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The use of tetramethylammonium hydroxide in the Zimmermann reaction

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SYNOPSIS The use of tetramethylammonium hydroxide (T.M.A.H.) in place of potassium hydroxide in the Zimmermann reaction has been investigated. Although various pure steroids have different colour equivalents, a comparison of the results of 17-ketosteroid and 17-hydroxycorticosteroid estimations on a series of urines showed that the differences for the two reagents were only small and may be ignored. Tetramethylammonium hydroxide has the considerable advantage over potassium hydroxide of stability and need not be prepared freshly. The correction procedures available are discussed.

The reaction described by Zimmermann (1935) is now widely used for the estimation of 17-ketosteroids, particularly in crude urine extracts, and depends upon the violet colour developed by mixing solutions of potassium hydroxide, dinitro-benzene, and ketosteroid. A careful study of the reaction was made by Callow, Callow, and Emmens (1938), and subsequently many modifications have been proposed with the object of simplifying the procedure or rendering it more specific. The main disadvantages of its use are that different steroids have different colour equivalents, and when used for crude biological extracts, materials other than steroids react to produce a non-specific interference. Wilson (1954) has suggested carrying out the reaction at a lower temperature to give more nearly equal colour equivalents, and various formulae have been proposed to correct for the interfering chromogens by the use of extinctions at two or three wavelengths. Alternatively, the chromogen may be extracted with organic solvent, which leaves a considerable proportion of the interfering material in the residual solution. Most of these modifications have retained the use of potassium hydroxide in the reaction, but more recently, Bongiovanni, Eberlein, and Thomas (1957) and Glenn (1959) have proposed the use of an organic base instead, and it was of interest to know if this offered any advantage in the estimation of ketosteroids. This paper reports some investigations made using tetramethylammonium hydroxide (T.M.A.H.), which is easily available, together with correction procedures which involve a minimum of labour.

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MATERIALS AND METHODS

m-DINITROBENZENE British Drug Houses, of a quality 'specially purified for the determination of 17-ketosteroids', dissolved in ethanol.

POTASSIUM HYDROXIDE AND SODIUM HYDROXIDE PELLETS Hopkin and Williams Analar.

ETHANOL was refluxed for two hours with 5 g./litre of dinitrophenylhydrazine and 10 ml./litre of concentrated hydrochloric acid, and distilled twice through a column.

ETHER (May and Baker) was used without purification.

TETRAMETHYLAMMONIUM HYDROXIDE Twenty-five per cent w/w aqueous, Hopkin and Williams.

ETHYLENE DICHLORIDE (British Drug Houses) was used without purification.

ETHANOLIC POTASSIUM HYDROXIDE 2·5 N was prepared as described by Wilson (1954) and stored under nitrogen in the refrigerator.

STEROIDS Solutions were prepared by weighing the steroids on a micro-balance and dissolving them in ethanol to give a concentration of approximately 50 mg./100 ml.; these solutions were kept in the refrigerator in stoppered tubes and allowed to warm to room temperature before use. For urine estimations a standard solution of dehydro*epi*androsterone (50 mg./100 ml.) was used. Twenty-four-hour urines were collected under toluene

(10 ml.) and stored at 4° until used. The volume was adjusted to 2,000 ml. with distilled water.

STOPPERED TUBES 'Excelo' 50 ml., with B.24 standard joint; 15 ml. with B.14 standard joint and calibrated to 10 ml. in 0·2 ml. divisions.

ANTI-BUMPING GRANULES British Drug Houses.

