THE CHEMICAL ESTIMATION OF OESTROGENS IN URINE.

by

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GENERAL INTRODUCTION.

When studying the behaviour of a substance in the body, once the qualitative responses have been investigated, a quantitative method for estimating the substance in the presence of body fluids is necessary before further information can be obtained. In the oestrogen field, a considerable amount is known about the qualitative response of the body to the oestrogens, but little is known concerning the actual mechanism by which they are metabolized. In clinical medicine, gross disorders of oestrogen production are easily recognized and if they are accompanied by a greatly increased production of oestrogens, they can be investigated by the crude quantitative methods at present available. However, little is known about the less obvious disorders. It is hoped that a more sensitive and precise method than is now available for estimating "natural" oestrogens in body fluids, will facilitate the investigation of oestrogen metabolism and of disorders which now are only suspected to be due to abnormalities of oestrogen metabolism. Such a method is probably the most important single requirement in the oestrogen field today. That this is universally appreciated is shown by the number of laboratories actively engaged in research on oestrogen methods, and it was this need which has also prompted the investigation described in this thesis.

A method for estimating natural oestrogens in urine was obviously the first to be developed, because urine is easily obtained in large amounts. However, methods are required for estimating oestrogens in blood and tissues and it is hoped that these will be developed in the near future. Methods for estimating oestrogens can be divided into two classes, one biological, the other chemical. Biological methods,

though specific, are tedious and lack the accuracy and convenience which are possible with chemical methods. Methods are required to isolate, identify and measure the small amounts of oestrogens which occur in urine. Even in pregnancy the urinary excretion seldom exceeds 20 mg. per day, and in non pregnancy the normal levels seem to be between 5 and 100 µg. per day. To measure the latter concentration, a method must be able to estimate one part of oestrogen in 500 million parts of urine, which appears at first to be an impossible task for a chemical method. Fortunately, however, when the natural oestrogens are heated with sulphuric acid under certain conditions, an intense red ("Kober") colour is produced which is almost specific for the natural oestrogens; under other conditions an even more intense greenish fluorescence is produced which, though not as specific as the red colour, is sensitive enough to estimate even the small amounts of oestrogens present in normal urine. A chemical method for measuring these amounts is therefore theoretically possible. Unfortunately, the methods which produce this colour and fluorescence also produce brown colours and bluish-white fluorescences with other urinary materials and these interfere in the measurement of the oestrogens. The limits of any method based on these colour or fluorescence reactions therefore depend upon the extent to which the interfering urine component can be removed. The investigation to be described deals with the development of a chemical method, and is divided into two sections. The first section describes the search for a stable colour method which is minimally affected by interfering urinary material; the other deals with the purification of urine extracts.

In outline, the method finally adopted consists of the following steps: acid hydrolysis of the urine, extraction of this with ether, separation of the phenolic fraction containing the oestrogens from the acid, polyhydroxy phenol and neutral fractions, methylation of the phenolic fraction, and chromatography of the phenol methyl ethers on alumina columns. Oestrogens in the purified urine extract were estimated colorimetrically. A spectrophotometric procedure was used to correct for residual interfering urinary material. Experiments to determine the recovery of oestrogens added to hydrolysed male urine indicated that this method is satisfactory for estimating oestriol, oestrone and oestradiol-17β at concentrations greater than 100 µg. of each in a 24 hour specimen of urine.

PART I

COLORIMETRIC METHODS

FOR ESTIMATING

OESTRIOL, OESTRONE AND OESTRADIOL—17β

INTRODUCTION.

Many colour reactions for oestriol, oestrone and oestradiol-17β have been described but few have been used for their quantitative estimation. These colour reactions can be classified broadly into two classes, namely those which develop colours with the common phenolic group and those grouped under the term "Kober reaction" which produce colours, usually red or yellow, with a green fluorescence, by heating with strong sulphuric acid or similar agent. Colour methods such as those described by Schmulovitz & Wylie (1936) who used diazotized sulphanilic acid, Talbot, Wolfe, MacLachlan, Karush & Butler (1940) who used diazotized dianisidine, and Chevallier, Cornil & Verdollin (1935) and Friedgood, Garst & Haagen-Smit (1948) who used ultra violet light absorption at 280 mμ., which depend upon the phenolic group, are unlikely to be reliable for the quantitative estimation of oestrogens in body fluids as their separation from other constituents also depends upon this phenolic group.

