# Some Errors in the Colorimetric Estimation of Oestriol, Oestrone and Oestradiol by the Kober Reaction

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In the course of an investigation of methods suitable for the accurate determination of oestriol, oestrone and oestradiol-17$\beta$ in the urine of human subjects, it was found that the colour produced in the Kober reaction by oestrogens was depressed by 15–40% in the presence of solvent residues. The particular modification of the Kober (1931) reaction which was principally used in this investigation was one using quinol and aqueous sulphuric acid (Brown, 1952), but the same effect was found with the original phenol reagent of Kober under the conditions described by Venning, Evelyn, Harkness & Browne (1937). The inhibition of colour production may readily be demonstrated by the addition to a pure oestrogen of the residue from distillation of ether which has been used to extract dilute mineral acid and washed with bicarbonate and water (as is required in the initial extraction of oestrogens from hydrolysed urine). The effect persisted even when the ether was purified by a variety of methods. The magnitude of error involved was sufficient to necessitate a thorough re-investigation of the colour reaction described by Brown (1952).

This reaction, like all modifications of the Kober method, is a two-stage process, in which the initial formation of a yellow compound with a green fluorescence is followed by its conversion into a pink, non-fluorescent complex on reheating in more dilute sulphuric acid. This pink colour fades if heated in still more dilute solutions. There are thus three possible forms of interference with the colour produced, namely: (1) failure to form the initial yellow complex (inhibition type I); (2) failure to convert the yellow complex fully into the pink (inhibition type II); (3) instability of the pink complex (fading).

## METHODS

*Purification of materials.* Ethanol (absolute) was purified by refluxing for 12 hr. with 5% (w/v) Zn dust and 5% (w/v) NaOH pellets, distilling, standing over *m*-phenylenediamine for at least 7 days and then distilling twice. The ether (A.R. grade) was purified as follows: 2 l. were shaken for 6 min. with 16 g. of $AgNO_3$ in 120 ml. of water and 200 ml. of N-NaOH (Werner, 1933), then washed with water and distilled.

The $NaHCO_3$ used was A.R. Two types of A.R. $H_2SO_4$ were used: type *A* was manufactured by British Drug Houses Ltd., type *B* by Hopkin & Williams Ltd.

*Apparatus.* Pyrex test tubes (18 × 150 mm.) were used initially for the colour reaction. The later experiments were done in Quickfit & Quartz test tubes (23 × 150 mm.) with *B*. 19 ground-glass sockets fitted with cones to which was attached glass tubing (5 mm. diam.) bent at an acute angle. These 'Kober tubes' were introduced to minimize changes in the volume of solution from condensation or evaporation of water during the heating stage.

Glassware was cleaned with chromic acid, rinsed with water and ethanol, washed with a vigorous stream of water for 2–3 min., and finally rinsed with distilled water.

In the preliminary experiments, optical densities were measured by a Spekker absorptiometer in 1 cm. cuvettes with Ilford Spectrum Green, Violet and Yellow (604, 601, 606) filters (transmission maxima at 520, 430 and 580 m$\mu$., respectively).

Absorption spectra and the colours of solutions obtained by the method finally chosen were measured in a Unicam diffraction-grating spectrophotometer (SP 600).

*Preparation of oestrogen solutions.* Standard solutions of pure crystalline oestrogens were prepared in ethanol (5 mg./100 ml.), the appropriate volumes were added to the reaction tubes and the solvent was removed by heating at 90–95° in a current of air. With aldehyde-free ethanol, no loss could be demonstrated by this procedure. The solutions were stored at 4°.

In order to demonstrate the effect of solvent residues, pure oestrogens were extracted with ether (1 × 150 ml., 3 × 125 ml.) from a mixture of 500 ml. of distilled water and 75 ml. of 30% (v/v) type *B* $H_2SO_4$. The combined ether extracts were washed with 8·5% (w/v) $NaHCO_3$ (3 × 35 ml.) and water (2 × 25 ml.). This corresponds to the initial stage of the method for the estimation of oestrogens in urine. The residues obtained after distillation of the ether were transferred with aldehyde-free ethanol to the tubes for the colour reaction. The solvent was removed either by heating in a stream of air or under reduced pressure in a stream of $N_2$, no significant difference between the two methods being demonstrable.

Oestrogen residues prepared by these methods are referred to as 'standard' and 'extract', respectively, throughout this paper.

*Colour reagents and reaction.* These were based on the conditions described by Brown (1952). The dilutions of $H_2SO_4$ employed for preparation of Kober reagents were 76, 66 and 60% (v/v) for oestriol, oestrone and oestradiol-17$\beta$, respectively. The term 'heating at 100°' means heating in a boiling-water bath.

*Colour-correction formulae.* Since solvent residues contribute a non-specific background colour in the reactions, it was necessary to correct for this enhancing effect. For measurements in the absorptiometer, an equation, devised by Dr J. B. Brown (to be published), was used.

$$D_{corr.} = (4D_{604} - 3D_{606} - D_{601})/3{\cdot}8,$$

where $D_{corr.}$ is the optical density corrected for the non-pink components, and $D_{604}$, $D_{606}$, $D_{601}$ are the observed optical densities with the Ilford filters indicated by the subscripts. For corrections of measurements in the spectrophotometer, as used in the final method, the equation of Allen (1950) was applied.

