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A Study of the Colorimetric Estimation of Oestradiol-17β, Oestradiol-17α, Oestrone, Oestriol and 16-*epi*Oestriol by the Kober Reaction

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In the course of applying the method of Brown (1955) for the estimation of urinary oestrogens, the Kober reaction employed in this method did not work well in our hands. The intensities of colours developed from pure crystalline oestrogens and their methyl ethers were considerably lower than those specified by Bauld (1954) and Brown (1955) (cf. Breuer, Nocke, Geissler & Mitchell, 1957). This was not rectified by addition of oxidizing agents to the reagents as recommended by Bauld (1954). In addition to the typical absorption maximum between 510 and 520 mμ there was a peak at 460–470 mμ, indicating that the yellow colour of the first stage of the reaction was not being completely converted into pink colour ('inhibition type II' of Bauld, 1954). Also, when compared with those of Brown (1955), there was a 4–6 mμ shift in the absorption peaks of the pink colours. The reproducibility of the results was satisfactory, but the sensitivity was low. The conditions of the reaction were therefore re-examined in an attempt to obtain the sensitivity reported by Bauld (1954) and Brown (1955). In this investigation, 16-*epi*oestriol and oestradiol-17α have been examined as well as the three 'classical' oestrogens, oestradiol-17β, oestrone and oestriol.

MATERIALS AND METHODS

Steroids. Standard solutions of pure crystalline oestrogens and their 3-monomethyl ethers were prepared in ethanol (absolute, 'pro analysi'; E. Merck, Darmstadt) with a concentration of 10μg./ml. and stored at 4°.

Colour reagents. The Kober reagents contained 2·0% (w/v) of quinol ('puriss.', E. Merck) dissolved in aqueous solutions of H_2SO_4 (sp.gr. 1·84; 'pro analysi', E. Merck). The final concentrations of H_2SO_4 were between 50 and 96% (v/v) and were calculated by assuming that the conc. H_2SO_4 was 96% (v/v). Solution of quinol was hastened by heating. The solutions were kept at least 1 week before use and stored in amber bottles at room temperature in the dark (cf. Brown, 1952*b*; Bauld, 1954); they were colourless and stable for months.

Kober reaction. The standard solutions (0·5–1·0 ml.) were pipetted into 'Kober tubes' (Jena glass; 175 mm. × 25 mm.) with standard NS 14·5 sockets fitted with cones. Quinol (4 mg.) in ethanolic solution (0·2 ml. of 2%, w/v) and two pieces of white Beauxilite abrasive (size no. 16; Universal Grinding Wheel Co. Ltd., Stafford) washed with ethanol

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Table 1. *Determination of the optimum sulphuric acid concentration for oestrone in the second stage of the Kober reaction*

Each tube contained the dry residue of 5 μg. of oestrone and 4 mg. of quinol. Colour reagent: 66% (v/v) H_2SO_4–2% quinol. Heating times: first stage 20 min., second stage 10 min. For the second stage, water was added so that the volume of water plus volume of reagent was 3·2 ml. in each tube. $E_{corr.} = 2E_{515} - (E_{474} + E_{556})$.

Kober reagent (ml.)	Concn. of H_2SO_4 in the second stage (%, v/v)	E 465 mμ	474 mμ	515 mμ ($\lambda_{max.}$)	556 mμ	$E_{corr.}$
1·5	30·9	0·060	0·082	0·188	0·015	0·279
1·7	35·1	0·071	0·099	0·223	0·016	0·331
1·8	37·1	0·073	0·102	0·235	0·014	0·354
1·9	39·2	0·080	0·109	0·252	0·018	0·377
2·0	41·2	0·085	0·116	0·266	0·015	0·401
2·1	43·4	0·085	0·118	0·275	0·016	0·416
2·2	45·4	0·090	0·122	0·275	0·015	0·413
2·3	47·5	0·100	0·128	0·275	0·016	0·406
2·7	55·7	0·155	0·162	0·243	0·025	0·299
2·8	57·7	0·161	0·166	0·238	0·021	0·289
2·9	59·8	0·170	0·174	0·247	0·027	0·293
3·2	66·0	0·200	0·192	0·237	0·032	0·250

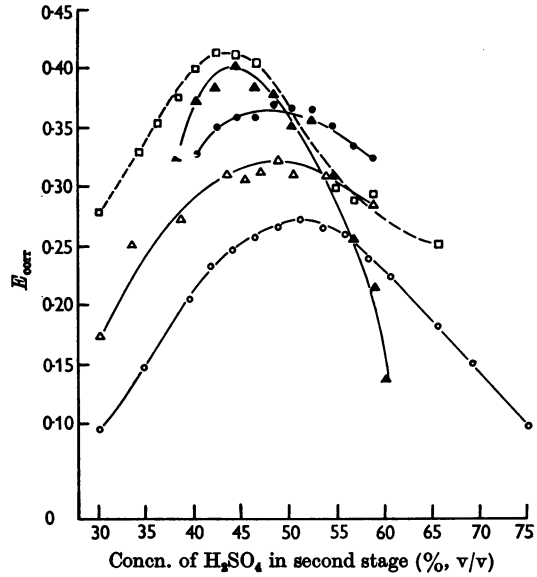

Fig. 1. Corrected extinctions ($E_{corr.}$) of the colour produced from 5μg. amounts of oestradiol-17β, oestradiol-17α, oestrone, oestriol and 16-*epi*oestriol as influenced by different sulphuric acid concentrations in the second stage of the Kober reaction.