Almost all of the other colour methods described produce colours with a green fluorescence and therefore belong to the second class described above. A number of reagents produce these colours and fluorescence when heated with the natural oestrogens. Bachman (1939), Finkelstein, Hestrin & Koch (1947) and Boscott (1948) used phosphoric acid, Clark & Thompson (1948) used phthallic or succinic anhydride and antimony trichloride, and Kleiner (1941) and Garst, Maron & Friedgood (1950), (who believed they were doing a phenolphthalein type condensation), used phthallic anhydride and zinc or stannic chloride, and Pincus, Wheeler, Young & Zahl (1936) used benzoyl chloride and sulphuric acid. However, sulphuric acid has been most commonly used for this purpose. Marrian

(1930) was the first to describe this colour reaction and reported that when oestriol was warmed with concentrated sulphuric acid, an orange colour with a green fluorescence was produced. Kober (1931) noted that when this orange fluorescent solution was diluted with water and warmed the colour changed to a clear red, also with a greenish fluorescence, and that this change was almost specific for the natural oestrogens. Phenol added to the reaction mixture quenched the final fluorescence making the red colour both more intense and suitable for visual colorimetry. Since Kober was the first to develop this type of colour reaction it is fitting that his name should be applied to the whole class. Kober's original method was performed in two stages, the first being the formation of the orange (or under certain conditions, yellow) intensely fluorescing colour and the second the conversion of this to the red colour.

Many colour and fluorescent methods employing sulphuric acid as the principal agent have been described. Many of the earlier workers, like Kober, added phenols to the sulphuric acid, phenol itself being most commonly used. **Cohen & Marrian** (1934) investigated the optimal proportions of phenol and sulphuric acid and these and other workers have modified the phenol-sulphuric acid ratios, the heating times and temperatures, the amounts of water added for the second stage of the reaction, and the final dilution technique recommended by Kober. For instance, see Cartland, Meyer, Miller & Rutz (1935), Bachman (1939), who described a one phase colour reaction and heated at 150°C, Pincus, Wheeler, Young & Zahl (1936) and Salter, Humm & Oesterling (1948). Venning, Evelyn, Harkness & Browne (1937) investigated the phenol-sulphuric acid colour method more thoroughly, and using the proportions

of phenol and sulphuric acid recommended by Cohen & Marrian (1934), devised a very satisfactory colour method which has been popularly used since. Other phenols used have been naphthol (Kober, 1938) and guaiacol (Szego & Samuels, 1940), but these have not been popular. Woker & Autener (1939) used furfural or ascorbic acid instead of phenols.

More recently colour methods using sulphuric acid without phenol have been described. Umberger & Curtis (1949) investigated the spectral characteristics of colours produced when oestrogens were heated with various concentrations of sulphuric acid and water, and Cohen & Bates (1947) described a two stage method for producing Kober colours by heating oestrogens with sulphuric acid and water. Their final colours had densities similar to those obtained by using the phenol-sulphuric acid method of Venning et al. (1937) and they suggested from this that the increase in colour density in the presence of phenol, reported by Kober, was only visually apparent through the quenching of the green fluorescence, and that it was not a real increase when measured photometrically.

In general these colorimetric methods have not been completely satisfactory. That of Venning et al. (1937) was probably the best and was used in this department for some time. However, the heating times were critical and results were not reproducible from day to day. Furthermore, Dr. Clayton, in this department, showed that when solutions of the oestrogens in organic solvents, especially in ether, were evaporated, the colours developed with the residues did not agree with those expected. She showed that it was the residue left after evaporation

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of the ether which caused the inconsistencies.

Recently, workers in this field have been attracted from these unsatisfactory colorimetric methods to fluorimetric methods, which measure the intense greenish fluorescence obtained in the first stage of the Kober reaction. Although these fluorimetric methods are not very specific they are very sensitive and therefore should be capable of estimating the small amounts of oestrogens which occur in non-pregnancy urine. Jailer (1947) and Bates & Cohen (1947) described methods using sulphuric acid and Finkelstein et al. (1947) used phosphoric acid. Bates & Cohen (1950) further investigated the optimum conditions for fluorescence production with sulphuric acid and the spectral characteristics of exciting and emitted light. However, these fluorimetric methods were still not entirely satisfactory since, besides lacking specificity, they were found to suffer from interference by solvent residues and urine extracts which was not completely due to quenching effects.

In the belief that these effects were not due to preliminary destruction of the oestrogens but to interference in the critical stages of the Kober reaction, it was hoped that a more complete assessment of the factors concerned in the Kober reaction might lead to specific colour methods which would not be so affected. As sulphuric acid appeared to be the most versatile agent for producing Kober colour and fluorescence, and the procedure of Cohen & Bates (1947) was the simplest application of sulphuric acid, it was decided that an investigation into their colour method was the logical starting point for this investigation.

EXPERIMENTAL/

EXPERIMENTAL.

Pure crystalline oestriol, oestrone and oestradiol-17β* and their methyl ethers (see appendix) were used throughout this work. The term "natural oestrogen" or "oestrogen" implies one of these three compounds. The term "oestradiol" when unqualified implies the -17β* isomer. Phenolic steroids other than these have not been studied.

