

## PROPOSED STANDARD METHOD OF 17-KETOSTEROID DETERMINATION

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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. He described (1936) a method applicable to pure substances and to urine extracts. Wu and Chou (1937) described a modification of this method using androsterone as reference substance. Oesting and Webster (1938) used the Zimmermann technique and roughly correlated the colorimetric assays with biological determinations on capons.

In a very careful and complete investigation of the Zimmermann reaction as applied both to pure substances and to urine extracts Callow et al. (1938) described a modified and improved method for determining urinary 17-ketosteroids. Many variations and modifications of the method described by Callow et al. have subsequently been published; but it probably still remains the most satisfactory and reproducible procedure. This method, called for convenience the Callow-Zimmermann method, is, therefore, the basis of the technique described below.

Laboratories now use various techniques to estimate 17-ketosteroids so that there is need for some standard by which all results can be made comparable. The committee therefore suggest that the following method be used as such a reference standard.

### The Method

#### COLLECTION OF URINE SPECIMEN

Since there is considerable diurnal variation in excretion of 17-ketosteroids (cf. Pincus 1943), complete 24-hour collections of urine are necessary. Subjects should be instructed to reject the first morning urine on the first day of collection and then to collect all urine up to and including the first morning urine on the second day. Addition of preservatives is usually unnecessary. Formation of inconvenient amounts of ammonium carbonate can be prevented by addition of a salt of a heavy metal (to inhibit urease activity)—e.g., copper sulphate 1 mg. per ml.

#### HYDROLYSIS AND EXTRACTION OF URINE

Published procedures for extraction and hydrolysis of urine are legion and have given rise to much controversy. The following method, based on the work of Robbie and Gibson (1943), has been found to be convenient and rapid. Direct comparison with other recommended procedures has shown close agreement in amounts of ketosteroid extracted.

A sample of urine (100 ml.) is brought to boiling under reflux over a bunsen flame. Concentrated HCl (10 ml.) is added down the condenser and boiling maintained for 10 minutes. The urine is then allowed to cool somewhat and 30 ml. of carbon tetrachloride added down the condenser. The contents of the flask are again maintained at the boil for 10 minutes. The flask is then cooled, the CCl<sub>4</sub> layer removed and replaced by the same volume of fresh solvent, and the mixture again refluxed for 10 minutes. The CCl<sub>4</sub> layer is then removed and added to the first extract.

The total CCl<sub>4</sub> extract (about 60 ml.) is washed successively with: (1) 20 ml. water, (2) 20 ml. 2N NaOH, (3) 20 ml. water, (4) 20 ml. water containing a pinch of sodium dithionite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>). The washed CCl<sub>4</sub> extract is then evaporated to dryness on a water-bath using water-pump vacuum to remove last traces.

The dry residue is dissolved in aldehyde-free absolute ethanol. The volume of ethanol may be varied according to the expected ketosteroid content. With normal urines 4 ml. of ethanol is convenient, but 2 ml. or even 1 ml. may be more appropriate for urines of low ketosteroid content. Ethanolic extracts thus prepared appear to be quite stable and need protection only from evaporation.

#### COLORIMETRIC ESTIMATION

##### Reagents

(1) *Ethanol*.—The suitability of the absolute alcohol is the most important factor in achieving satisfactory results. Some grades of commercial "absolute" alcohol can be used without preliminary purification but usually some treatment is neces-

sary. The following method described by Callow et al. (1939) may be employed.

Commercial "absolute" alcohol is treated with 4 g. per litre of *m*-phenylenediamine hydrochloride, allowed to stand in the dark for a week, with occasional shaking, and then distilled, the head and tail fractions being rejected.

This purified alcohol should be used for all purposes in connection with the estimation of 17-ketosteroids.

(2) *m*-Dinitrobenzene.—A well-crystallised and fairly pure specimen is further purified thus: 20 g. is dissolved in 750 ml. of 95% ethanol, warmed to 40°C, and 100 ml. of 2N NaOH is added. After five minutes, the solution is cooled and 2500 ml. of water is added. The precipitated *m*-dinitrobenzene is collected on a Buchner funnel and washed very thoroughly with water, sucked dry, and recrystallised twice in succession from 120 ml. and 80 ml. of absolute ethanol. The material should be well crystallised in almost colourless needles m. pt. 90.5–91°C.

The reagent is a 2% *w/v* solution of this material in absolute ethanol. It is stored in a brown bottle with a glass stopper and kept in the dark. Under these conditions it is stable for 10–14 days.