Steroid	Symbol	Concn. of H_2SO_4 in the first stage (%, v/v)	Wavelengths (mμ) E_1	E_2 ($E_{\lambda max.}$)	E_3
Oestradiol-17β	△	60	480	518	556
Oestradiol-17α	▲	66	486	521	556
Oestrone	□	66	474	515	556
Oestriol	○	76	472	514	556
16-*epi*Oestriol	●	66	472	514	556

Heating times: first stage, 20 min.; second stage, 10 min. Final volume of all reaction mixtures: 3·2 ml.

$E_{corr.} = 2E_2 - (E_1 + E_3)$.

were added and the solvent was evaporated by heating on a water bath under reduced pressure in an atmosphere of nitrogen. Blank tubes containing quinol only were prepared at the same time. After adding the appropriate colour reagent to the dry residue the yellow fluorescent colour was developed by heating the tubes with stoppers in place on a boiling-water bath (first stage). The tubes were shaken 12 times under identical conditions after 2 and 5 min. of heating. The solutions were then cooled in a bath of ice–water (5 min.). Water was added in amounts to give the required concentration of H_2SO_4 and the tubes were shaken vigorously. After development of the pink colour by further heating the tubes with stoppers in place, the mixtures were cooled again for 5 min. in a bath of ice–water and subsequently allowed to stand at room temperature for about 10 min. before reading. The mixtures were protected from bright light at all stages of the reaction. The sum of the volumes of colour reagent and water added in the second stage was kept constant within each series. The final volumes and the final concentrations of H_2SO_4 were calculated from the sum of these volumes and no allowance was made for the volume contraction of about 7%.

Spectrophotometry. Extinction ($\log I_0/I$) was measured in a Beckman DU spectrophotometer against similarly treated reagent blanks in 10 mm. glass cells. The readings were corrected for unspecific background colour by applying the equation of Allen (1950).

RESULTS

Influence of sulphuric acid concentration in the second stage of the reaction

Kober reagents with the following sulphuric acid concentrations were employed: 60% (v/v) for oestradiol-17β, 76% (v/v) for oestriol and 66% (v/v) for oestradiol-17α, oestrone and 16-*epi*-oestriol. In the first stage of the reaction the oestrogens were heated with the reagent for 20 min. on a boiling-water bath. After cooling, water was added to give the required final concentration of sulphuric acid. The mixtures were then reheated for 10 min. and cooled again. The final volume of all reaction mixtures was 3·2 ml. and the volumes of reagent and water were adjusted so that the sulphuric acid concentrations in the second stage were 30·9–60·0% for oestradiol-17β, 39·2–61·0% for oestradiol-17α, 30·0–66·0% for oestrone, 30·8–76·0% for oestriol and 41·3–59·5% for 16-*epi*oestriol. An example of this type of experiment carried out with oestrone is given in Table 1. This and similar experiments with the other four steroids are summarized in Fig. 1.

As found by Brown (1952*a*, *b*), there was an optimum concentration of sulphuric acid for each compound. Less of the first-stage yellow colour was converted into pink when the acid concentration in the second stage was too high. This effect is shown for oestrone by the data in Table 1 and Fig. 2. When the second-stage sulphuric acid concentration was 57·7%, which corresponds to that used by Brown (1955), the conversion of yellow colour into pink was incomplete, giving an absorption curve which indicates a distinct 'inhibition type II' of Bauld (1954). At a concentration of 66%, i.e. when reheating in the second stage was carried out without previous dilution of the reaction mixture with water, the inhibition resulted in an absorption curve with two peaks: the first, near 510 mμ, resembled the pink Kober colour and a second, near 565 mμ, was due to the residual first-stage yellow colour. When the second-stage sulphuric acid concentration was 31%, i.e. far below the optimum, there was a complete conversion of yellow into pink, as indicated by the reading at 465 mμ (Table 1) and by the single absorption maximum at 515 mμ. But the pink colour was unstable and faded (cf. Brown, 1952*b*; Bauld, 1954). For the optimum compromise between 'inhibition type II' and 'fading' the following con-

