impurities, it might well be expected that even if the natural urine contained a simple mixture of androgens in constant proportions, the inherent correlation between colorimetric and biological assays would be blurred beyond recognition. It has been shown that a significant degree of correlation does exist. Thus, if it is a question of deciding, on theoretical grounds of chemical metabolism or physiological relationship, whether chemical or biological assay is the better, we can make the best of both worlds, and employ either. Neither

form of assay is specific, but the chemical one is more susceptible to investigation and improvement in this respect, and has the added advantage of rapidity and smaller inherent error: it would seem at least as likely to give diagnostically significant results.

The contributions of this investigation to the question of the exact composition of the urinary excretory transformation products of male hormones are an improved chemical test for a type of these products, but not for any individual compound, and an indication of a relationship of chromogenic to biological activity concordant with a simple working hypothesis without giving it preferential support. The relationship $y=4{\cdot}392x-7{\cdot}74$, which gives the most likely estimate of the biological assay equivalent to a given colorimetric reading, is intermediate between that which would be given by androsterone and dehydroandrosterone. In practice pure androsterone would give an equation close to $y=10x$, and pure dehydroandrosterone one approximating to $y=3{\cdot}3x$, since the activity of the latter on capons is about one-third of that of androsterone. It might be assumed that the androgenic and chromogenic material in urine extracts is a mixture of androsterone and dehydroandrosterone, the two substances which have actually been isolated from normal male urine, an assumption which has been made, on other grounds, by Dingemanse & Laqueur [1938]. However, the intermediate position of the experimentally determined line is not in itself proof of the presence of a mixture of androsterone and dehydroandrosterone in the average urine dealt with. It could result from the presence of androsterone and biologically inactive chromogen only. The presence of some inactive chromogen is indicated by the constant 7·74, which is a measure of the amount found when little active substance is present, whilst there may be more inactive chromogen in urines with higher biological activity. Moreover, there is a wide discrepancy between the amounts of androsterone and dehydroandrosterone recorded as actually isolated (about 1 mg. per litre of each) and the amounts indicated by biological assay. From the evidence now presented, no more definite conclusion can be drawn, than that the content of biologically active material is proportional to the content of 17-ketosteroid compounds.

Summary

1. A description is given of a modified and improved method of determining androsterone and other 17-ketosteroids by the colour reaction with *m*-dinitrobenzene and potassium hydroxide in alcoholic solution.

2. The sources of error and the factors influencing this determination have been investigated.

3. Examination of the behaviour of other methyleneketones has shown that the absorption spectrum of the coloured solution produced and the rapidity of development of the colour vary according to the structure of the compound.

4. The neutral fractions of extracts of hydrolysed urine give the colour reaction characteristic of 17-ketosteroids, although with some indication of the presence of other chromogenic compounds. Further, biological activity is associated with the ketonic fraction. These facts justify the use of the reaction for estimating the excretory transformation products of male hormones.

5. A comparison of the chemical with the biological method of determining the excretory products by their activity in the capon comb-growth test has been made with 59 urines from a variety of sources. A significant degree of correlation is found between the colorimetric assay expressed in chromogenic equivalents

of androsterone ("sterone") per litre of urine and the biological assay expressed in international units of male hormone activity per litre.

6. It is concluded that colorimetric assay can replace biological assay provided that due regard is paid to the occasional presence of interfering substances, which may be revealed when the approximate absorption spectrum is examined.

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