(3) *Potassium hydroxide*.—The reagent solution is 2.5N KOH in absolute ethanol. KOH (9 g.) is dissolved with shaking or mechanical stirring in 50 ml. of absolute ethanol and the solution filtered through a hardened filter paper. The concentration is checked by titration with acid (methyl orange indicator) and adjusted between the limits 2.48–2.52N. The solution is stored in a refrigerator and must be discarded as soon as the faintest colour is perceptible.

#### Mode of Operation

The following tubes should always be set up:

(1) *Reagent Blank*.—0.2 ml. ethanol, 0.2 ml. *m*-dinitrobenzene reagent (D.N.B.), 0.2 ml. KOH.

(2) *Urine Extract*.—0.2 ml. urine extract, 0.2 ml. D.N.B., 0.2 ml. KOH.

(3) *Standard*.—0.2 ml. standard, 0.2 ml. D.N.B., 0.2 ml. KOH.

A convenient standard is one containing 0.1 mg. androsterone or dehydroepi-androsterone in 0.2 ml. absolute ethanol.

The tubes are stoppered and placed in a thermostat at 25° ± 1°C for 60 minutes. During incubation the tubes should be protected from bright light. It is most convenient to keep them in complete darkness.

At the end of 60 minutes 10 ml. of absolute ethanol is added to each tube and the contents mixed. The urine extract and the standard are then read in a photo-electric colorimeter against the reagent blank tube.

Colorimeter readings should always be made with two different filters, one green (approximate wave-length maximum 5200 Å), and one blue or violet (approximate wave-length maximum 4300 Å). These correspond to 'Ilford Spectrum' filters nos. 604 and 601 respectively. Readings with both filters are necessary for correcting for interfering chromogens by the method given below.

There is a straight-line relation between extinction and amount of ketosteroid up to about 0.1 mg. of androsterone. With larger quantities of sterone the calibration deviates from a straight line. If very high readings are obtained it is necessary to dilute the original urine extract and repeat colour development. Dilution of the final coloured solution should not be employed as it gives erroneous results.

#### CORRECTION FOR INTERFERING CHROMOGENS

Substances other than 17-ketosteroids develop colour with the reagents. Talbot et al. (1942) suggested the use of the following formula for correcting the observed green extinction values for interfering chromogens:

$$\text{Corrected Green} = \frac{\text{Observed G} - 0.6 \text{ V}}{0.73}$$

That is, from the observed green extinction subtract  $\frac{1}{10}$  of the observed violet extinction and divide by 0.73. The corrected green extinction is converted into mg. of standard by comparison with the extinction of the latter with the green filter. The validity of this correction has been checked by comparison with values obtained on urinary neutral ketone fractions obtained by the use of Girard's reagent (Talbot et al. 1942) and by simultaneous determinations by colorimetric and polarographic methods (Butt et al. 1951).

*N.B.*—If the original paper of Talbot et al. is consulted it should be noted that there is a confusing misprint in discussing the ratios of the extinctions in green and blue light. They are reversed in the text. The preface to vol. 143 of *J. biol. Chem.* carries a corrigendum on this point.

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## NORMAL EXCRETION OF 17-KETOSTEROIDS

Barnett et al. (1946) have tabulated most of the data for excretions by normal subjects published from 1939 to 1946. Younger and middle-aged subjects showed an average excretion per 24 hours of about 13 mg. for men and 9 mg. for women. A later report by Forbes et al. (1947) gave an average of 12.5 mg. for 73 young men and 8.2 mg. for 65 young women. Variations in excretion of ketosteroids by normal subjects with special reference to age and sex are discussed in publications by Robinson (1948a) and by Hamburger (1948).

## Evaluation of Colorimetric Determination

In a review of ketosteroid excretion in health and disease, Robinson (1948b) concludes: "With the advent of a colorimetric method a wide survey of normal and pathological urines became possible and during the past ten years many thousands of specimens have been examined in laboratories in all parts of the world. At first much painstaking work was done in simultaneous comparisons of androgenic potencies as determined by capon assay with the results of chemical estimations. Correlation between the two methods was fairly good in general but not invariably so. But it soon became apparent that, at least in some clinical conditions, there were important correlations between the chemically determined values and the disease. The value of the chemical determination today rests upon these empirically established relations rather than upon their more doubtful value as measures of biologically potent androgens."