## RESULTS

### *Type I inhibition. Failure to form initial yellow complex*

Typical examples of the incomplete recoveries obtained by extracting oestriol (25 $\mu$g.) from dilute mineral acid with ether are shown in Table 1*A*. The oestrogen content was determined by heating the extraction residue at 100° for 20 min. with 4 ml. of 2% quinol in 76% (v/v) sulphuric acid, diluting with 1 ml. of water, reheating for 5 min., and diluting to 15 ml. with a solution comprising 70 ml. water and 30 ml. $H_2SO_4$ (Brown, 1952). The increase in non-specific background colour and the depression of pink are evident. Addition of certain reducing agents to the reagent immediately before use improved the recovery values. The results obtained with *m*-cresol are shown in Table 1*B*.

*Sulphonation of the reagent as cause of type I inhibition.* These findings suggested that the reagent had insufficient reducing power in the presence of oxidants in the solvent residues. Pinnow's (1915, 1917) work suggests that quinol would be almost quantitatively sulphonated, and thus its reducing power diminished, during the preparation and ageing of the reagent. Pinnow's experiments were repeated in part, and it was found that a reagent prepared by dissolving 2 g. of quinol in 100 ml. of 76% (v/v) sulphuric acid at room temperature contained more than 90% of the phenol unchanged at 24 hr. but less than 2% after 3 weeks. Moreover, solution of the quinol by heating to 35–40° and 70–80° caused sulphonation to the extent of 50 and 97%, respectively. It seemed that the yields of oestriol (determined colorimetrically) obtained by extraction from aqueous mineral acid with ether might vary with the method of preparation and age of the reagent. The results of experiments demonstrating this are shown in Table 2. No *m*-cresol was added, and the reaction was carried out as described by Brown (1952).

An attempt was made by substitution of duroquinol (2:3:5:6-tetramethylquinol) for quinol to obtain a reagent which would be stable on ageing, but the duroquinol was found to be too strong a reducing agent since oxidation to intensely coloured products occurred merely on heating in 76% (v/v) sulphuric acid in absence of oestrogen.

In the absence of solvent residues, the reducing power of the sulphonated quinol appears to be adequate for satisfactory colour development. However, for the estimation of oestriol in the presence of oxidizing contaminants such as may arise from urine or solvent residues, the reagent must contain free quinol. This requirement is not met in reagents prepared and used as suggested by Brown (1952).

*Comment.* Chamot & Pratt (1909, 1910) found irregular results in the colorimetric determination of nitrates owing to changes in the composition of their phenol:sulphuric acid reagent. Clayton (1949) noted that the best results in the determination of oestriol in the presence of solvent residues were obtained with a reagent prepared as suggested by Venning *et al.* (1937) if used within 48 hr. Marlow (1950) also used a freshly prepared reagent.

Table 1. *Effect of ether residues on the development of colour by oestriol* (25 $\mu$g.) *in the quinol:sulphuric-acid reaction*

(In this and subsequent tables recovery values are 100 $D_{corr.}$ for extract/$D_{corr.}$ for standard.)

| Expt. | Oestriol used | $D_{604}$ | $D_{601}$ | $D_{606}$ | Recovery (%) |
|---|---|---|---|---|---|
| | | *A.* No *m*-cresol added | | | |
| 1 | Standard | 0·160 | 0·047 | 0·002 | — |
| | Extract | 0·118 | 0·058 | 0·018 | 61 |
| 2 | Standard | 0·168 | 0·028 | 0·004 | — |
| | Extract | 0·125 | 0·059 | 0·025 | 57 |
| 3 | Standard | 0·177 | 0·032 | 0·004 | — |
| | Extract | 0·121 | 0·055 | 0·018 | 57 |
| | *B.* *m*-Cresol (0·1 ml.) added to each sample at start of colour reaction | | | | |
| 1 | Standard | 0·150 | 0·033 | 0·0 | — |
| | Extract | 0·168 | 0·060 | 0·025 | 94 |
| 2 | Standard | 0·165 | 0·022 | 0·0 | — |
| | Extract | 0·192 | 0·081 | 0·038 | 90 |
| 3 | Standard | 0·165 | 0·028 | 0·003 | — |
| | Extract | 0·176 | 0·081 | 0·030 | 87 |

Table 2. *Effect of age and method of preparation of reagent on colour production in the presence of ether residues by oestriol (25 μg.)*

(Reagent 1: quinol (2 g.) dissolved at room temperature in 100 ml. of 76 % (v/v) $H_2SO_4$ and used within 12 hr. Reagent 2: as for reagent 1; but heated to 40–50° during solution of quinol. Reagent 3: as for reagent 1, but 3 weeks old.)