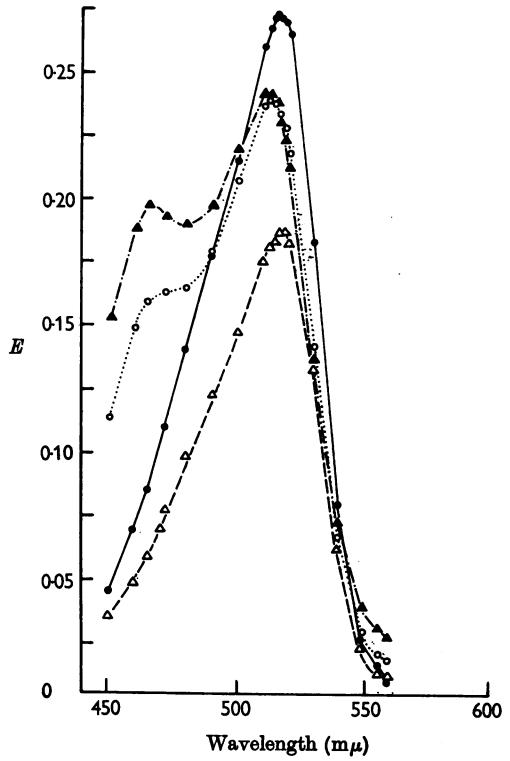

Fig. 2. Spectral characteristics of the colour produced from 5μg. of oestrone as influenced by different sulphuric acid concentrations in the second stage of the Kober reaction. Concentrations of sulphuric acid: △, 30·9% (fading); ●, 43·4% (optimum acid concentration); ○, 57·7% (moderate inhibition, type II); ▲, 66·0% (heavy inhibition, type II). For further experimental details see Table 1.

centrations were obtained: 48 % for oestradiol-17β, 45 % for oestradiol-17α, 43 % for oestrone and 50 % for oestriol and 16-*epi*oestriol. Under these conditions the absorption maxima of the final pink colour were 518 mμ for oestradiol-17β, 521 mμ for oestradiol-17α, 515 mμ for oestrone and 514 mμ for oestriol and 16-*epi*oestriol. The specific extinction coefficients ($E^{1\%}_{1\,cm.}$) of the colour produced by each of the compounds may be seen from Table 3.

Influence of heating time in the second stage of the reaction

Heating times in the second stage were varied between 0 and 30 min. after the first stage of the reaction had been carried out with 5 μg. each of the five compounds as described above. Sulphuric acid concentrations of the second stage were optimum. Some results are given in Fig. 3. The colour production of oestradiol-17α, oestrone and 16-*epi*oestriol exhibits a definite dependence upon the heating time, the optima being 6 min. for oestradiol-17α and oestrone and 5 min. for 16-*epi*oestriol. With oestradiol-17β there is a less sharp but distinct peak at 10 min. The intensity of the colour complex produced by oestriol showed a more delayed increase up to 12 min. and was stable on continued heating without formation of a peak.

Influence of sulphuric acid concentration in the first stage of the reaction

A series of colour reagents with sulphuric acid concentrations of 50–96 % were heated with 10 μg. each of the oestrogens for 20 min. in the first stage. In the second stage the sulphuric acid concentrations and the heating times were optimum for each oestrogen. The final volumes of the reaction mixtures were 3·2 ml. for oestradiol-17β, oestradiol-17α and 16-*epi*oestriol, 3·5 ml. for oestrone and 4 ml. for oestriol. In Fig. 4 the corrected extinctions of the colour produced by the five oestrogens are plotted against the sulphuric acid concentration of the various Kober reagents used in the first stage. For each of the compounds there is an optimum sulphuric acid concentration and the shape of the curves is characteristic. There was a tendency for the absorption maxima to shift to lower wavelengths with increasing sulphuric acid concentration. With oestrone and oestriol, for example, the range of the maxima was 508–520 mμ and 508–515 mμ respectively. With oestra-

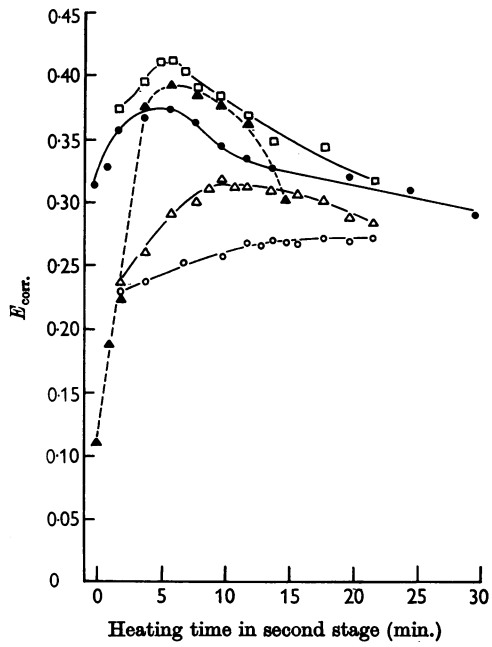

Fig. 3. Corrected extinctions ($E_{corr.}$) of the colour produced from 5 μg. amounts of oestradiol-17β, oestradiol-17α, oestrone, oestriol and 16-*epi*oestriol as influenced by different heating times in the second stage of the Kober reaction. Sulphuric acid concentrations in the second stage were optimum. For symbols and further experimental details see Fig. 1.