Quantities of oestrogens were measured in the appropriate volumes of standard solutions in ethanol prepared by dissolving approximately 5 mg. (accurately weighed) of pure oestrogen or oestrogen methyl ether in redistilled 95% ethanol and making the final volume 100 ml. Although these standard solutions did not contain exactly 5.0 mg. per 100 ml., they were treated as if they did, so that exact comparisons can only be made between results obtained with the same standard. As only comparative results were required, no attempt has been made in this work to distinguish between different standard solutions or to give their exact concentrations.

Solvents were removed from oestrogen solutions in "Kober" tubes (6 x $\frac{3}{4}$ in. Pyrex test tubes) by heating in a stream of air or in later work under nitrogen and reduced pressure.

No special precautions were taken to prevent entrance of water vapour or air during colour development. Throughout this paper, the term "heating" implies "heating in a boiling water bath". All reagents were A.R. quality.

Colour densities were measured against a reagent blank in a Spekker absorptiometer using 1 cm. cuvettes. Ilford spectrum green no.603 and no.604 light filters were used, according to the wavelength of maximum absorption, which varied with the reagent used for

developing the colour.

Density-wavelength curves were measured in a Unicam diffraction grating or U.V. spectrophotometer.

*This nomenclature follows that used by Fieser & Fieser (1949) and replaces the older term "α" oestradiol.

THE PROCEDURE OF COHEN & BATES AND THE INFLUENCE OF ETHANOL IN THE KOBER REACTION.

Cohen & Bates (1947) developed the red Kober colour in two stages. In the first stage, the oestrogen dissolved in 0.4 ml. of absolute ethanol was heated 6 min. with concentrated sulphuric acid (2 ml.) without removal of the ethanol. In the second stage 25% (v+v) sulphuric acid (8 ml.) was added and the solution was heated again for 3 min.. The solutions were cooled and the colours measured in a colorimeter. Preliminary experiments in this laboratory showed that when the method was applied to small amounts of pure oestriol in the absence of ethanol, no red colour was obtained. It was also found that water added with the sulphuric acid for the first stage caused increased colour densities, and that the 25% (v+v) sulphuric acid diluent was not optimal.

Sulphuric acid concentrations for the second stage.

The second stage of the Kober reaction is usually performed by diluting the products of the first stage with water and heating, during which process the characteristic red colour appears. Cohen & Bates investigated this factor by heating oestrone with 2 ml. of sulphuric acid for the first stage and then adding 8 ml. of a series of dilutions of sulphuric acid and water before heating for the second stage. Diluents more concentrated than 50% (v+v) sulphuric acid did not permit the formation of any red colour from the yellow colour formed in the first stage, whereas those containing 25% (v+v) or less of sulphuric acid caused a complete transition from yellow to red. This change was followed with a spectrophotometer which showed the transition by a shift in the wavelength of maximum light absorption from 460 to 505 mμ.

