XLVIII. THE COLORIMETRIC ESTIMATION OF THE OESTROGENIC HORMONES

II. OESTRONE

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THE colorimetric estimation of oestrone, based on treatment of oestrone with conc. H_2SO_4 or a phenolsulphonic acid, followed by dilution with water and by heating the solution to develop the colour, was described by the present author in 1931. It has since been modified and its applications critically examined by a number of other workers [Cohen & Marrian, 1934; Cartland *et al.* 1935; Pincus *et al.* 1936; Venning *et al.* 1937]. In the meantime the method has been further developed by the author and the present paper is an account of the current methods in use for oestrone and urine extracts: only a brief account of these has been published hitherto [Kober, 1936].

The principal change which has been made is the substitution of β -naphthol-sulphonic acid for phenolsulphonic acid as the reagent. Secondly, changes have been made in the technique of developing the colour, and thirdly, methods for estimation of oestrone in urine extracts have been elaborated. The detailed technique now used is described in the first section below and in subsequent sections the systematic investigations which form the basis of this technique are described. Finally, a description is given of investigations leading to a method of determination of oestrone in extracts of mares' urine, and the possibility of applying this to human urine is discussed. It should be emphasized that this paper deals only with determination of oestrone; the same reaction however, although in a modified manner, is given by all the known oestrogenic hormones: their behaviour will be described in a later publication.

I. *Technique of determination of oestrone*

(a) *Apparatus.* Test tubes, 7–8 mm. $\times$ 70 mm. with rubber caps. Glycerol baths, kept at 100° and 120°. Absorption cells of Zeiss Stuphometer, 1 and 2.5 mm. Stop watch. Photoelectric colorimeter with Lifa Filter, No. 210, or a spectrophotometer. Pipettes, etc.

(b) *Reagents.* β -Naphtholsulphonic acid, prepared by dissolving 2.5 g. of β -naphthol in 100 g. of H_2SO_4 (93–96%) in the cold. Distilled water (acidified with 0.5% H_2SO_4 to prevent growth of mould). 50% acetone (mixture of equal parts of chemically pure acetone and water).

Aqueous-alcoholic hydrogen peroxide solution (concentration 0.33%): freshly prepared by mixing equal volumes of 96% alcohol and 0.67% aqueous hydrogen peroxide.

(c) *Preparation of mixture A.* A quantity of the material to be tested which contains 2.5–12 γ of oestrone, dissolved in alcohol (acetone sometimes gives irregular results) is evaporated to dryness in a test tube. To the residue is added 0.2 ml. of the β -naphthol reagent, the tube is shaken thoroughly and heated for 2 min. at 100°, shaking in the bath 45 and 90 sec. from the start: it is then cooled in ice water. At this stage, mixture A, the tubes can be stored in the dark for several hours.

(d) *Mixtures B₀ and B₁*. 0.2 ml. water is added to mixture A, the contents are well stirred while the tube is immersed in ice water, and the tube is then heated for 30 sec. at 120° or for 90 sec. at 100°, shaking it after 15 or 45 sec. heating respectively. The tube is then cooled in ice water for not more than 10 sec. It has been found possible to handle up to 16 tubes at the same time by this method in our laboratory routine determinations. The mixture (B₀) is then transferred to the 1 mm. cell for colorimetry, or is diluted with 0.6 ml. of 65 % H₂SO₄ (1 : 1 by volume) (mixture B₁) and transferred to the 2.5 mm. cell.

(e) *Mixtures B₂ and B₃*. When, as in the case of urine extracts, measurements have to be made of the "residual colour" after treatment with H₂O₂, mixture B₂ is prepared from mixture B₀ (referred to in previous papers as B) as follows: the mixture B₀ is covered with 0.6 ml. of ice-cold acetone and the tube itself is cooled in ice water for about 10 sec. The tube is closed with a rubber cap, the contents mixed by inverting the tube four times, and then cooled in ice water until 20 sec., timed by a stop watch, after beginning the mixing. The liquid cell of the colorimeter is then filled and the reading is taken 60 sec. after the start. The "residual colour" is determined in a second tube of mixture B₀ treated with 0.6 ml. of alcoholic H₂O₂ and shaken for 10 sec. by inverting it about 20 times (mixture B₃).