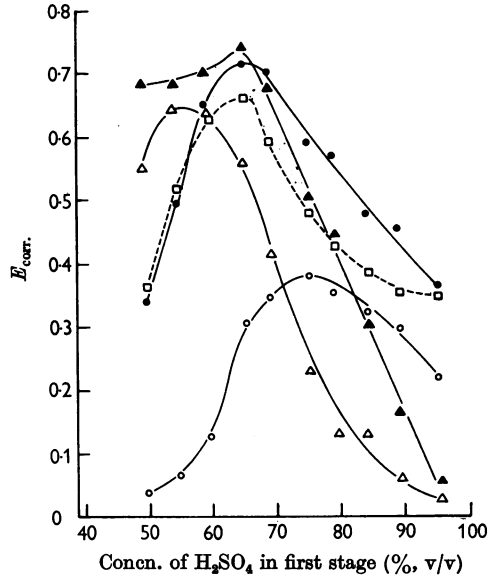

Fig. 4. Corrected extinctions ($E_{corr.}$) of the colour produced from 10 μg. amounts of oestradiol-17β, oestradiol-17α, oestrone, oestriol and 16-*epi*oestriol as influenced by different sulphuric acid concentrations in the first stage of the Kober reaction. Sulphuric acid concentrations and heating times in the second stage were optimum. The final volumes of the reaction mixtures were as follows: oestradiol-17β, oestradiol-17α and 16-*epi*oestriol, 3·2 ml.; oestrone, 3·5 ml.; oestriol, 4·0 ml. For symbols and further experimental details see Fig. 1.

diol-17β, the range was 512–520 mμ, the lower value occurring at an acid concentration of 76%. When the acid concentration was above 76%, oestradiol-17β did not produce any pink colour, and only small amounts of first-stage yellow colour were formed, the absorption maxima of which were between 460 and 470 mμ (combined inhibition types I and II of Bauld, 1954). However, for reasons of comparability, the extinctions of the reaction mixtures of one steroid have been measured at the same wavelength and the corrected extinctions have been calculated by applying the same correction formula. The optimum sulphuric acid concentrations for the first stage as concluded from these experiments are: 66% for oestrone, oestradiol-17α and 16-*epi*oestriol, 76% for oestriol and 55% for oestradiol-17β. The optima for oestrone and oestriol confirm the data reported by Brown (1952*a*, *b*, 1955) and Bauld (1954), whereas the optimum for oestradiol-17β is somewhat lower than that of these authors (60%). This may be due to the higher resolving power of the spectrophotometer used in this study.

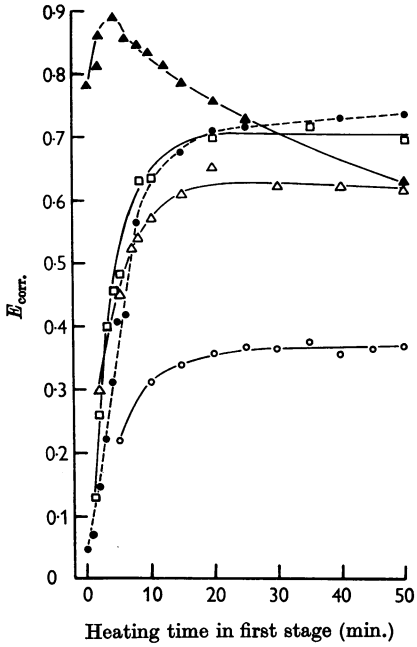

Fig. 5. Corrected extinctions ($E_{corr.}$) of the colour produced from 10 μg. amounts of oestradiol-17β, oestradiol-17α, oestrone, oestriol and 16-*epi*oestriol as influenced by different heating times in the first stage of the Kober reaction. Sulphuric acid concentrations in the first stage and heating times in the second stage were optimum. Final volumes of the reaction mixtures were as given in Fig. 4. Sulphuric acid concentrations (%) in the second stage were (optimum concentrations in parentheses): oestradiol-17β, 46·4 (49·8); oestrone, 40·5 (43·4); oestriol, 49·4 (52·2). Final acid concentrations of oestradiol-17α and 16-*epi*oestriol were optimum (see Fig. 1). For the remaining experimental details and symbols see Fig. 1.

Influence of heating time in the first stage of the reaction

Heating times of the first stage were varied stepwise between 0 and 50 min. with 10 μg. amounts of the steroids and colour reagents containing the optimum concentration of sulphuric acid. The first-stage fluorescence colour was converted into pink under optimum conditions in the second stage. The final volumes of the reaction mixtures were the same as those in the previous section. As shown in Fig. 5, similar effects of heating time were found with oestradiol-17β, oestrone, oestriol and 16-*epi*oestriol: the colour intensity increased for the first 20 min. of heating and was practically stable on further heating. The rate of increase was greatest with oestrone and lowest with oestriol. Oestradiol-17α behaves very differently from the others. Without any heating in the first stage, it produced about 90% of the Kober colour that is obtainable under optimum conditions; the yellow fluorescent complex is visible after standing for a few minutes with the reagent at room temperature. The intensity of the pink colour is greatest after heating for 4 min. in the first stage but is less with continued heating.

Stability of colours

Coloured mixtures obtained under optimum conditions from the crystalline oestrogens and their methyl ethers and from urinary fractions obtained according to Brown (1955) were kept in the dark at room temperature. The extinctions (read after 6, 12 and 24 hr.) showed a slight tendency to decrease, but $E_{corr.}$ values were stable to within ± 3%. Crystalline oestradiol-17α (5 μg.) gave a pink colour stable to within ± 1% after standing for 10 days.