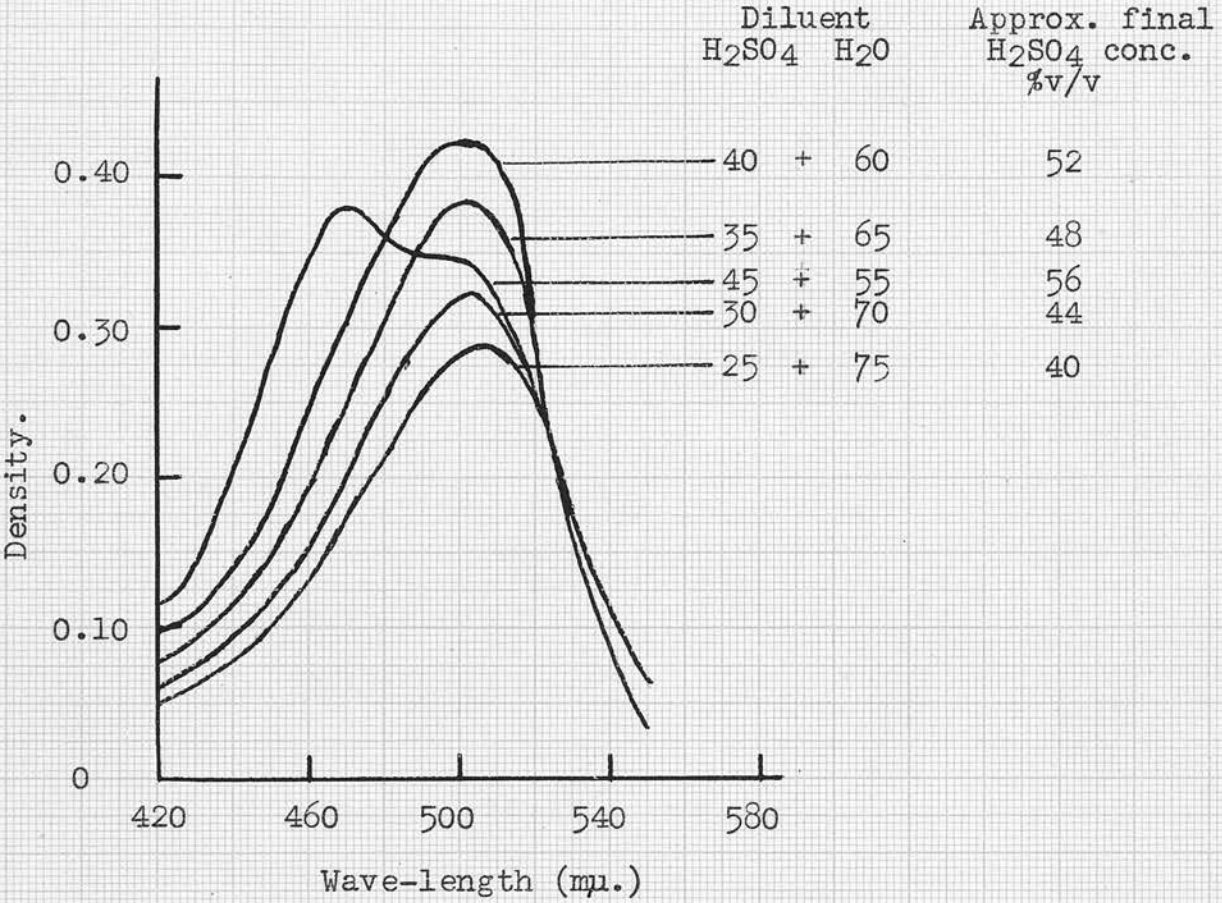


Fig. 1. The influence of sulphuric acid concentration at the time of the second heating upon colour formation with oestrone (50 μg.), showing the spectral characteristics of the colours produced.

On the basis of these results, Cohen & Bates chose 25% (v+v) sulphuric acid for their diluent at the second stage, but did not study diluents with sulphuric acid concentrations between 25 and 50%. This sulphuric acid range was therefore investigated using their procedure (see fig.1). The density of red colour increased as the sulphuric acid concentration of the diluent was increased to 40% (v+v), but decreased thereafter owing to incomplete conversion of the yellow colour. The effect of smaller increments in acid concentration was further investigated in the same manner but by reading the colour densities in a Spekker absorptiometer using Ilford No. 603 filters.

% (v+v) sulphuric acid	Actual sulphuric acid concentration (v+v) in the second stage	D_{603}
35	48	.523
37.5	50	.550
40	52	.550
42.5	54	.410
45	56	.374

The important point appeared to be the final sulphuric acid - water ratio. In this case the optimum was 52%.

The influence of water and ethanol added before the first stage on the optimum sulphuric acid concentration for the second stage was next investigated. Water or ethanol followed by concentrated sulphuric acid (3 ml.) were added to oestriol or oestrone (50 µg.) and heated for 20 min.. After cooling, water was added, the mixture was heated another 4½ minutes, cooled, diluted to 15 ml. with 25% (v+v) sulphuric acid, and the colour density measured either in a Unicam diffraction grating spectrophotometer or in a Spekker absorptiometer. The data obtained is summarized in the table.

Oestrone 50 µg.

Addition to 1st stage.

Water (ml.)	Ethanol (ml.)	Max. light absorption (mµ.)	Extra water (ml.) added for 2nd stage					
Nil	Nil	500 D_{603}	3.8 .183	3.4 .210	3.0 .230	2.6 .230	2.2 .240*	1.8 .200
0.4	Nil	500 D_{603}			3.0 .290	2.6 .290*	2.2 .290	
1.0	Nil	500 D_{603}				2.4 .270	2.0 .280*	1.6 .260
2.0	Nil	500 D_{603}				1.4 .258	1.0 .258	0.6 .270*
Nil	0.6	505 D_{603}	3.2 .240	2.8 .268	2.4 .288*	2.0 .274	1.6 .242	
Nil	1.4	510 D_{603}	2.6 .235	2.2 .220	2.0 .240	1.8 .248*	1.6 .239	1.2 .219
Nil	3.0	515 D_{604}				2.0 .239	1.6 .245*	1.2 .220

* Denotes maximum colour density for the particular experiment.

Oestriol 50 µg.

Addition to 1st stage.

Water (ml.)	Ethanol (ml.)	Max. light absorption (mµ.)	Extra water (ml.) added for 2nd stage				
Nil	Nil	495	4.5	3.7	3.0*	2.5	2.0
	Max. D.Unicam		.162	.216	.244	.240	.230
Nil	0.6	504	2.8	2.4*	2.0	1.6	
	Max. D.Unicam		.348	.367	.348	.329	
Nil	1.4	508	2.4	2.0*	1.6	1.2	
	Max. D.Unicam		.338	.342	.338		
Nil	3.0	525	2.2	1.8	1.4	1.0	
	Max. D.Unicam		.200	.194	.205	.194	
0.4	Nil	495	3.2	2.8	2.4	2.0	
		D_{603}	.100	.100	.102*	.092	
1.0	Nil	495	2.6	2.2	1.8	1.4	
		D_{603}	.116	.120*	.120	.109	

* Denotes maximum colour density for the particular experiment.