(f) *Colorimetry*. Since the time factor is most important in obtaining reproducible results, the use of a photoelectric colorimeter or spectrophotometer is essential. The original apparatus designed for this purpose [Kober, 1933] has been modified by using 5 % Cu(NO₃)₂ solution instead of alum solution to filter off infra-red (I am indebted to Dipl. Ing. St. Gradstein for suggesting this improvement), and by using a blue-green Lifa Filter, No. 210 with a maximum transmission at 5000 Å.

This filter has the following characteristics:

λ (Å.)	I/I_0
4700	0.000
4800	0.011
4900	0.040
5000	0.055
5100	0.036
5200	0.009
5300	0.000

The readings of the instrument in % of the total potentiometer resistance give directly the percentage absorption (% $A = 100 - D$ %; $K = -\log D = \log I_0/I$). A powerful monochromator has now been substituted for the light source and filter, resulting in a great improvement. The region 5050 ± 100 Å. is employed.

II. The reagent

The original phenol reagent of Kober [1931] is very viscous, whilst the β -naphthol reagent is comparable with H₂SO₄. We found with the phenol reagent that intensity of colour was not directly proportional to the oestrone concentration [cf. Cohen & Marrian, 1934], and it was necessary to construct a calibration curve with known amounts of oestrone, although Pincus *et al.* [1936] appear to have found conditions under which proportionality held good. Under the conditions described here with the β -naphthol reagent a calibration curve is superfluous.

With the phenol reagent it is difficult to obtain the same results with different batches [cf. Cohen & Marrian, 1934, and Pincus *et al.* 1936], partly, at least, because of the influence of varying amounts of water in the acid. Results are

highly reproducible with the β -naphthol reagent, as illustrated by the figures in Table I. (All data given here and in the rest of the paper are, unless otherwise stated, of estimations made as described above with 1 ml. of mixtures B_2 or B_3 . The values of the absorption coefficient $K = \log I_0/I$, measured at $5050 \pm 100 \text{ \AA}$., refer either to the coloration of 10γ of oestrone in 1 ml. of mixture B_2 (after dilution) in a 2.5 mm. layer or to 0.4 ml. of mixture B_0 (before dilution) in a 1 mm. layer. $E_{1\text{ cm.}}^{1\%}$ is obtained by multiplying these figures by 4000.) All measurements were made without a compensating cell and, therefore, the "reflection value" of the cell filled with the reagent only ("blank") has always to be subtracted.

Table II shows the influence of water content of the reagent on the coloration, which is decidedly smaller than that found for the phenol reagent by Cohen & Marrian [1934].

Table I. *Reproducibility of the β -naphthol reagent*

Reagent no.	K_{blank}	$K_{10\gamma \text{ oestrone}}$
18/5	0.056	0.279
19/5	0.059	0.284
20/5	0.058	0.282
21/5	0.053	0.286
27/5	0.055	0.272
2/6	0.059	0.269
14/6	0.051	0.277
	Mean	0.2796

The reagents are each separate, freshly-prepared samples. The figures for the blank, and the figures for oestrone are means of from 2 to 4 and from 4 to 8 separate measurements, respectively.

Table II

Water added %	% H_2SO_4	$K_{10\gamma \text{ oestrone}}$
None	95	0.279
1	94	0.272
2	93	0.270
4	91	0.265
8	87	0.252

Oestrone values are means of 3 separate measurements.

It seemed, from the data in Table II, that a further increase in coloration might be obtained by further lowering the water content, but the figures in Table III, obtained with an acid prepared by admixture of oleum and H_2SO_4 , show that there is an actual decrease, with a marked change between 98.5 and 99.5 % acid. Reagent prepared from oleum with 20 % SO_3 gave $K_{10\gamma} = 0.125$. Apparently the mechanisms of the reaction in the presence and in the absence of water are different.