Influence of ultraviolet light on the conversion of the first-stage yellow colour into pink

In Fig. 6 the wavelength/absorption curves of the colours obtained from 5 μg. amounts of oestradiol-17β, oestrone and oestriol after the first stage of the Kober reaction are shown. These indicate that transformation of the initially formed yellow colour into pink is already started during heating in the first stage, and the second stage of the reaction deals only with the completion of the conversion of residual yellow colour. The spectral characteristics of the first-stage colour given in Fig. 6 indicate that the proportion of residual yellow is greatest in the colour mixture produced from oestriol, whereas the oestradiol-17β mixture contains only a small amount of yellow. The

colour mixture formed by oestrone is composed of about equal parts of yellow and pink.

In the experiments described in Table 2 the coloured complexes formed in the first stage of the Kober reaction were subjected to ultraviolet irradiation which was found to induce the conversion of the yellow into the pink colours. The colour of oestriol was the most influenced by irradiation, as shown by an increase of the $E_{corr.}$ value by 79%; oestrone is less affected, the increase being 36%. The oestradiol-17β colour mixture had very little of the residual yellow colour and showed only a 6% increase in the pink colour.

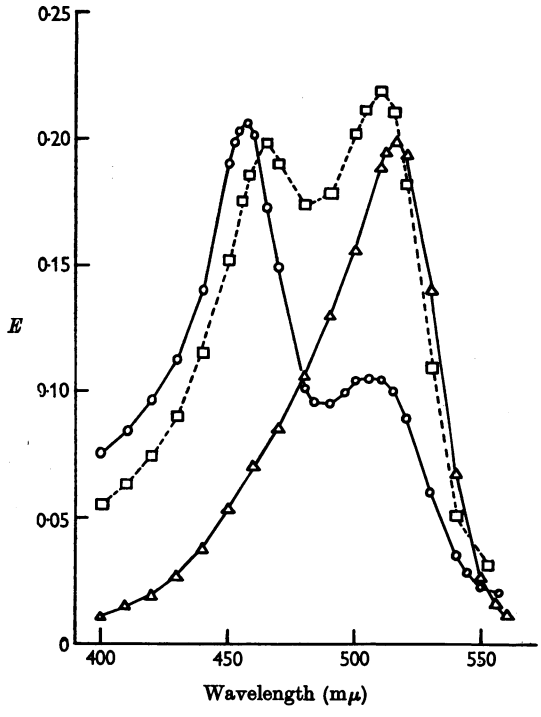

Fig. 6. Spectral characteristics of the colour complex produced from 5 μg. amounts of oestradiol-17β, oestrone and oestriol in the first stage of the Kober reaction. For experimental details of the colour reaction see Table 3. Readings were taken immediately after dilution of the reaction mixtures with water without heating in the second stage. Symbols are as identified in Fig. 1.

Properties of the reaction under optimum conditions

The present study resulted in a modified Kober reaction, the details of which are summarized in Table 3 for each of the five phenolic steroids investigated. As shown in Fig. 7, the corrected extinctions obey Beer's law closely in the range 2–10 μg. The tolerances which may be allowed in reaction conditions vary somewhat between the individual steroids and are to be deduced from Fig. 1 and Figs. 4 and 5.

This method has been in constant use in this laboratory for a period of more than 2 years and has always given reproducible results in the hands of five different workers. It has been applied to the routine clinical determination of urinary oestrogens by Brown's (1955) method (cf. Breuer, Nocke & Bayer, 1958) as well as to the estimation of several oestrogen metabolites in extracts from incubations of a variety of human and animal tissues *in vitro* (cf. Breuer & Nocke, 1958; for more detailed references see Breuer, 1959, 1960*b*). The colour reaction for 16-*epi*oestriol has been incorporated into a method for measuring 16-*epi*oestriol as both the methyl ether and the acetone derivative (Nocke, 1961). Fifteen different batches of Merck sulphuric acid have been used during this time. The corrected extinctions per unit weight of crystalline steroids obtained with the same batch of colour reagent remained constant for several months provided that the precautions outlined above were observed. The wavelength of maximal absorption of the colour produced by the individual compounds was constant with different batches of colour reagents, whereas corrected extinction coefficients varied slightly from batch to batch. Therefore the preparation of new reagents necessitates the construction of new calibration curves, but no modification of the colour-correction formulae.

Table 2. *Production of pink colour from oestradiol-17β, oestrone and oestriol as influenced by ultraviolet irradiation after the first stage of the Kober reaction*

Kober reactions were performed in duplicates with 5 μg. amounts of free oestrogens. Water was added as described in Table 3. The second heating was replaced (*a*) by allowing the mixtures to stand in the dark at room temperature for 30 min. and (*b*) by exposing them in 10 mm. quartz-glass cells to the H_2 lamp of a Beckman DU spectrophotometer for 30 min. from a distance of 25 cm. Readings were taken against similarly treated reagent blanks at the wavelength specified in Table 3. $E_{corr.} = 2E_2 - (E_1 + E_3)$.