METHOD OF ESTIMATING URINARY 17-KETOSTEROIDS The urine was well shaken and two 10 ml. aliquots were pipetted into stoppered 50 ml. tubes. Then 3 ml. of concentrated hydrochloric acid was added and the tubes were shaken and placed in a boiling water bath for 10 min. They were then transferred to a cold water bath and when cool, 10 ml. of ethylene dichloride was added, the tubes stoppered and agitated for 15 min. in a horizontal shaker. The tubes were centrifuged for 10 min. at 2,000 r.p.m. and the urine layer removed by suction. The solvent was then filtered through a No. 1 Whatman filter paper into a clean stoppered 50 ml. tube and approximately 20 pellets of sodium hydroxide added. The tubes were stoppered and again shaken for 15 min. The solution was then filtered through Whatman No. 1 filter paper into calibrated tubes, and the volume reduced to 7 ml. with a fine pipette. A few anti-bumping granules were added and the tubes placed in a water bath at 90° in a fume cupboard, until the solvent had evaporated. The residue was finally dried by brief application of a vacuum or nitrogen, and the extract dissolved in 0·2 ml. of ethanol.

COLORIMETRY Colorimetry was carried out in potassium hydroxide and in T.M.A.H.

Potassium Hydroxide Five tubes were used containing 0, 0·05, 0·10, 0·15, and 0·2 ml. of the standard steroid solution. The volumes were adjusted to 0·2 ml. with ethanol. To each tube, including the urine extracts, was added 0·4 ml. of a 1 : 1 mixture of 2% dinitrobenzene and potassium hydroxide solution. The tubes were then stoppered, the contents mixed and kept at 25° in the dark for one hour. After this time, 2 ml. of aqueous ethanol (30% ethanol in water) was added, followed by 5 ml. of ether. The tubes were then shaken vigorously for 15 sec., and the ether layer decanted into a covered cuvette (1 cm. light path). Tests and standards were read at 515 mμ in the Unicam SP 600 against the blank tube. A calibration curve was then drawn and used to calculate the amount of steroid in the urine extracts. If a urine blank correction was to be made, one of the two extracts was used for this purpose, and was dissolved in 0·4 ml. of ethanol instead of 0·2 ml. A tube containing 0·4 ml. of ethanol was used as a reagent blank, and 0·2 ml. of potassium hydroxide solution was added to all tubes. After incubation and extraction as above, the urine blanks were read at 515 mμ against the reagent blank The optical density obtained was deducted from that of the appropriate test, and this corrected figure was used to calculate the amount of steroid present.

Tetramethylammonium hydroxide The procedure followed was exactly as described above, except that various concentrations of T.M.A.H. were substituted for the 2·5 N potassium hydroxide, and the alkali and 1% or 2% dinitrobenzene solutions were added separately, or mixed before addition, depending on the concentration used.

17-HYDROXYCORTICOSTEROIDS These were estimated as described by Appleby, Gibson, Norymberski, and Stubbs (1955), using 5 ml. of urine. Colorimetry was carried out as described above.

RESULTS

EXTINCTION MAXIMUM Using potassium hydroxide solution, the absorption maximum of the colour developed with dinitrobenzene and steroid was at 515 mμ in alcohol or ether. For the six ketosteroids examined, this was unaltered by substituting T.M.A.H., and all optical densities were therefore read at this wavelength. Fig. 1 shows calibration curves for dehydro*epi*androsterone using potassium hydroxide (2·5 N) and T.M.A.H. (2·7 N), and these are seen to be linear up to at least 100 μg. of steroid; using T.M.A.H., however, the colour developed by the alcohol blank was considerably lower. Furthermore, the colour developed by the T.M.A.H. for identical weights of steroid was 34% greater than with potassium hydroxide.

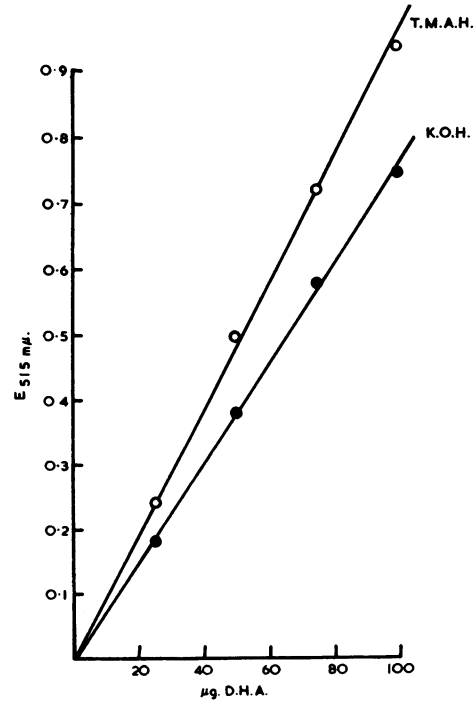

FIG. 1. *Calibration curves for dehydroepiandrosterone using KOH (2·5 N) and T.M.A.H. (2·7 N).*