Table III

Water added %	% H_2SO_4	$K_{10\gamma \text{ oestrone}}$
None	99.5	0.162
1	98.5	0.246
2	97.5	0.251
4	95.5	0.269
8	91.5	0.260

In Table IV are given figures illustrating the stability of the reagent under different conditions of storage. It is essential to exclude light and air, and even the use of a dark-coloured bottle is advisable. Irradiation of the reagent

sealed in an ampoule under CO_2 with a Hanau quartz lamp for 1 hr. had an effect similar to that of storage in daylight for a long period.

Table IV. *Stability of the reagent*

Days after preparation	K_{blank}			$K_{10\gamma} \text{ oestrone}$		
	(a)	(b)	(c)	(a)	(b)	(c)
0	0.053	(0.053)	(0.053)	0.278	(0.278)	(0.278)
1	0.070	0.061	0.060	0.277	0.274	0.276
2	0.074	0.060	0.060	0.281	0.275	0.269
4	0.073	0.059	0.059	0.295	0.268	0.271
8	0.084	0.058	0.056	0.294	0.275	0.283
16	0.103	0.055	0.058	0.289	0.279	0.274
32	0.133	0.055	0.054	0.293	0.275	0.280

Reagent stored (a) in a half-filled bottle in daylight, (b) in a waxed, brown glass bottle in refrigerator, (c) in a waxed, brown glass bottle in the laboratory cupboard. The blanks and the oestrone values are means of 2-3 and of 4 separate determinations, respectively.

No substance superior to β -naphthol has been found. α -Naphthol behaves similarly, but is impracticable because a 2.5% solution crystallizes. Anthrol, 1:5-, 1:4-, 2:6- and 2:7-dihydroxynaphthalenes all give coloured solutions without the addition of oestrone, and even with the least coloured, the 2:7-compound, the oestrone colour is less intense than with β -naphthol.

III. Preparation of mixture B

The method of developing the colour by heating mixture A with water to give mixture B_0 has been modified considerably with a view to making the process as reproducible as possible. Mixture A is diluted with water while the tube is cooled in ice water. The effect of the subsequent time of heating is shown in Table V.

Table V. *Effect of time of heating at 120° of mixture B_0*

Time ...	5 sec.	15 sec.	30 sec.	45 sec.	60 sec.
$K_{10\gamma} \text{ oestrone}$	0.185	0.291	0.285	0.282	0.260

K values are means of 3 determinations.

Since the change in colour between periods of 15 and 45 sec. heating is small, 30 sec. are chosen as the best time in practice. Heating at 100° for 1.5 min. gives equally satisfactory results. Once the mixture has been cooled the colour remains stable at room temperature for 30 min., and the practical limit to storage of the mixture at this stage is generally crystallization of the β -naphthol-sulphonic acid.

Dilution of mixture B_0 to a volume of 1 ml. was originally made [Kober, 1931] with water, but some impure preparations became cloudy. It has not been possible to find the ideal, indifferent solvent which does not cause this effect and is equally suitable for "residual colour" determinations. A mixture of equal parts of acetone and water is the most satisfactory, but immediately after dilution the colour begins to fade, and does so more rapidly at higher temperatures. The mixture is therefore kept as cold as possible and a rigid time schedule is adhered to for measurement, since in the routine conditions described the value of K falls to 94-95% of the initial measured value 2 min. after mixing and to 88-90% of this value after 5 min.

IV. *Absorption curves of the mixtures obtained with the reagent and oestrone or mares' urine extract free from oestrone*

Using the photoelectric spectrophotometer mentioned above, readings have been taken at intervals of 100 Å. Since a sensitivity of 0.5–1 % absorption (at 50 % absorption) is only attainable with this instrument with a spectral width of 50 Å., any finer structure of the absorption bands is not detectable. The measurements were carried out on mixture B₀, owing to the instability of mixture B₂, and only a few points were checked with the diluted mixture to assure ourselves that both values were identical. The curve obtained is shown in Fig. 1, curve *a*.