For amounts of water or ethanol ranging from 0 to 1.4 ml. in 3 ml. of sulphuric acid in the first stage, the optimum total water plus ethanol in the second stage was approximately 3.0 ml..

To find the optimum amounts of water and ethanol in the first stage when the optimum volumes were used in the second stage.

Measured amounts of water and ethanol followed by 3 ml. of concentrated sulphuric acid were added to oestrone and oestriol (50 µg.) and heated 20 min.. After cooling, water was added so that the total volume of water plus ethanol was 3.0 ml., the mixture was heated again

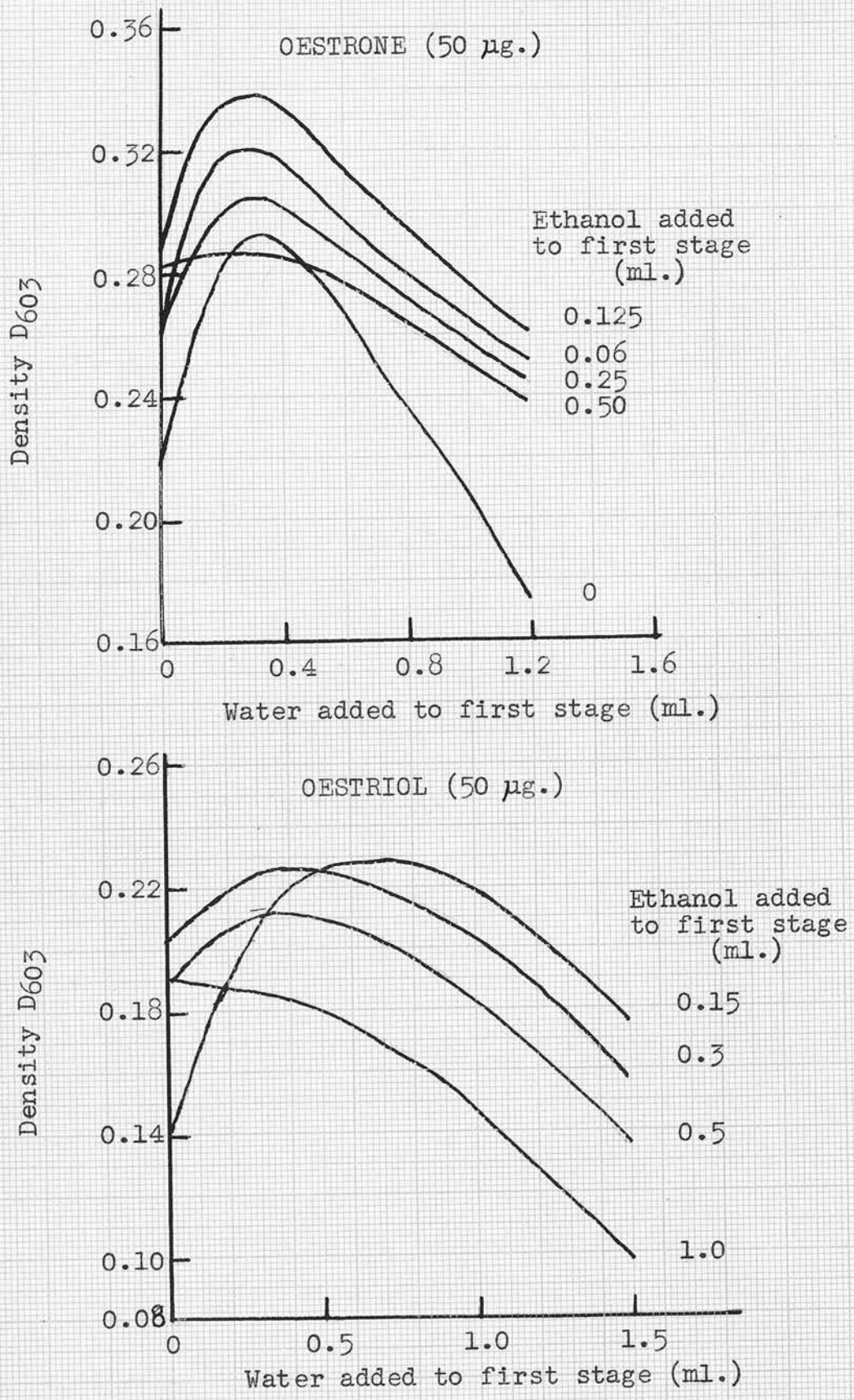


Fig. 2. The effect of water and ethanol in the first stage of the Kober reaction on the density of colour developed.
The optimum amount of water was added for the second stage.