	(*a*) Control expt.				(*b*) U.v.-irradiation expt.				Percentage increase of $E_{corr.}$ after irradiation
Steroid	E_1	E_2	E_3	$E_{corr.}$	E_1	E_2	E_3	$E_{corr.}$	
Oestradiol-17β	0·080	0·185	0·007	0·283	0·080	0·194	0·010	0·298	6
Oestrone	0·142	0·208	0·018	0·256	0·149	0·257	0·018	0·347	36
Oestriol	0·115	0·129	0·018	0·125	0·127	0·186	0·021	0·224	79

Table 3. *Optimum reaction conditions, results and intensities of the colour production from oestradiol-17β, oestradiol-17α, oestrone, oestriol and 16-epioestriol in the modified Kober reaction*

For preparation of colour reagents, colour reaction and measurement of extinctions see the Materials and Methods section. $E_{corr.} = 2E_2 - (E_1 + E_3)$.

Standard deviations were calculated according to: S.D. $= \sqrt{\frac{\Sigma(\bar{x}-x)^2}{N-1}}$. Number of estimations are given in parentheses.

Steroid	Kober reaction: First stage: Kober reagent (ml.)	First stage: Concn. of H_2SO_4 (%, v/v)	First stage: Heating time (min.)	Second stage: Water added (ml.)	Second stage: Final Concn. of H_2SO_4 (%, v/v)	Second stage: Heating time (min.)	Wavelengths (mμ): E_1	Wavelengths (mμ): E_2 ($\lambda_{max.}$)	Wavelengths (mμ): E_3	$E_{corr.}$/10 μg. ± S.D.	$E^{1\%}_{1cm.}$
Oestradiol-17β	2·8	55	20	0·4	48·1	10	480	518	556	0·677 ± 0·007 (15)	1520
Oestradiol-17α	2·2	66	4	1·0	45·4	6	486	521	556	0·838 ± 0·011 (15)	1790
Oestrone	2·1	66	20	1·1	43·4	6	474	515	556	0·872 ± 0·010 (12)	1840
Oestriol	2·1	76	20	1·1	49·8	14	472	514	556	0·563 ± 0·010 (14)	1200
16-*epi*Oestriol	2·4	66	20	0·8	49·5	5	472	514	556	0·735 ± 0·013 (17)	1550

When the method of Brown (1955) is used, the optimum reaction conditions as developed for free oestrogens may be adopted for the colorimetric measurement of the methylated oestrogens, but the absorption maxima of oestrogen methyl ethers occur at longer wavelengths (cf. Table 4) and this must be allowed for in the Allen (1950) correction.

Reliability in the presence of urinary contaminants

The specificity of the Kober reaction has been well established by Kober (1931) and Marlow (1950).

As shown in Tables 3 and 4 for 10 μg. amounts of the free compounds or the methyl ethers, the standard deviations within replicate estimates were between 0·6 and 1·8% (mean 1·3%). With 5 μg. amounts (cf. Table 5) the mean standard deviation is 1·7%, with a range 1·1–2·2%.

Urinary samples from oophorectomized patients with mammary cancer were processed according to the method of Brown (1955) including the modification of Brown, Bulbrook & Greenwood (1957).

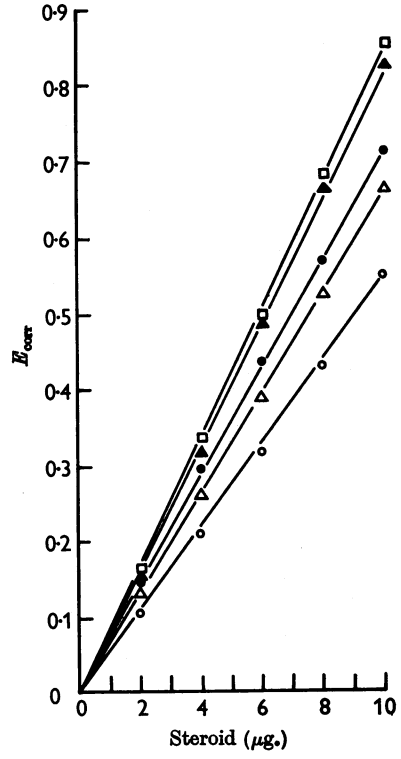

Fig. 7. Calibration curves for oestradiol-17β, oestradiol-17α, oestrone, oestriol and 16-*epi*oestriol in the modified Kober reaction. All reaction conditions were optimum. Values given are founded on duplicate estimations. Final volume of all reaction mixtures was 3·2 ml. For symbols and calculation of corrected extinctions ($E_{corr.}$) see Fig. 1.

Table 4. *Results and intensities of the colour production from the methyl ethers of oestradiol-17β, oestrone, oestriol and* 16-epi*oestriol in the modified Kober reaction*

Reaction conditions for the methyl ethers are those which are optimum for the corresponding free oestrogens as shown in Table 3.