| Type of reagent | Oestriol used | $D_{604}$ | $D_{601}$ | $D_{606}$ | Recovery (%) |
|---|---|---|---|---|---|
| 1 | Standard | 0·180 | 0·032 | 0·004 | — |
| | Standard | 0·186 | 0·033 | 0·005 | — |
| | Extract | 0·179 | 0·053 | 0·018 | 89 |
| | Extract | 0·173 | 0·061 | 0·019 | 84 |
| 1 | Standard | 0·179 | 0·031 | 0·004 | — |
| | Standard | 0·175 | 0·025 | 0·0 | — |
| | Extract | 0·165 | 0·052 | 0·013 | 85 |
| | Extract | 0·174 | 0·068 | 0·019 | 85 |
| 2 | Standard | 0·179 | 0·035 | 0·0 | — |
| | Extract | 0·128 | 0·040 | 0·009 | 65 |
| | Extract | 0·143 | 0·047 | 0·006 | 74 |
| 3 | Standard | 0·192 | 0·048 | 0·0 | — |
| | Standard | 0·190 | 0·048 | 0·0 | — |
| | Extract | 0·140 | 0·078 | 0·029 | 55 |
| | Extract | 0·150 | 0·089 | 0·031 | 58 |
| | Extract | 0·157 | 0·088 | 0·032 | 60 |
| | Extract | 0·151 | 0·088 | 0·032 | 59 |

Ageing of the reagent did not interfere with colour production by oestrone or oestradiol-17β in the presence of solvent residues. This was to be expected for oestrone, since Brown (1952) showed that this oestrogen does not require a reducing agent in the first stage of the Kober reaction. Consideration of the findings of previous workers suggests a possible explanation for the anomalous behaviour of oestriol.

It appears that oestriol when heated in sulphuric acid can undergo at least two different reactions. The first is apparently an oxidation and is not undergone by oestrone (Marrian, 1938). The second, the Kober reaction, requires a reducing agent and is undergone much less readily than by oestrone (Cartland, Meyer, Miller & Rutz, 1935; Bachman, 1939; Brown, 1952). The fact that oestriol requires longer heating and higher acid concentration led Szego & Samuels (1940) to suggest that in the first stage of the Kober reaction, oestriol is dehydrated to oestrone, a possibility which is supported by the similarity of the absorption spectra of the two coloured products (cf. Fig. 5). The fact that 16-epioestriol (oestra-1:3:5(10)-triene-3:16β:17β-triol) gives a more intense colour under these conditions than does oestriol (Marlow, 1950) is also suggestive.

It is possible that in the Kober reaction for oestriol, a reducing agent is required to prevent the alternative oxidative reaction, thus permitting quantitative dehydration to oestrone. Under these circumstances it would be expected that oxidants arising from solvent residues might tend to prevent full formation of the yellow precursor of the Kober pink (type I inhibition), and that the addition of an effective reducing agent would exert a stabilizing action.

*Type II inhibition (failure to convert yellow into pink colour) and fading*

In agreement with Brown's (1952) results, it was found that concentrations of 76, 66 and 60 % (v/v) of sulphuric acid were the most satisfactory ones for the first stage of the reaction with oestriol, oestrone and oestradiol-17β, respectively. That a concentration of 60 % was the best for the second stage could not, however, be demonstrated (see Fig. 1). Samples of oestriol (25 μg.) were heated for 20 min. at 100° with 4 ml. of 2 % quinol in 76 % (v/v) sulphuric acid. The cooled solutions were diluted with either 1 ml. or 2 ml. of water to give second-stage concentrations of approximately 60 % or 50 %, respectively, mixed, reheated for 0–10 min., and diluted to a final concentration of 40 % (15 ml.). Optical densities were measured with the Ilford 604 and 601 filters after 30 min. and again after 90 min.

Fig. 1 shows that dilution with 1 ml. of water and 5 min. reheating were insufficient to cause full conversion of yellow into pink (low $D_{604}$ value), prolonged standing at room temperature being required. Dilution with 2 ml. of water to give a second-stage concentration of approximately 50 % permitted complete development of colour after reheating for 5 min., but the pink tended to fade on dilution of the solution.

It was observed that the development of colour was aided by sunlight and it was considered that

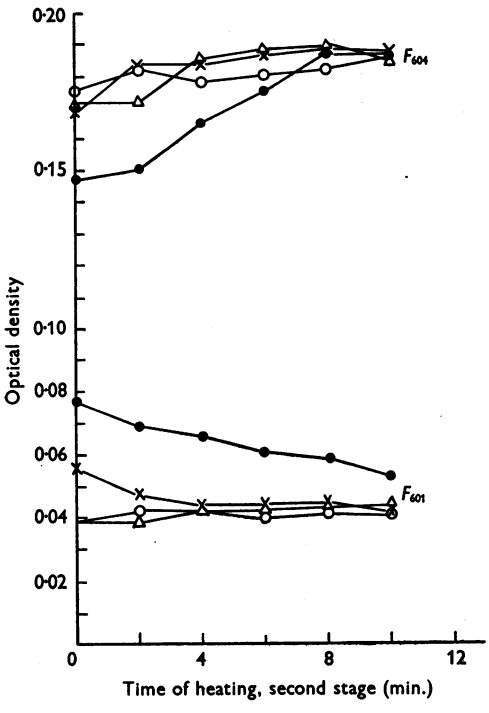


Fig. 1. Effect of variation of $H_2SO_4$ concentration of the second stage, and of standing at room temperature after final dilution to 40%, on intensity of colour produced by oestriol (25 $\mu$g.). Second-stage concentration of 60% (diluent, 1 ml. of water): ●—●, after 30 min. standing; △—△, after 90 min. standing. Second-stage concentration of 50% (diluent, 2 ml. of water): ×—×, after 30 min. standing; ○—○, after 90 min. standing.