Fig. 1. (a) Absorption curve of 10 γ oestrone/ml.; thickness of layer = 2.5 mm.
(b) Absorption curve of 500 γ oestrone-free extract from mares' urine.

The curve *b* in Fig. 1 shows the absorption of mixture B prepared from an oestrone-free benzene extract of the urine of non-pregnant mares, purified by treatment with aqueous Na₂CO₃ and 65 % H₂SO₄ (method II, described below); a weight of 500 γ , equivalent to about 1 ml. of urine, was taken. In the region 5100–5200 Å. the absorptions of 500 γ extract free from oestrone or of 10 γ of oestrone are equal, but the urine extract differs in that the value decreases markedly after dilution with 50 % acetone, e.g. from $K = 0.315$ to $K = 0.185$ (the diluted solution being measured in a correspondingly thicker layer).

V. *Measurement of "residual colour" and its use for determination of oestrone in urine extracts*

As the example given in section IV shows, a simple photoelectric measurement of absorption would lead to the assumption of the presence of 10 mg. of oestrone per l. in a urine free from oestrone. When it was necessary simply to determine whether samples of urine (in a series amounting to tens of thousands) were worth extraction on a technical scale, it was sufficient to determine the total solids and to neglect any colorimetric "oestrone" value under 4 % of the total solids (e.g. 2 % in the above case by determination in B₀, and 1 % in B₂). Purification by such a process as that of Cohen & Marrian [1934] was impracticable in these circumstances.

For determination of oestrone in extracts of mares' urine of low content or in extracts of human urine (less than 2 mg./l.), purification must be followed by a determination of the "residual colour" based on the observation that

H_2O_2 destroys the characteristic oestrone colour selectively [Kober, 1931; Cohen & Marrian, 1934]. A new technique suitable for use with the β -naphthol reagent has had to be elaborated, since the Cohen & Marrian method leads to the formation of brown naphthoquinone in warm solutions, whilst no bleaching occurs in the cold.

Two requirements must be fulfilled by the diluent, viz. (1) with pure oestrone the colour should be reduced to that of the blank mixture, and (2) with a urine extract free from oestrone, the colorimetric value should be the same as with 50 % aqueous acetone. The alcoholic H_2O_2 solution used as described above is satisfactory in these respects. The following example demonstrates the degree of recovery of oestrone added to a fairly crude oestrone-free mares' urine extract obtained by method II (see below, section VI).

$$\begin{array}{ll} 175\gamma \text{ extract} + 0.5\gamma \text{ oestrone: } 50\% \text{ acetone: } K_1 = 0.207, \\ 175\gamma \text{ extract} + 0.5\gamma \text{ oestrone: } \text{H}_2\text{O}_2: K_2 = 0.195, \end{array}$$

$K_1 - K_2 = 0.011$, equivalent to 0.4γ oestrone, i.e. 80 % recovery in a 1 : 350 mixture. The amount of extract used here corresponds to 0.25 ml. of urine, and the limit of sensitivity of the reaction with such an extract is about reached at this concentration, i.e. 2 mg./l.

VI. Purification of urine extracts for colorimetry

Purification of urine extracts is necessary, not only because of the effect of high residual colours, but also, in human urine, because of the presence of inhibitors such as cholesterol in the neutral fractions. Thus the addition of 10, 20 or 40γ of cholesterol to 10γ of oestrone reduces the amount found colorimetrically to 7.8, 7.0 and 5.5γ , respectively.

The extraction of the "weak phenol" fraction by the method of Cohen & Marrian [1934] is valuable: this we have designated as method I below.

Our routine method for extracting mares' urine, designated method II, is as follows.