STABILITY To ascertain the stability of the colour developed, readings were taken immediately after extraction of the colour, one hour later and at 17 hours. The results are shown in Table I, and indicate that after one hour the optical density has decreased by 2%, and fades linearly at 17 hours. Since readings are invariably made in under one hour, even with a large batch, this may be considered insignificant.

TABLE I

OPTICAL DENSITY OF ETHER SOLUTION OF ZIMMERMANN COMPLEX AT VARIOUS TIMES AFTER EXTRACTION

	Optical Density (515 mμ)
Immediate extraction	0·463
1 hour	0·454
17 hours	0·314

EFFECT OF CONCENTRATION OF T.M.A.H. The amount of colour developed at a given time and temperature depends upon the concentration of alkali. Fig. 2 shows standard curves obtained with T.M.A.H. at various concentrations, and demonstrates a progressive increase in colour with concentration. At lower alkali strengths, the curves become non-linear, due to the relatively lower amount of colour developed by the higher standards. Concentrations greater than 25% have not been studied, but the increase from 20 to 25% produced only a small change in the slope of the curve and 25% may well be near the maximum possible under these conditions.

EFFECT OF CONCENTRATION OF DINITROBENZENE The effect of concentration of dinitrobenzene at an alkali concentration of 25% is shown in Fig. 3. Below 1%, there is a sharp fall in the amount of colour developed, but from 1% to 2% this is practically unchanged. Calculation shows that the amount of dinitrobenzene present at a concentration of 1% represents an excess of 32 moles for 100 μg. of steroid. However, the nature of the reaction is as yet not understood, although it has been postulated by Zimmermann (1944) that the chromogen contains 1 mole of steroid to 1 of dinitrobenzene. The use of 1% dinitrobenzene would therefore appear to give maximum colour formation, and a linear calibration curve up to at least 100 μg. of steroid. It is advantageous to use this concentration since it can be

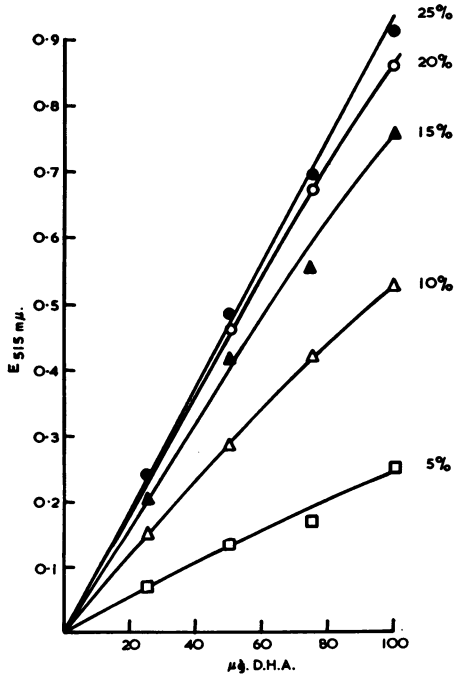

FIG. 2. *Standard curves for dehydroepiandrosterone at different concentrations of T.M.A.H. (2% dinitrobenzene).*