In this and subsequent figures, the symbols $F_{604}$ and $F_{601}$ denote the Ilford filters used in the measurement of optical density.

Table 3. *Effect of variation of* $H_2SO_4$ *concentration of second stage and of illumination on stability of final colour intensity obtained with oestriol* (25 $\mu$g.)

| Time of reading (min.) | 2nd stage concn. 60% | | 2nd stage concn. 50% | |
|---|---|---|---|---|
| | $D_{604}$ | $D_{601}$ | $D_{604}$ | $D_{601}$ |
| *A.* Solutions exposed to sunlight, in a brightly lit laboratory during the development of the colour, at all stages except during heating | | | | |
| 5 | 0·170 | 0·044 | 0·151 | 0·048 |
| 15 | 0·160 | 0·042 | 0·148 | 0·036 |
| 90 | 0·153 | 0·043 | 0·140 | 0·025 |
| 120 | 0·153 | 0·041 | 0·132 | 0·030 |
| *B.* Reactions carried out at night in dimly lit laboratory | | | | |
| 5 | 0·178 | 0·079 | 0·198 | 0·059 |
| 15 | 0·189 | 0·072 | 0·200 | 0·053 |
| 90 | 0·194 | 0·065 | 0·199 | 0·049 |
| 120 | 0·198 | 0·057 | 0·199 | 0·048 |

differences in illumination in the two laboratories might account for the discrepancy between the results reported here and those of Brown (1952). The results of an experiment demonstrating this effect are shown in Table 3. The colour reaction was carried out as in the experiment shown in Fig. 1, except that the period of reheating in all cases was 5 min. The data are typical of a large series of experiments conducted with oestriol, oestrone and oestradiol-17$\beta$ with reagents both freshly prepared and aged. It will be seen that either a decrease in the second-stage concentration or an increase in illumination was required for full conversion of yellow into pink, as shown by low $D_{601}$ values. When both these conditions were present, there was a marked tendency to a fading of the pink.

*Comment.* The conversion of yellow into pink in the second stage of the Kober reaction is apparently an oxidation, since it is aided by ferric ions (Haenni, 1950), hydrogen peroxide (Brown, 1952) and cupric ions (present investigation). If the oxidizing power of the system is too small, there will be a tendency for incomplete conversion of yellow into pink in the second stage, rendering the reaction susceptible to interference by contaminants arising from the extraction procedure. Reactions such as those described by Bachman (1939) and Brown (1952), in which the completion of the colour reaction occurs at room temperature after final dilution, may be unstable because of this effect. Brown (1953) found that the time required for complete formation of the pink compound was altered by urinary residues.

On the other hand, it has long been known that the pink may be converted into a colourless compound by hydrogen peroxide (Kober, 1931). Thus, when conditions are such as to facilitate the conversion of yellow into pink, the reaction will be susceptible to fading. Cohen & Marrian (1934) found this to occur in the presence of urinary residues and corrected it by increasing the sulphuric-acid concentration of the second stage. The effect of sunlight in modifying the conditions of the reaction might have been expected from the known photosensitivity of the sulphuric-acid colour reaction for cholesterol (cf. Kenny, 1952) and in this connexion it is perhaps significant that Szego & Samuels (1943) kept the solutions in the dark at one stage of their modified Kober reaction.

### *Elimination of interference with colour production in the Kober reaction*

*Elimination of type I inhibition.* In order to avoid the inconvenience of using freshly prepared reagent, quinol was added at the start of the colour reaction.

*Elimination of type II inhibition.* Brown (1952) showed that the pink colour obtained with pure oestrogen was stable at 100° for 2 hr. at a concentration of 60% (v/v) sulphuric acid. Accordingly, it

was decided to eliminate the final dilution, thereby minimizing the possibility of fading and doubling the sensitivity of the reaction. The solutions were protected from bright light at all stages. The optimum sulphuric acid concentration for the second stage was reinvestigated.

The appropriate quinol:sulphuric acid reagent (4 ml.) was heated at 100° with eleven samples of 25 $\mu$g. of oestrogen in Kober tubes for 20 min. The solutions were cooled, diluted with 3·5 ml. of sulphuric acid of various concentrations, and reheated, samples being withdrawn at 2 min. intervals from 0 to 20 min. In all cases the final colour was stable at room temperature for at least 12 hr. The results obtained with oestrone and its reagent (2 % quinol in 66 % (v/v) sulphuric acid) are shown in Fig. 2.

With a second-stage concentration of 60 %, prolonged heating was required to effect full conversion of yellow into pink, whereas with 40 %, prolonged heating caused fading. Similar results were obtained with oestriol and oestradiol-17$\beta$. In all cases the best compromise between the two opposing tendencies to type II inhibition and fading was obtained with second-stage concentrations of 50–55 %. When the colour reaction was carried out in test tubes (18 × 150 mm.), it was found that continual stirring during the reheating increased the pink, but did not alter the relationship between time of heating and sulphuric acid concentration. The use of the larger bore Kober tubes gave optimum $D_{604}$ values when the usual procedure of shaking the tube 5–10 times before reheating was adhered to.