Methyl ether	Wavelengths (mμ) E_1	E_2	E_3	$E_{corr.}/10\,\mu g. \pm$ S.D.	$E^{1\%}_{1cm.}$
3-Methoxyoestradiol-17β	488	522	556	0·584±0·003 (9)	1390
3-Methoxyoestrone	482	519	556	0·729±0·012 (11)	1660
3-Methoxyoestriol	478	517	556	0·540±0·006 (9)	1250
3-Methoxy-16-*epi*oestriol	478	517	556	0·680±0·009 (9)	1530

Table 5. *Precision of estimation and recovery in the presence of urinary fractions*

Kober reactions were performed under optimum conditions. For experimental details see Tables 3 and 4. Number of estimations are given in parentheses.

Steroid	Crystalline compounds $E_{corr.}/5\,\mu g. \pm$ S.D. (A)	Crystalline compounds added to urinary fractions* $E_{corr.}/5\,\mu g. \pm$ S.D.† (B)	Percentage recovery $\left(\frac{B_n}{A} \times 100 \pm \text{S.D.}\right)$
Oestradiol-17β	0·339±0·004 (15)	0·312±0·010 (14)	92·4±3·0
Oestrone	0·436±0·005 (12)	0·410±0·016 (7)	94·0±3·6
Oestriol	0·282±0·005 (14)	0·266±0·008 (15)	94·6±3·0
3-Methoxyoestradiol-17β	0·284±0·006 (7)	0·263±0·007 (7)	92·8±2·3
3-Methoxyoestrone	0·383±0·006 (5)	0·372±0·009 (9)	97·1±2·3
3-Methoxyoestriol	0·267±0·006 (7)	0·255±0·008 (7)	95·6±3·1

* Urines from oophorectomized patients with mammary cancer were processed according to Brown (1955), including the modification of Brown *et al.* (1957). Steriods were added to the appropriate column fractions before evaporation of the solvents. Different urines of two patients were used for the free oestrogens. Portions of pooled urine samples from one patient were used for the methyl ethers.

† Data are corrected for endogenous blank values.

Amounts (5 μg.) of oestradiol-17β, oestrone and oestriol and their methyl ethers were added to the resulting column fractions before evaporation of solvents. The results of the colour reactions carried out on these fractions in comparison with those of pure crystalline standards obtained with the same batch of Kober reagent are presented in Table 5. The precision of the method when applied to urinary fractions with the 5 μg. amount is indicated by a mean standard deviation of ±3·1 % (range ±2·4–3·9 %) from the mean $E_{corr.}$ value, thus being somewhat less favourable than for pure crystalline standards. The percentage recoveries of the added steroids were between 92·4 and 97·1 %. Borth (1957) has defined the sensitivity of a method as the smallest amount which can be distinguished from zero with 95 % significance by a single determination. The standard deviations shown in Table 5 indicate that the sensitivity of the method at the 5 μg. amount is about 0·2 μg. for the pure compounds and about 0·3 μg. in the presence of the urinary fractions.

DISCUSSION

The optimum reaction conditions for oestradiol-17β, oestrone and oestriol in the first stage of the Kober reaction found in this study were, with a minor exception, exactly those described by Brown (1955). The important point for complete conversion of yellow into pink, however, is the final sulphuric acid concentration in the second stage. Concentrations considerably lower than those reported by Brown (1955) were found to be optimum in this investigation, the differences being 8·1 % for oestradiol-17β, 13·3 % for oestrone and 7·2 % for oestriol. Data have been published indicating that conversion of yellow into pink is accelerated by lowering the second-stage acid concentration (Brown, 1952*a*, *b*; Bauld, 1954) and by exposing the reaction mixtures to sunlight, and that when both these conditions are in excess a tendency to fading of the pink occurs (Bauld, 1954). It was proved in this study that decreasing the second-stage acid concentration favours the conversion of yellow into pink and that a concentration beyond the optimum causes fading of the final pink colour. Furthermore, it was shown that colour transformation is aided to a marked extent by ultraviolet light. The percentage increase of the colour intensity (cf. Table 2) fits well with the spectral characteristics of the colour mixtures formed by the three steroids in the first stage of the reaction. Apparently, acid concentrations which are optimum when the second stage of the reaction is performed under artificial light may cause

fading of the pink colour when the reaction is performed in direct sunlight. Therefore the colour reaction should be performed under standardized illumination. The final Kober colour, once formed under optimum conditions and protected from bright light, is stable even in urinary fractions for at least 24 hr.

The conversion of yellow into pink is assumed to be an oxidation step (Brown, 1952*b*; Bauld, 1954). Failure to reproduce colour formation with different types of sulphuric acid led Bauld (1954) to add trace amounts of oxidizing agents (sodium nitrate, 1 mg./100 ml., and quinone, 2 mg./100 ml.) to the colour reagents. From this point of view it is of interest to compare the limits of trace impurities as given by the manufacturers of the two types of sulphuric acid in question here (cf. Table 6). It would appear from the Table that there are considerable differences in the purity of the two acids. The most striking difference is in the figure for nitrate, which is 100 times as high in Merck acid, i.e. more than twice the amount added by Bauld (1954) to his 'modified reagent'. This probably explains the fact that the colour intensities were not improved by adding oxidizing agents, as recommended by Bauld (1954), to Kober reagents prepared with Merck acid. Under these conditions the greatly enhanced oxidation power, while causing a rapid colour conversion, also caused a rapid fading of the pink colour as reflected by an $E_{corr.}$ value of less than 0·100 obtained from 10 μg. of oestradiol-17β.