20 ml. of urine, acidified with 1 ml. of 65 % H_2SO_4 , are boiled for 2 min. After addition of 10 ml. of 96 % alcohol the warm solution is shaken for 4 min. with 30 ml. of benzene and the benzene layer separated, after filtration through a filter-aid if the emulsion is persistent. The benzene is washed successively with 1 ml. of 65 % H_2SO_4 , water, 5 ml. of 10 % Na_2CO_3 and finally with water; it is evaporated, and the residue dissolved in 4 ml. of acetone (1 ml. $\equiv$ 5 ml. urine).

Method III is as follows: 200 ml. of mares' urine are hydrolysed as in method II and then boiled for 3 hr. with 300 ml. of benzene. The benzene layer is extracted three times with 20 ml. of *N* NaOH and then discarded. The combined alkaline extracts are treated with 5 g. of NaHCO_3 , and then with sufficient HCl to give pH 8–9 (phenolphthalein colourless, but litmus blue). This solution is then extracted twice with 20 ml. of benzene; this is evaporated and taken up in 4 ml. of acetone (1 ml. $\equiv$ 50 ml. urine). The whole of the phenolic fraction is isolated in this way and the purification of the extract takes half an hour. Whilst method II enables determinations to be made down to 2 or 3 mg./l., method III brings this limit down to 0.3 mg./l.

Table VI gives examples of the recovery of oestrone added to various urines and extracts submitted to methods I, II or III. In Exps. 1–5 only 80–90 % of oestrone added after purification by method II is recovered. This is evidently due to the presence of inhibitors, and observed oestrone values should be divided by 0.85. After applying this correction it is seen that in Exps. 6–9

Table VI

Exp. no.	Nature of urine	Purification method	Wt. of dry extract mg./l. urine	Ratio of total extract to oestrone in extract	Added oestrone		Recovered		When oestrone was added*
					γ per tube	mg./l. urine	mg./l. urine	%	
1	Non-pregnant mares	II	575	14.5	5	40	35	87	a.p.
2	"	II	575	29	5	20	16	80	"
3	"	II	575	57	5	10	9	90	"
4	"	II	575	115	5	5	4.2	84	"
5	"	II	700	350	0.5	2	1.6	80	"
6	"	II	ca. 600	15	5; 10	40	28	70	b.e.
7	"	II	600	30	5; 10	20	14	70	"
8	"	II	600	60	2.5; 5	10	7.3	73	"
9	"	II	600	120	2.5; 5	5	3.5	70	"
10	"	III	90	180	2.5	0.5	0.37	74	b.p.
11	"	III	72	144	2.5	0.5	0.35	70	b.e.
12	"	III	85	170	2.5	0.5	0.34	68	"
13	Men	I	5.3	222	1.0	0.025	0.016	67	a.p.
14	"	I	1.4	68	1.0	0.025	0.019	75	"
15	"	I	1.4	34	2.0	0.05	0.036	72	"

* b.e. = before extraction; b.p. = before purification; a.p. = after purification.

80 % of the added oestrone was extracted. The same applies to method III, as shown by Exps. 11 and 12. In the purification alone, as Exp. 10 shows, the loss is slightly lower. In Exps. 13–15 the lower limit of sensitivity of the reaction with human urine is demonstrated by recovery of 75 % of an added amount of 25 γ , but in these cases, instead of subtracting the "residual colour", the value for the extract without added oestrone was subtracted; these differ by 10 γ /l.

Actually a further correction should be applied when, as in these cases, only 1 γ of oestrone is used for the determination, for the variation of K with concentration is not linear with small amounts of oestrone, and the values of $K_{10\gamma}$ oestrone calculated in this region are 10–20 % lower than the standard value. An empirical factor must be determined, by which the oestrone value is multiplied, to correct for this effect and the presence of inhibitors: this is of the order of 1.25. Whilst the final error may be about 20 %, it is still less than that of bio-assay and application to estimations on urine of non-pregnant individuals is worth investigation.