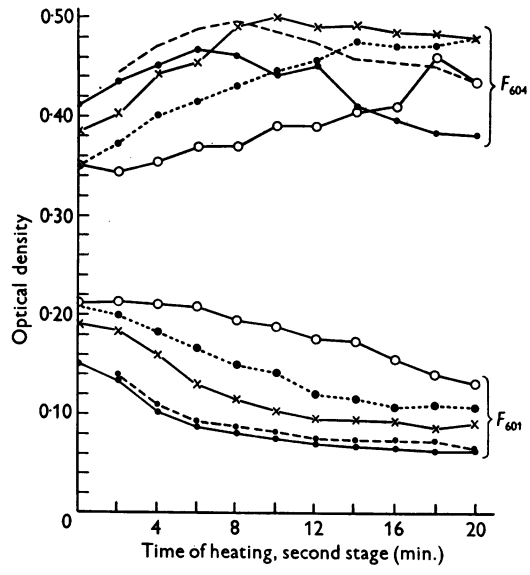


Fig. 2. Effect of variation of $H_2SO_4$ concentration of the second stage (oestrone 25 $\mu$g.).

| | Diluent, $H_2SO_4$ (%, v/v) | Second-stage concn. (%) |
|---|---|---|
| ●—● | 10 | 40 |
| ●– – –● | 20 | 45 |
| ×—× | 30 | 49 |
| ●. . .● | 40 | 54 |
| ○—○ | 53 | 60 |

### *Variation of colour reaction with type of sulphuric acid*

The experiments described above were conducted with reagents made from type *B* sulphuric acid. There are some indications in the literature that the make of sulphuric acid used profoundly affects the course of the Kober reaction (cf. Engel, Slaunwhite, Carter & Nathanson, 1950; Venning, 1952). Before the conditions of the present colorimetric method were standardized, two types of sulphuric acid were compared. The results of oestradiol-17$\beta$ with a second-stage concentration of 60 % are shown in Fig. 3. The marked difference in optical densities obtained with reagents made from types *A* and *B*, as well as the strikingly different rates of colour formation, is apparent. It was clear that these findings rendered the colour method unsatisfactory.

The effect of the addition of trace amounts of oxidizing agents to the diluting acid is also shown. The indication is that in neither reagent were conditions optimum; trace contaminants could cause falsely high results.

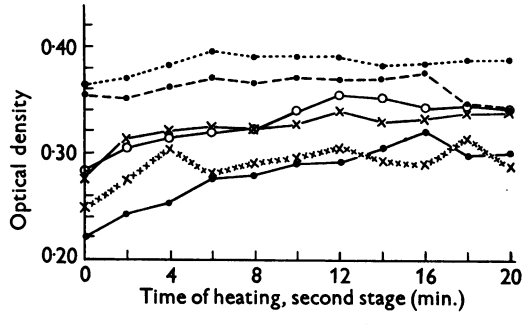


Fig. 3. Effect of type of $H_2SO_4$ used in preparation of reagent, and of oxidants added in the second stage, on the colour intensity produced by oestradiol-17$\beta$ (25 $\mu$g.).

| | $H_2SO_4$ type | Additions in diluent (mg./100 ml.) |
|---|---|---|
| ●—● | *B* | Nil |
| ×—× | *B* | $Cu^{2+}$ (8·0) |
| ○—○ | *B* | $NO_3^-$ (0·1) |
| × × × | *B* | $Fe^{3+}$ (3·5) |
| ●– –● | *A* | Nil |
| ●. . .● | *A* | $Cu^{2+}$ (8·0) |

*Variation of reagent with type of sulphuric acid*

The effects of the addition of trace amounts of an oxidant (hydrogen peroxide) are shown in Table 4. The reaction was carried out by heating 4 ml. of 2% (w/v) quinol in 60% (v/v) sulphuric acid (type *B*) with 50 $\mu$g. of oestradiol-17$\beta$ at 100° for 20 min., diluting with 3·5 ml. of 60% (v/v) sulphuric acid (type *B*) and reheating for 15 min. Greater intensities were produced when the oxidant was added in the first stage of the reaction and when the reagent was freshly prepared, suggesting that the oxidizing agents were acting indirectly by an oxidation of the quinol. With ageing of the reagent, the quinol was mostly sulphonated, more resistant to oxidation (Pinnow, 1917) and less effective in development of colour. Moreover, it was preferable to have the oxidized form present in the first stage of the reaction.

Table 5 shows typical results obtained with reagents containing varying amounts of quinol and its oxidation products. The solvent in all cases was 60% (v/v) sulphuric acid (type *B*) and the reaction was as described for the experiment shown in Table 4. It is apparent from reagents 1–3 that a poised system of quinol:quinone is not desirable, a large excess of the former being required for maximum colour production. The difference in behaviour of reagents 4 and 5 is not readily explained. The increased colour found with reagent 5 has been repeatedly confirmed during the past year, not only for the oestradiol-17$\beta$ reagent but also for the reagents for oestrone and oestriol. There is a marked difference in appearance: reagent 4 is colourless, reagent 5 pink. It is possible that the effective agent is an oxidation product intermediate between quinol and quinone. Solutions prepared as for reagent 5 are subsequently referred to as 'modified reagents', while those prepared by dissolving 2 g. quinol in 100 ml. aqueous sulphuric acid are referred to as 'pure reagents'.