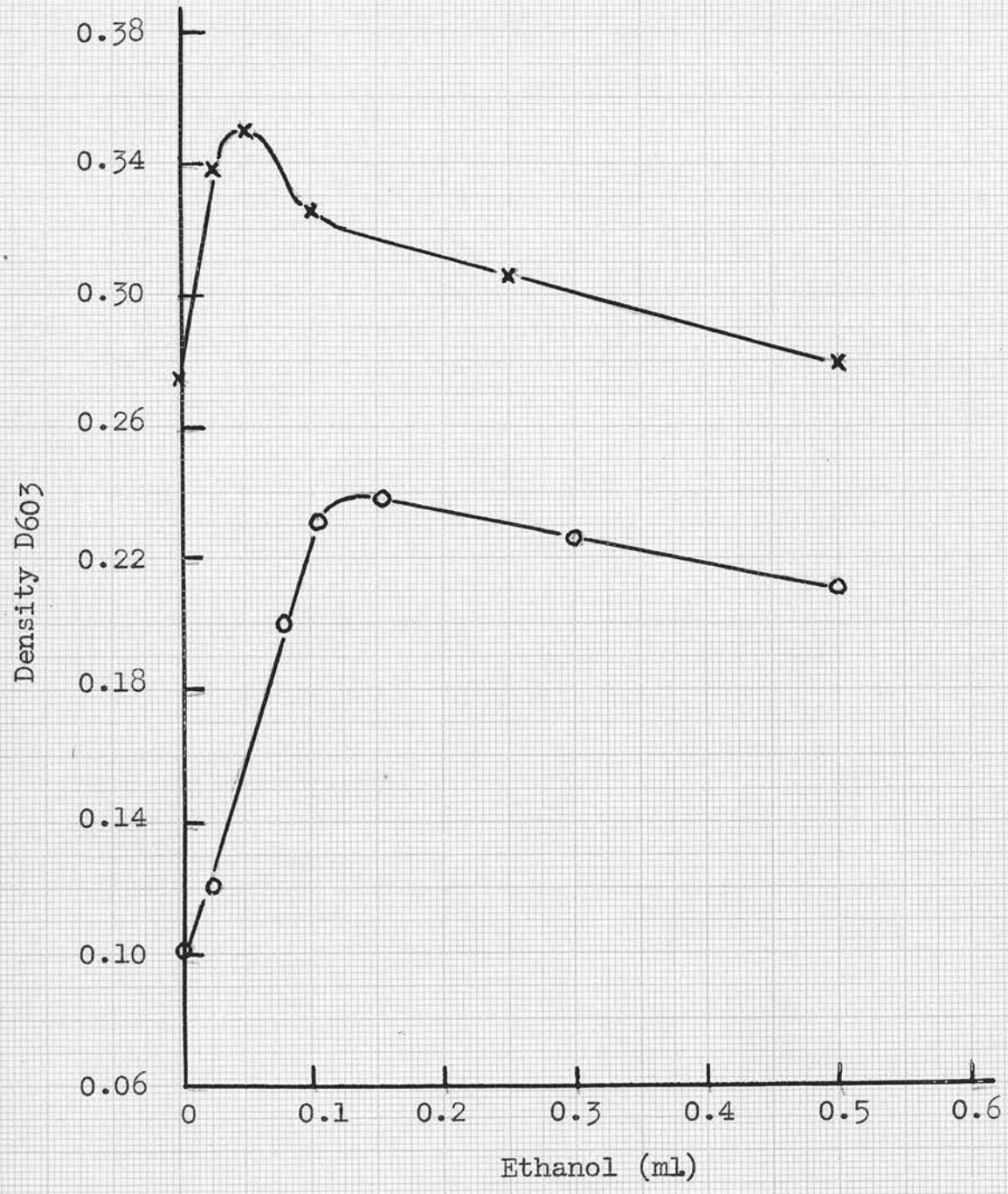


Fig. 3. The influence on Kober colour production of varying amounts of ethanol added to oestriol and oestrone and heated in the first stage of the colour reaction. x—x oestrone, 50 µg.; 0—0 oestriol 50 µg.

4 min., cooled and diluted to 15 ml. with 30% (v+v) sulphuric acid. Colour densities were measured in a Spekker absorptiometer.

See figure 2.

Maximum colour densities were obtained with oestrone when 0.25 ml. of water was present in the first stage and with oestriol when the volume of ethanol plus water was 0.8 ml. in the first stage. Using these optimum proportions of ethanol and water the optimum amount of ethanol was sought in a similar manner, with the results shown in figure 3.

Using 20 min. heating for the first, and 4 min. for the second stage of colour production, oestrone produced maximum colour densities with 0.05 ml. of ethanol, 0.25 ml. of water and 3 ml. of concentrated sulphuric acid in the first stage and a further 2.7 ml. of water in the second. Oestriol produced maximum colour with 0.15 ml. of ethanol, 0.65 ml. of water and 3 ml. of concentrated sulphuric acid in the first stage and a further 2.2 ml. of water in the second.