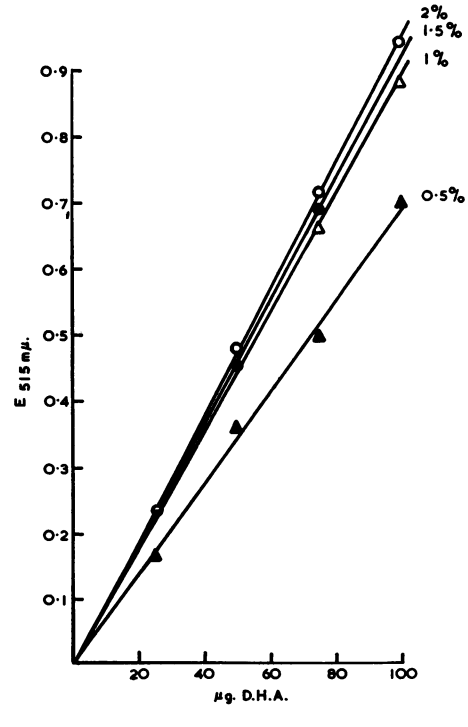

FIG. 3. *Standard curves for dehydroepiandrosterone at different concentrations of dinitrobenzene (2·7 N T.M.A.H.).*

mixed with 25% T.M.A.H. and added directly to the steroid solution, whereas at higher concentrations of dinitrobenzene, precipitation occurs, and it is necessary to add the reagents separately.

TIME OF DEVELOPMENT Fig. 4 shows the development of colour with time at room temperature and at 25°. In both cases the optical density increases only slowly after 60 min., although to obtain maximum colour it appears necessary to incubate for two hours. However, for routine estimations, a period of 60 min. is convenient and produces a large proportion of the total colour which can be developed. Also, after ether extraction at 60 min., the optical density alters by less than 2% up to one hour; it is necessary to use glass caps for the cuvettes to prevent evaporation of the ether.

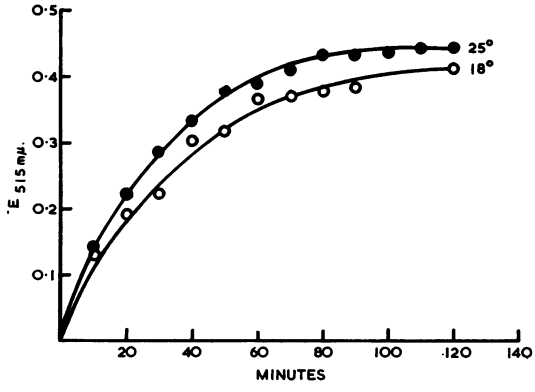

FIG. 4. *Development of Zimmermann colour with time at 18° and 25°.*

SENSITIVITY For the estimation of smaller quantities of steroid, it is convenient to employ half quantities of reagents. This gives a final volume of ether of 2·5 ml. and 5μg. of dehydro*epi*androsterone produces an optical density of 0·100. Quantities of steroid less than 5 μg. can be estimated with some accuracy, and the use of micro-cells would offer even greater sensitivity.

COMPARISON OF COLOUR EQUIVALENTS OF DIFFERENT KETOSTEROIDS, PURE STEROIDS, AND URINE EXTRACTS Different ketosteroids, such as those normally excreted in human urine, are chromogenic to different degrees in the Zimmermann reaction, and a knowledge of these colour equivalents is necessary to calculate absolute amounts of steroid from one standard. Alternatively a different standard may be required in each case. The colour equivalents will vary with different conditions of development, and a comparison was therefore made of the optical densities obtained by using potassium hydroxide and T.M.A.H. for pure reference steroids. The results are shown in Table II and are relative to dehydro*epi*androsterone. It will be seen that quite different

TABLE II

ZIMMERMANN COLOUR EQUIVALENTS OF EQUAL WEIGHTS OF VARIOUS 17-KETOSTEROIDS

Steroid	*DHA Colour Equivalent*	
	T.M.A.H.	*Potassium Hydroxide*
Dehydro*epi*androsterone	100	100
Aetiocholanolone	97	108
Androsterone	98	99
11β-Hydroxyaetiocholanolone	74	96
11β-Hydroxyandrosterone	65	73
11-Ketoaetiocholanolone	113	122

relative results were obtained using T.M.A.H. and potassium hydroxide, and it might therefore be anticipated that a difference would be found for the crude mixture of steroids obtained from human urine. A similar comparison was therefore made of 12 ketosteroid extracts obtained from normal urines. Table III shows these results, and in general it

TABLE III

17-KETOSTEROID CONTENT (MG./24 HR.) OF 12 NORMAL URINES USING KOH AND T.M.A.H.