*Comparison of pure and modified reagents.* Fig. 4 compares the effectiveness of pure and modified reagents, prepared from different types of sulphuric acid, in the formation of colour with oestriol. Water was used as the diluent. Preliminary experiments established that the concentration of approximately 55%, which has been used in the second stage for both the pure reagents, was required in order to permit full conversion into pink in 15–20 min. heating with type *B* sulphuric acid. These conditions were imperfect, and the colour was liable to be enhanced by trace contamination. No significant change occurred on ageing. Application of the same conditions to a pure reagent prepared from type *A* sulphuric acid gave maximum optical densities in 5 min. heating and a tendency to fading. It is clear then that 'pure' (unmodified) reagents can be used only when the conditions for the second stage of the reaction are developed for each type (and possibly each batch) of sulphuric acid.

Table 4. *Quinol:$H_2SO_4$ reaction with oestradiol-17$\beta$ (50 $\mu$g.) in the presence of $H_2O_2$ (50 $\mu$g.): effect of time of addition of $H_2O_2$ and age of reagent*

| | $D_{604}$ | $D_{601}$ |
|---|---|---|
| $H_2O_2$ (50 $\mu$g.) added in first stage | | |
| 5-week-old reagent | 0·682 | 0·161 |
| | 0·697 | 0·192 |
| Fresh reagent | 0·770 | 0·165 |
| | 0·762 | 0·166 |
| $H_2O_2$ (50 $\mu$g.) added in second stage | | |
| 5-week-old reagent | 0·613 | 0·190 |
| | 0·698 | 0·176 |
| Fresh reagent | 0·666 | 0·169 |
| | 0·687 | 0·170 |
| No $H_2O_2$ added | | |
| 5-week-old reagent | 0·558 | 0·208 |
| | 0·551 | 0·208 |
| Fresh reagent | 0·611 | 0·201 |
| | 0·620 | 0·206 |

Table 5. *Effect of quinol and its oxidation products on the colour produced by oestradiol-17$\beta$ (50 $\mu$g.) in 60% (v/v) $H_2SO_4$*

(Reagent 4 was prepared by mixing solutions of quinol and quinone; reagent 5 was prepared by adding quinol to a solution of quinone.)

| Reagent no. | Quinhydrone (mg./100 ml.) | Quinol (mg./100 ml.) | Quinone (mg./100 ml.) | $D_{604}$ | $D_{601}$ |
|---|---|---|---|---|---|
| 1 | 2 | — | — | 0·553 | 0·243 |
| | | | | 0·553 | 0·243 |
| 2 | 2 | 500 | — | 0·638 | 0·174 |
| | | | | 0·658 | 0·179 |
| 3 | 2 | 2000 | — | 0·694 | 0·170 |
| | | | | 0·684 | 0·170 |
| 4 | — | 2000 | 2 | 0·685 | 0·183 |
| | | | | 0·674 | 0·192 |
| 5 | — | 2000 | 2 | 0·775 | 0·159 |
| | | | | 0·761 | 0·162 |

The 'modified' reagent prepared from type *A* or type *B* sulphuric acid gave maximum optical densities and no tendency to fading or type II inhibition with a second-stage concentration of approximately 60%. (Virtually identical results were obtained with the two types of sulphuric acid.) As the reagent aged, there was slight diminution (3–5%) in the formation of pink and a corresponding increase in the residual yellow. This was eliminated, as shown, by the addition of 100 mg. of quinol in the second stage of the reaction.

Exactly comparable results were obtained with pure and modified reagents for oestrone and oestradiol-17$\beta$. The stability of the reagents in the presence of solvent residues is attested by the typical experiments in Table 6.

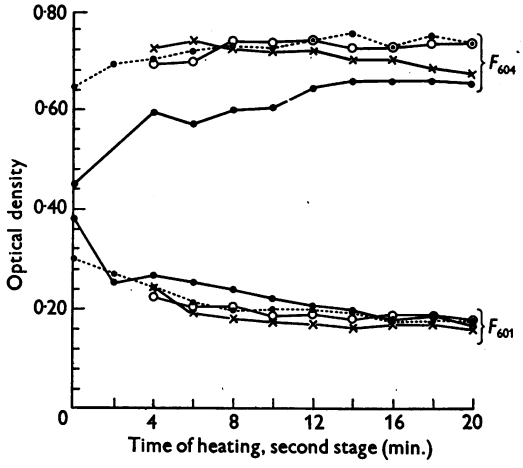


Fig. 4. Development of colour by oestriol (50 $\mu$g.) with pure and modified reagents. ●—● Pure reagent, type *B* $H_2SO_4$; ×—×, pure reagent, type *A* $H_2SO_4$; ●···●, modified reagent, 1-day old; ○—○, modified reagent, 10-days old, quinol (100 mg.) added at second stage.