Diczfalusy *et al.* (1958) have presented evidence suggesting that certain methylated urinary oestriol fractions obtained according to Brown *et al.* (1957) contain contaminants which diminish the final colour of added crystalline oestriol methyl ether considerably. In this study the behaviour of both the free compounds and their methyl ethers in the presence of urinary fractions was investigated with the modified Kober reaction. It was shown that there are in fact small losses of Kober chromogenicity although in no case were these of the magnitude reported by Diczfalusy *et al.* (1958). Losses are reproducible in a very narrow range and do not affect the reliability of the Kober reaction when used in connexion with the Brown method. However, the Kober reaction when combined with insufficient purification procedures yields unreliable results.

Table 6. *Limits of trace impurities in two different types of analytical reagent-grade sulphuric acids*

Contaminant	Supplier: British Drug Houses Ltd. ($10^6 \times$ Concn. of impurity (%))	Supplier: Merck ($10^6 \times$ Concn. of impurity (%))
Non-volatile matter	2500	500
Chloride (Cl)	200	60
Nitrate (NO_3)	20	2000
Selenium (Se)	1000	300
Heavy metals (as Pb)	200	500
Lead (Pb)	Not stated	250
Iron (Fe)	100	100
Ammonium (NH_4)	500	300
Arsenic (As)	10	3
Oxygen absorbed (O)	150	200

Marlow (1950) has compared the Kober chromogenicity of several phenolic steroids and reported $E_{\lambda max.}$ values for oestriol and 16-*epi*oestriol representing 42 and 45% of the corresponding values found in this study. Although working under non-optimum conditions he found that 16-*epi*oestriol was more chromogenic than oestriol by about 39%. The corresponding difference in the present data is 27%. Marrian & Bauld (1955), using the oestriol reagent (76% sulphuric acid) of Bauld (1954), have found that 16-*epi*oestriol is about 13% more chromogenic than oestriol. Furthermore, it was shown by these authors that with the oestriol reagent maximum colour was developed by 16-*epi*oestriol after a much shorter heating time in the first stage than by oestriol. The present data confirm that the pink colour is developed more readily by 16-*epi*oestriol than by oestriol, as indicated by the optimum acid concentration of 66% instead of 76% in the first stage (Fig. 4) and a more rapid development of colour in both the first (Fig. 5) and the second (Fig. 3) stages. These data fit well with the hypothesis of Brown (1952*b*) and Bauld (1954) that the first stage of the Kober reaction with oestriol involves a dehydration to oestrone which is more readily undergone by the *cis*-glycol configuration than by the *trans*-glycol configuration. Oestrone itself produces the most intense Kober colour of all phenolic steroids so far investigated. Further evidence is given (W. Nocke, unpublished work) by the behaviour of the other naturally occurring *cis*-glycolic compound, i.e. 17-*epi*oestriol (cf. Breuer, 1960*a*; Nocke & Breuer, 1960; Nocke, Breuer & Knuppen, 1961).

The behaviour shown by oestradiol-17α in the Kober reaction is strikingly different from that of its 17β-epimer or of the other oestrogens. It undergoes the first stage of the reaction at room temperature within a few minutes and it very readily gives an intense Kober colour, which is particularly susceptible to variation in the reaction conditions.

SUMMARY

1. The influence of various reaction conditions on the colour production by five naturally occurring oestrogens in the Kober reaction was studied.

2. Optimum conditions for the formation of the yellow colour from oestradiol-17β, oestrone and oestriol were almost identical with those of Brown (1955), whereas those for the conversion of yellow into pink differed considerably. The differences are most likely due to the different commercial types of sulphuric acid used in the two methods.

3. Transformation of yellow colour into the pink colour is aided by (*a*) decreasing the sulphuric acid concentration, (*b*) increasing the heating time, (*c*) the presence of oxidizing agents and (*d*) ultraviolet light. Excess of these influences results in fading of the final pink colour.

4. The pink-colour complex once formed under optimum conditions from both pure crystalline compounds and urinary fractions is stable for at least 24 hr. in the absence of bright light.

5. Urinary contaminants reduce the chromogenicity of phenolic steroids. The effects are small and reproducible in a very narrow range when the colour reaction is used in connexion with the Brown purification procedure.

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A New Chromatographic Method for the Determination of Thiamine and its Mono-, Di- and Tri-Phosphates in Animal Tissues

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Some methods hitherto used for the determination of thiamine and its phosphoric acid esters in animal tissues only allow a distinction between free and phosphorylated thiamine (by using thiochrome methods before and after enzymic digestion) (see Association of Vitamin Chemists, 1951), or measurement of cocarboxylase activity (Westenbrink & Steyn-Parvé, 1949; Kaziro, 1957), displayed by thiamine diphosphate alone.

Paper chromatography (Spadoni & Tecce, 1950; Rossi-Fanelli, Siliprandi & Fasella, 1952; Kiessling & Lindahl, 1953; Bartley, 1954; Bernabei & Wildemann, 1959) and paper ionophoresis (Gurtner, 1957) have been widely employed, but usually only