	2·5 N KOH	*25% T.M.A.H.*	Δ
1	8·8	8·6	−0·2
2	2·9	2·4	−0·5
3	3·0	4·1	+1·1
4	5·5	5·5	0
5	2·0	2·5	+0·5
6	8·8	8·4	−0·4
7	4·2	4·3	+0·1
8	5·9	5·6	−0·3
9	3·7	3·9	+0·2
10	5·2	4·5	−0·7
11	9·8	8·5	−1·3
12	12·3	11·0	−1·3

appears that T.M.A.H. gives slightly lower values than potassium hydroxide, although this is not invariably true, and the difference is small. Similar results (Table IV) were obtained for 17-hydroxycorticosteroids estimated on 12 urines from patients with various disorders.

STABILITY OF T.M.A.H To investigate the stability of the T.M.A.H., a comparison was made of two batches, one obtained freshly from the manufacturers, and a second which had been already used and kept in the laboratory for one year. There was no significant difference in the calibration curves obtained or in the low reagent blanks; this is in

TABLE IV

17-HYDROXYCORTICOSTEROID CONTENT (MG./24 HR.) OF 12 URINES USING KOH AND T.M.A.H.

	2·5 N KOH	*25% T.M.A.H.*	Δ
	(*a*)	(*b*)	(*b*−*a*)
1	23·9	24·2	+0·3
2	32·8	31·4	−1·4
3	30·0	31·1	+1·1
4	31·4	30·1	−1·3
5	34·5	37·1	+2·6
6	27·6	27·2	−0·4
7	9·5	10·6	+1·1
8	12·3	9·9	−2·4
9	19·4	19·2	−0·2
10	15·7	16·1	+0·4
11	13·6	14·5	+0·9
12	15·0	15·3	+0·3

striking contrast to potassium hydroxide solution, which, however carefully preserved, will quickly become unusable, since the reagent blank increases rapidly.

DISCUSSION

A major source of error in the estimation of urinary ketosteroids is the presence of pigment and other material which contribute colour to that produced by the Zimmermann reaction. In general, two methods for obviating this error are in use. The first involves the application of a mathematical correction, and the equation proposed by Talbot, Berman, and MacLachlan (1942) has been widely used. Alternatively the formula deduced by Allen (1950) may be applied from optical densities obtained at three wavelengths. Both these formulae make the basic assumption that the interfering chromogens behave in a consistent and known fashion, and this is difficult to ascertain for individual extracts and may not be true. In the estimation of 17-hydroxycorticosteroids, the chemical manipulations involved are likely to alter the composition of the non-steroidal material, and the application of correction formulae would not be justified without prior investigation of the spectral characteristics. Braunsberg (1957) and later O'Sullivan (1958) devised tests for justifying the application of the Allen correction to crude extracts and such tests should undoubtedly be made before a correction may be considered valid. In any event, the time required to obtain further data for correction and their application will lengthen the time required for assay.