Using oestriol and the optimum amounts of water and ethanol, i.e. ethanol 0.15 ml., water 0.6 ml., sulphuric acid 3 ml., in the first stage and a further 2.2 ml. of water in the second stage, the effect of the times of heating were investigated.

The heating time for the first stage using 4 min. for the second.

Heating time (min.)	6	12	20	40	60	120
D_{603}	.248	.239	.239	.212	.207	.193

The heating time for the second stage using 20 min. for the first.

Heating time (min.)	2½	4	6	9	12
D_{603}	.150	.239	.235	.230	.239

It was found in this and in other experiments that in the first stage 20 minutes heating was the best, since small variations in water and

ethanol concentration had less effect than when shorter heating times were used. The second heating time was not critical and 5 min. was selected as the best.

OTHER SUBSTANCES WITH COLOUR ENHANCING PROPERTIES.

Only small volumes of ethanol were required for maximum colour production. Larger volumes caused a shift in the wavelength of maximum light absorption but the colours so produced were not as intense and faded rapidly. Ethanol did not act merely as a solvent which helped the oestrogens to dissolve in the sulphuric acid since it still enhanced the red Kober colour when added after the sulphuric acid.

A search was made for other substances which would similarly enhance the Kober colour. Substances investigated included other organic hydroxyl compounds stable to sulphuric acid. Since many of these have reducing properties, some reducing agents and their oxidized forms were also investigated. The possibility that impurities such as peroxides in the ethanol might catalyse the reaction was also investigated.

Substances were tested by adding them to oestrone (50 µg.) and water (0.25 ml.) before the sulphuric acid (3.0 ml.) and 20 min. heating of the first stage of colour development. The second stage was performed by adding water (2.7 ml.), and heating another 4 min.. The reaction products were cooled, diluted to 15 ml. with 30% (v+v) sulphuric acid and the colour densities measured against 30% (v+v) sulphuric acid blanks in a Spekker absorptiometer, using both Ilford No. 603 and 604 filters.

Substance	Amount	D_{603}	D_{604}
Ethanol purified by NaOH	0.05 ml.	.345	.250
Ethanol aldehyde free from m-phenylene diamine	0.05 ml.	.330	.240
Di-ethyl ether peroxide free	0.05 ml.	.340	.249
Di-ethyl ether with peroxides	0.05 ml.	.318	.230
Hydrogen peroxide (1 vol%)	0.05 ml.	colour destroyed	
Methanol	0.05 ml.	.160	.145
Acetone	0.05 ml.	.358	.270
Ferrous sulphate	few crysts.	.376	.260
Ferric sulphate	" "	.092	.083
Copper metal		.292	.210
Cupric sulphate	few crysts.	.279	.208
Arsenious oxide		no red colour formed	
α-naphthol	few crysts.	.405	.295
β-naphthol	" "	.404	.287
Hydroquinone	" "	.392	.285
Pyrocatechol	" "	.413	.296
Phloroglucinol	" "	.350	.250
Guaiacol sulphonic acid	" "	.370	.265
Resorcinol	" "	.355	.250
Phenol	" "	.300	.217
p.Cresol	" "	.385	.274
Pyrogallol	" "	.405	.290
Orcinol	" "	.394	.286

All substances which increased the colour density were potential reducing agents. Ferrous sulphate was active in this respect even when it contained small amounts of ferric sulphate, although a larger proportion of the latter caused rapid fading of the red colour. Trihydroxy, dihydroxy and mono-hydroxy phenols were active as also were ethanol and diethyl ether. Arsenious oxide and sulphites prevented the formation of the red colour in the second stage but did not interfere in the first stage.

The stage of the Kober reaction at which these compounds had their effect was next investigated. Hydroquinone (60 mg.) was added (a) dissolved in the 3 ml. of sulphuric acid used for the first stage (b) dissolved in the water added for the second stage and (c) dissolved in the 30% (v+v) sulphuric acid used for diluting the final colour.

Colours were produced with oestriol and oestrone (50 µg.) by the same optimal procedure as when ethanol was used, (i.e. oestriol, 0.8 ml. of water in the first stage and 2.2 ml. in the second; oestrone, 0.25 ml. of water in the first stage and 2.75 ml. in the second).

Stage of addition of reducing agent. (hydroquinone 60 mg.)	Drum reading (D_{604})	
	Oestrone (50 µg.)	Oestriol (50 µg.
In sulphuric acid for the first stage	0.378	0.289
In water for the second stage	0.364	0.120
In 30% sulphuric acid for final dilution	0.275	0.116
None added	0.276	0.116

When reducing agents were omitted from the first stage but were added with the water for the second, oestriol did not form any red colour, while oestrone formed almost the same density of colour as when the agent was added in the first stage. It appears that when oestriol is heated with sulphuric acid in the absence of reducing agents a yellow colour forms which does not undergo the second stage of the Kober reaction.