Table 6. *Recovery of oestrogens* (25 $\mu$g.) *on extraction from aqueous* $H_2SO_4$ *with modified reagents and colour reaction*

| Oestradiol-17$\beta$ | $D_{604}$ | $D_{601}$ | $D_{606}$ | Recovery (%) |
|---|---|---|---|---|
| Extract | 0·362 | 0·087 | 0·010 | 100 |
| | 0·365 | 0·092 | 0·014 | 100 |
| Standard | 0·345 | 0·070 | 0·0 | — |
| | 0·349 | 0·071 | 0·0 | — |
| Oestrone | | | | |
| Extract | 0·445 | 0·108 | 0·009 | 98 |
| | 0·452 | 0·110 | 0·013 | 99 |
| Standard | 0·444 | 0·087 | 0·002 | — |
| | 0·442 | 0·090 | 0·003 | — |
| Oestriol | | | | |
| Extract | 0·393 | 0·170 | 0·042 | 92 |
| | 0·400 | 0·188 | 0·050 | 90 |
| Standard | 0·385 | 0·110 | 0·013 | — |
| | 0·390 | 0·112 | 0·016 | — |

*An improved method for the colorimetric determination of oestriol, oestrone and oestradiol-17$\beta$*

Since residues from the phenolic fraction of urine give a background colour which is not linear over the wavelength range covered by the 601 (violet) and 606 (yellow) filters, optical densities are now determined over a narrower range in the Unicam SP-600 instrument which permits measurement of 3·3 ml. of solution in a cell of 1 cm. optical thickness. The volumes of solution have been modified to suit these new conditions, the final reaction being described below.

*Preparation of reagents*

*Reagent for oestradiol-17$\beta$.* $NaNO_3$ (A.R. grade, 10 mg.) was added to 1 l. of $H_2SO_4$ (A.R., 60%, v/v) and 20 mg. quinone were dissolved with warming (types *A* and *B* acid give similar results). The solution turned yellow, changing to a light green with a definite opalescence. Quinol (20 g. of B.D.H. Laboratory Reagent) was added and dissolved by warming and shaking. The solution lightened in colour, becoming pale pink but still opalescent. After filtration through sintered glass (porosity no. 4, fine) the reagent was stored in an amber bottle. Occasionally opalescence reappeared with ageing and a second filtration was required. Certain batches of $H_2SO_4$ caused the appearance of brown oxides of nitrogen in small quantities during the dissolution of the quinol. These were removed during the filtration. At this low concentration of $H_2SO_4$, the quinol occasionally crystallized on cooling and had to be reheated.

*Reagent for oestrone.* This reagent was prepared as above but with 66% (v/v) sulphuric acid as the solvent. The final reagent was yellow with a trace of pink.

*Reagent for oestriol.* This was prepared similarly to the oestradiol-17$\beta$ reagent but with 76% (v/v) sulphuric acid as the solvent. Less opalescence formed and the final solution was yellow with no trace of pink.

Satisfactory reagents can be prepared exactly as recommended by Brown (1952) with certain batches of type *A* $H_2SO_4$ provided quinol is added to the oestriol reagent before use and provided all reagents are allowed to stand at room temperature for at least 1 week before use. Such reagents are highly coloured, being pink, yellowish pink, and yellow, for oestradiol-17$\beta$, oestrone, and oestriol, respectively. Satisfactory reagents cannot be prepared in this manner from type *B* $H_2SO_4$.

*Colour production.* The appropriate reagent (2·6 ml. for oestradiol-17$\beta$ and oestriol, 3·0 ml. for oestrone) was added to dry residues of oestrogens in Kober tubes. Quinol (50 ± 5 mg. from a calibrated measure) was added and the tubes, with tops in place, were heated for 20 min. in a bath of boiling water, with shaking after 2 min. and 5 min. to ensure adequate mixing and the dissolution of the quinol. The solutions were then cooled in a bath of cold water (approx. 15°), 50 mg. of quinol were added and diluted as follows: oestradiol-17$\beta$, with 0·7 ml. of reagent; oestriol, with 0·7 ml. of water; oestrone, with 0·3 ml. of water. The tubes were then shaken 5–10 times and reheated, with the tops in

place, for 15 min. with two shakings during this period to ensure dissolution of the quinol.

*Measurement of optical densities.* The cooled solutions (which are stable for several hours) were transferred to 1 cm. cuvettes and their optical densities measured at 480, 512·5 and 545 m$\mu$. for oestrone and oestriol and at 480, 515 and 550 m$\mu$.

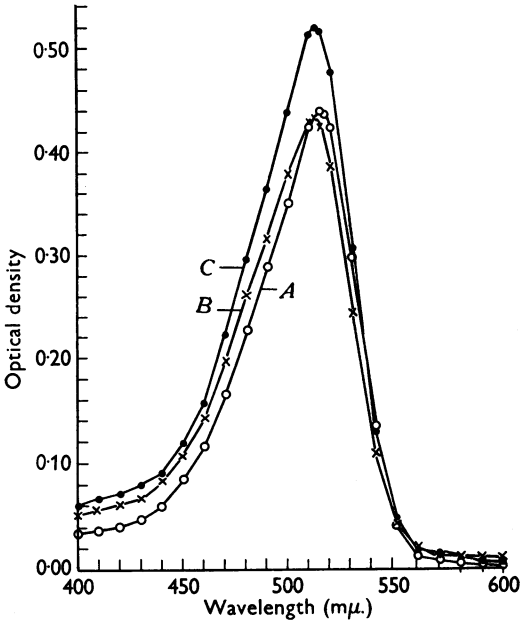


Fig. 5. Absorption spectra of the first coloured products obtained from pure oestrogens (10 $\mu$g.) with the modified reagents and reactions. *A*, oestradiol-17$\beta$; *B*, oestriol; *C*, oestrone.