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## THE ADRENAL CORTEX AND RHEUMATOID ARTHRITIS

## THE HEBERDEN ORATION

ON Sept. 19, in London, Prof. E. C. KENDALL, D.Sc., delivered the annual Heberden oration before the Heberden Society. He recalled that it was three years since the first injection of cortisone (then known as Compound E) was given with successful results to a patient with rheumatoid arthritis at the Mayo Clinic. It had been given, in fact, seven days before his colleague, Dr. Philip Hench, left for England to deliver a previous Heberden oration. Dr. Hench's script, which had been prepared some weeks previously, had contained no reference to this trial. Instead, Dr. Hench had set out his reasons for believing that rheumatoid arthritis was a reversible disease—that there was, in fact, some substance  $\alpha$ , produced in the body during pregnancy and in jaundice, which could cause complete remission of the activity of the rheumatoid process. One could imagine the conflict in Dr. Hench's mind when he was stating his reasons for believing in the existence of a certain substance but was unable to disclose its discovery because this had not been confirmed. The startling efficiency of cortisone in causing a remission in rheumatoid arthritis and many other diseases was now known, and it seemed possible that cortisone may be the substance  $\alpha$  of pregnancy, although the substance  $\alpha$  of jaundice remains obscure.

Tracing the history of investigations of the adrenal hormones, Dr. Kendall divided it into two ten-year

periods. In the first ten years Reichstein and his colleagues at Zürich, his own team at the Mayo Clinic, and Wintersteiner and Pfiffner of Columbia University, made fundamental contributions to the detection, isolation, and identification of the adrenal steroids. By 1940 it was known that 4 groups of steroids could be isolated from adrenal glands. The first group were sex hormones, not peculiar to the adrenal; the second group were inactive steroids found only in the adrenals; the third group were steroids with adrenal hormonal activity; while the fourth group was an unidentified "amorphous residue," probably of steroids, which also possessed considerable hormonal activity.

But the world supply of adrenal glands was limited. To apply these hormones to clinical investigation it was necessary to develop methods for their synthesis from non-adrenal sources. From 1940 onwards the various workers concentrated on this aspect of the problem, which was full of difficulty. On paper the transition from the steroid acids of bile to cortisone might appear easy, but in fact over 30 difficult chemical steps are required. However, each year brought improvements and alternatives, and there were now easier and more fruitful methods: indeed, so much knowledge had accrued that R. B. Woodward had recently reported the total synthesis of cortisone from simple aromatic compounds. Tribute must be paid to the workers in the laboratories of Messrs. Merck, who had made many important advances. Chemical manipulation of the steroid nucleus could now be controlled in many ways, and ring C was now in reach of the chemists, thus opening the way for the production of active adrenal hormones from plant steroids. In addition, organic chemists had produced 40 or more steroids closely related to cortisone. Other chemists had invented better methods for the detection and assay of steroids in blood and urine. These chemical methods had also enabled physiologists to study accurately the hormones released from the adrenal gland under stimulation with adrenocorticotrophic hormone (A.C.T.H.). The discovery of Sept. 21, 1948, had proved to be a great stimulus to further work in this field.

Was cortisone specific? Apparently it was. No simple substitute (analogous to stilboestrol as a substitute for oestradiol) was known. Other steroids had been claimed as having antirheumatic activity, but their evaluation, which must be in patients and not in animals, had sometimes failed to take into account the psychic factors in patients. In this way desoxycorticosterone (deoxycortone) and ascorbic acid had enjoyed a brief spell of popularity. In fact, so specific was the structure of cortisone that two otherwise identical isomers—one with no double bond in the 4-5 position, and one with an extra double bond 6-7—were both devoid of physiological activity. Furthermore, the effects were not simply those of toxic overdosage, since the dose of cortisone required to produce remissions of rheumatoid arthritis was within the physiological range—i.e., the normal human gland could produce enough hormones to keep the symptoms of rheumatoid arthritis from appearing. Under normal conditions human urine might contain 30-60 mg. of steroids of adrenal origin every day, as determined by the methods of Venning, Corcoran, Mason, and others. Furthermore, under conditions of stress the output of steroids increased considerably. When cortisone was given therapeutically, doses of less than 100 mg. a day were effective, and as it was probable that the endogenous contribution of adrenal steroids is suppressed the total available cortisone was thus in the normal range. That the adrenals could, but do not, produce enough hormones to ensure clinical remission, pointed to a failure in the control of adrenal output. Dr. Kendall thought it might be profitable to investigate pituitary factors in rheumatic diseases.

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