The method is also probably applicable to rabbits' urine, although Pincus *et al.* [1936] speak of a "complete overestimation" of the oestrone content, whilst Marrian (private communication) finds that the "residual colour" method of Cohen & Marrian [1934] appears to fail. We have found, using method I on 10 ml. samples of a mixture of rabbits' urine yielding 1 mg. of extract per l. in two separate estimations, the following values: (1) acetone, 2.2 γ , peroxide, 2.1 γ ; diff. (oestrone) = 0.1 γ , and (2) 3.7 γ – 3.7 γ = 0. The urine therefore certainly contains less than 200 i.u./l. Using method III, 70 mg./l. of extracted solids had an apparent oestrone content of 0.3 mg./l. and this process of purification is evidently not a suitable one.

VII. Calibration

The calculation of the concentration of oestrone in an unknown sample is based on the existence of a constant value of the absorption coefficient $K_{10\gamma}$ oestrone, since Beer's law holds over a wide range. This coefficient is measured directly or

calculated, according to the method of calibration of the instrument, after subtraction of a blank value for the reagent alone, which is obtained by extrapolation of the curve of concentration/ K to zero concentration.

In Table VII the results are given of determinations of oestrone in four different concentrations (expressed as γ oestrone in mixture B_0), each determination being repeated 22–24 times. From the values at each of these concentrations values of $K_{10\gamma}$ oestrone are calculated, which are nearly constant, with a mean value of 0.274.

Table VII

Oestrone γ	No. of deter- minations	% light absorption	Standard deviation for a single measured value	Absorption coefficient	Absorption coefficient after subtraction of blank	Absorption coefficient calculated for 10 γ	γ Oestrone calculated from mean absorption coefficient	% standard deviation of K or C
c	n	% A	σA	K	$K - K_0$	$K_{10\gamma}$	c from (k)	σc %
1	2	3	4	5	6	7	8	9
4	24	31.3	0.53	0.1633	0.1073	0.269	3.9	3.2
6	23	40.2	0.56	0.2233	0.1673	0.279	6.1	2.4
9	22	50.2	0.60	0.3028	0.2468	0.274	9.0	2.1
13	23	61.45	0.83	0.414	0.3580	0.275	13.0	2.7
Mean						0.274		

VIII. Errors and reproducibility

In Table VII the standard deviations of the measurements have been given in the fourth and ninth columns, calculated for the measurements and as percentages of the concentration, respectively. Within this range of concentration, which is that generally used, the standard deviation for a single measurement is 2–3%. With 8 or 9 determinations the standard deviation of the mean is approximately 1%, and hence the greatest error to be expected is about 2%. Further increase in the number of measurements is not useful, for deviations from linearity of the K/c curve then become of the same order. It will readily be understood that when total colour and "residual colour" measurements are made, the errors in each measurement must be added together. Thus the standard deviation of the difference may be so large that equality of total and residual colours does not necessarily imply absence of oestrone, and it is only strictly correct to express the result as indicating less than a certain amount of oestrone, e.g. 9 determinations of total colour of 300 γ of extract equivalent to 0.5 ml. urine gave $A = 36.13\% \pm 0.28\%$, and 13 determinations of residual colour gave $A = 36.20\% \pm 0.52\%$, i.e. corresponding to the apparent oestrone contents of $(5.15 \pm 0.075\gamma)$ and $(5.15 \pm 0.13\gamma)$, respectively. The standard deviation of the difference is 0.15 γ in a single determination, and a maximum oestrone content of 6000 I.U./l. is possible. With 4 determinations the maximum oestrone content is still 3000 I.U./l. Further purification of the extract will, of course, yield greater accuracy.

SUMMARY

1. A modification of the author's original method for the colorimetric determination of oestrone is described. A 2.5% solution of β -naphthol in concentrated sulphuric acid is used instead of the original phenol reagent.
2. The effects of various factors on the reaction are discussed.
3. Urines containing little oestrone can also be assayed colorimetrically by determining the "residual colour" due to impurities after destroying the

characteristic oestrone colour; the method being based on that of Cohen & Marrian.

4. Suitable methods of purification of urine extracts are described.
5. The error and reproducibility of the measurements are discussed.

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