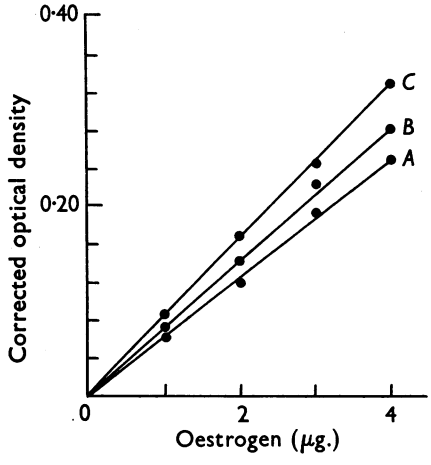


Fig. 6. Relationship between concentration of oestrogen and corrected optical density with the modified reagents and reactions. *A*, oestriol; *B*, oestradiol-17$\beta$; *C*, oestrone.

for oestradiol-17$\beta$. The colour correction formula of Allen (1950) was then applied.

*Characteristics of the colour reaction.* The absorption spectra of the final coloured products are shown in Fig. 5 ($\lambda_{max.}$, 512·5 m$\mu$. for oestrone and oestriol, 515 m$\mu$. for oestradiol-17$\beta$). The sensitivity, as shown by $E^{1\%}_{1\,cm.}$, is approximately 1700 for oestrone and 1400 for oestriol and oestradiol-17$\beta$. Proportionality between concentration of oestrogen and corrected optical density is shown in Fig. 6.

## SUMMARY

1. Solvent residues interfere with the production of colour by oestriol, oestrone and oestradiol-17$\beta$ in the reaction with the quinol:sulphuric acid reagents of Brown (1952). There are three different kinds of interference with the colour reaction.

2. The first of these (failure to form the initial yellow complex; type I inhibition) applies only to oestriol and is due to diminished reducing power of the reagent as a result of sulphonation of the quinol. This has been overcome by the addition of quinol immediately before colour development.

3. The other two types of interference depend upon the fact that the second stage of the Kober reaction, involving a conversion of the yellow complex into pink, appears to be an oxidation which, if excessive, causes fading. Under the conditions described by Brown (1952) the conversion of yellow into pink and the stability of the latter are influenced by the degree of illumination of the laboratory, size of the tube used and degree of stirring. Elimination of the final dilution stage, the use of larger-bore tubes and modification of the conditions for the second stage removed these variable factors.

4. The optimum reaction conditions depend upon the make of analytical reagent grade sulphuric acid used; this variable can be eliminated by inclusion of trace amounts of sodium nitrate and quinone in the reagent.

5. The modified reaction is unaffected by solvent residues and gives colour intensities which are reproducible and obey Beer's law over a suitable range of concentration.

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# Continuous Direct Photometry of Dyed Materials in Filter Paper with Special Reference to the Estimation of Proteins Separated by Electrophoresis

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The estimation of protein fractions after their separation by electrophoresis in filter paper and subsequent staining may be carried out by excision, elution, and colorimetric estimation (Cremer & Tiselius, 1950) or by direct photometry, either intermittent (Grassmann, Hannig & Knedel, 1951) or continuous. Direct photometric methods have the advantage of being less laborious and also of requiring less material.

A brief account of an apparatus and technique for direct continuous photometry has already been given (Crook, Harris & Warren, 1952). In the present paper the underlying problems involved in such a technique are discussed, and a detailed account is given of an apparatus which has given satisfactory results in practice.

## CONSIDERATIONS IN DESIGN

In designing an apparatus and a technique of measuring continuously the optical-density distribution in a strip of filter paper, many decisions have to be made between possible alternative solutions of the problems that arise. Some of these are straightforward, such as the choice of moving part, since it is obvious that movement of the paper past a fixed optical system and photocell is the simplest arrangement. Similarly, although a cylindrical paper carriage is more compact, a flat carriage is much simpler to construct and to retain the paper upon.

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At first sight, measurement of the amount of colour by reflexion from the dry paper might appear preferable to the measurement of transmission, since it avoids all the complications of the scattering of light by the fibres of the paper and the consequent necessity for an immersion fluid. However, although it is easy to perceive qualitative differences in reflexion by the unaided eye, preliminary experiments showed that there were quantitative irregularities in the amount of light reflected, due presumably to variations in the surface texture of the paper and to differences in the degree of penetration of protein and dye into the paper. Thus a transmission system which allows the light to pass through all the dyed protein is the only admissible type.

The paper must, however, be rendered as translucent as possible to reduce light scattering, i.e. it must be made to approach an optically homogeneous medium by the use of an immersion fluid. Theoretically, except for a small residual effect due to differences in the dispersion of immersion fluid and paper which would cause the combination to act as a Christiansen filter, an immersion fluid with a refractive index equal to that of the paper would reduce the scatter to zero provided the paper was homogeneous. However, since the paper is not homogeneous and scatter can only be reduced to a minimum, the refractive index of the immersion medium is not critical provided it is in the neighbourhood of 1·55, which is the approximate refractive index of cellulose. On trial, media with refractive indices in this region were found to be