The second group of methods has the object of removing the interfering chromogens either before or after colorimetry, and the separation of ketonic and non-ketonic components by Girard's method is of this type. This is a lengthy process, requiring careful manipulation for quantitative results. and is not entirely suitable for routine estimations. Furthermore, the process often fails to eliminate non-steroidal 'ketonic' material. A different process was adopted by Drekter, Heisler, Scism, Stern, Pearson, and McGavack (1952) who used sodium hydroxide pellets to adsorb interfering pigments, and showed that unlike some other adsorbents, *e.g.*, charcoal, this material did not alter the recovery of keto-steroid. Alternatively, it is possible to purify the chromogen developed in the Zimmermann reaction by partitioning between an organic solvent and aqueous alcohol (Cahen and Salter, 1944; Henry and Thevenet, 1951; Zimmermann, Anton, and Pontius, 1952; Masuda and Thuline, 1953; Werbin and Ong, 1954; Crépy, Meslin, and Desgrez, 1956) and much yellow-brown pigment is then left in the aqueous layer of the reaction mixture. This component occurs even with pure ketosteroids, and is greatly increased with urine extracts. The colour due to remaining non-steroidal material is corrected for by the use of a parallel extract developed without dinitrobenzene. In our experience, these procedures are highly satisfactory, and treatment with sodium hydroxide pellets is often sufficiently effective to obviate the need for a urine blank when combined with solvent extraction of the Zimmermann chromogen. However, this blank correction is somewhat variable, and, where considerable quantities of pigment appear after hydrolysis, they may account for approximately 10% of the uncorrected optical density. It is preferable to adopt this correction routinely, otherwise some slight overestimate may be obtained. In the estimation of 17-hydroxycorticosteroids, the problem of interfering material is less important, since the preliminary reduction-oxidation process appears to alter the pigments chemically and later interference is small. Examination of the results from a series of urines from patients showed that the blank correction was negligible, and it was not considered necessary to adopt this for routine estimations[1].

At present, it is not easy to assess the extent to which any method for estimation of urinary ketosteroids approximates the true value, although purification by Girard separation is most likely to give fractions of reasonable purity. It has been shown that ether extraction of the Zimmermann complex gives values for urines which are in close agreement

[1]It should be noted that the ether extraction of the Zimmermann chromogens is less effective with polyketonic steroids. Thus the derivative of androstan-3, 11, 17-trione is only poorly extracted under these conditions. It has been observed (M.I. Stern, personal communication) that if the original ketogenic procedure is adopted for urine estimations, in which prior reduction with borohydride is omitted, the Zimmermann chromogens obtained are not extracted completely. This is probably due to the formation of polyketonic steroids in the oxidation procedure which are not formed when borohydride is employed.

with those obtained by Girard purification, whilst mathematical corrections still produce higher results (Zimmermann *et al.*, 1952). In the present investigation, urines were examined from a series of 20 women, of ages 18 to 47; the range of steroid excretion found was 4·7 to 16·8 mg./24 hr. with a mean of 11·3. The same urines were fractionated by enzyme hydrolysis and paper chromatography (James, 1961) into the component ketosteroids, and the total obtained was expressed as a percentage of the total estimated as described. This percentage varied from 50 to 108 with a mean of 70. Whilst this would appear to indicate that the crude method produces an overestimate, it should be borne in mind that the acid hydrolysis used will alter most of the naturally occurring steroids, which will then be estimated as various steroid compounds of which the colour equivalents may be highly variable. Thus, the total estimated will vary with the composition and will not necessarily bear any strict relationship to the total originally present and isolated by milder procedures. Any method employing vigorous hydrolysis will therefore produce results which at best are only indicative of the total steroid originally present, and if more accurate information is required, it is essential to employ more gentle methods. For routine clinical purposes, it is usually sufficient to obtain an indication of the total produced, and simple methods are therefore adequate.

Regarding the use of T.M.A.H. in place of the conventional potassium hydroxide in the Zimmermann reaction, it is clear that this introduces no gross error into the estimation, and it may therefore be considered as a suitable substitute. It has the advantage of producing more colour at the same normality, and this would give somewhat greater sensitivity. If pure steroid fractions are being estimated, it is necessary to remember that the colour equivalents of the various ketosteroids are different with the two reagents.

For routine ketosteroid and 17-hydroxycorticosteroid estimations, T.M.A.H. offers the considerable advantage of stability and even after one year at room temperature in the laboratory no detectable change in reagent blanks or calibration curve was observed. By contrast, potassium hydroxide rapidly becomes useless, since it produces increasing reagent blanks and has to be made freshly for each batch. Even when prepared as described by Wilson (1954) the reagent will keep only one month, is tedious to make, requires refrigeration, and has to be kept carefully under nitrogen. Tetramethylammonium hydroxide therefore appears to be well suited for use in a laboratory which undertakes only occasional steroid estimations, where the preparation of a large quantity of stabilized reagent is uneconomical.

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