Further work on these reducing agents is summarized in the next table which shows the effect of various concentrations of reducing agents upon the Kober colour formed by oestrone (50 µg.) under the conditions which were optimal when ethanol was present (i.e. heating 20 min. with water (0.25 ml.) and sulphuric acid (3 ml.) for the first stage and 4 min. with a further 2.7 ml. of water for the second and diluting to 15 ml. with 30% (v+v) sulphuric acid). Colour densities were measured against equivalent reagent blanks and within 5 min. of dilution.

Reducing agent	mg. added	D_{603}	D_{604}	D_{603}/D_{604}
none		.280	.200	1.40
acetone	.05 ml.	.344	.262	1.31
	.10 ml.	.358	.283	1.27
	.25 ml.	.359	.337	1.07
reagent blank	.25 ml.	.058	.043	
o-cresol	8 mg.	.355	.252	1.41
	62 mg.	.346	.251	1.38
	145 mg.	.314	.249	1.26
	474 mg.	.280	.267	1.05
reagent blank	500 mg.	.082	.074	
m-cresol	.05 ml.	.349	.255	1.37
	.10 ml.	.322	.254	1.27
	.25 ml.	.339	.286	1.18
	.50 ml.	.307	.296	1.04
reagent blank	500 mg.	.008	.008	
p-cresol	.05 ml.	.340	.260	1.30
	.10 ml.	.349	.270	1.30
	.25 ml.	.325	.280	1.16
	.50 ml.	.300	.284	1.05
reagent blank	500 mg.	.000	.001	
phenol	.05 ml.	.305	.217	1.41
	.25 ml.	.355	.290	1.22
	.50 ml.	.309	.292	1.06
	2.0 ml.	.205	.291	0.70
reagent blank	500 mg.	.000	.000	
guaiacol (potassium salt of sulphonic acid)	13 mg.	.332	.235	1.41
	32 mg.	.349	.249	1.40
	155 mg.	.338	.244	1.39
	416 mg.	.337	.275	1.23
reagent blank	500 mg.	.016	.018	
hydroquinone	5 mg.	.402	.282	1.42
	16 mg.	.401	.288	1.39
	36 mg.	.389	.286	1.36
	86 mg.	.401	.297	1.35
reagent blank	50 mg.	.029	.026	
pyrocatechol	7 mg.	.397	.287	1.38
	15 mg.	.406	.294	1.38
	31 mg.	.392	.288	1.36
	72 mg.	.364	.264	1.38
reagent blank	50 mg.	.019	.018	

Reducing agent	mg. added	D_{603}	D_{604}	D_{603}/D_{604}
pyrogallol	4 mg.	.391	.280	1.40
	10 mg.	.386	.274	1.41
	19 mg.	.385	.279	1.38
	49 mg.	.336	.236	1.46
reagent blank	50 mg.	.127	.117	
orcinol	9 mg.	.366	.264	1.39
	18 mg.	.352	.263	1.34
	39 mg.	.330	.259	1.27
	80 mg.	.306	.256	1.19
reagent blank	50 mg.	.078	.058	
α-naphthol	8 mg.	.353	.256	1.38
	16 mg.	.343	.265	1.29
	34 mg.	.352	.283	1.24
	55 mg.	.319	.286	1.11
reagent blank	50 mg.	.056	.040	
β-naphthol	8 mg.	.381	.282	1.35
	17 mg.	.345	.273	1.27
	30 mg.	.328	.275	1.19
	61 mg.	.285	.269	1.06
reagent blank	50 mg.	.090	.050	
ferrous sulphate	10 mg.	.360	.247	1.46
	20 mg.	.382	.269	1.42
	50 mg.	.368	.357	1.43
	100 mg.	.364	.254	1.43
reagent	50 mg.	.000	.000	

On the basis of these results the following were selected for more thorough investigation; hydroquinone and pyrocatechol because they caused most enhancement of the colour, ferrous sulphate because its reagent blank was very low, and phenol and p.cresol because mixtures containing a large proportion of these in sulphuric acid produced low blanks in the colour reaction and caused a marked shift in the wavelength of maximum light absorption of the final red colour. This shift is desirable since it allows a better spectroscopic separation from the yellow material given by urine extracts.

FERROUS SULPHATE IN THE KOBER REACTION.

As shown in the preceding section the amount of ferrous sulphate used in the Kober reaction was not critical. Ferrous sulphate is poorly soluble in strong sulphuric acid and it was found that 50 mg. was about the greatest amount which would dissolve in 3 ml. of sulphuric acid. Accordingly this amount was used throughout this work.

Ferrous sulphate was dissolved in very dilute sulphuric acid and a measured volume of this solution containing ferrous sulphate (50 mg.) was added to the oestrogen before the sulphuric acid (3 ml.).

The effect of water in the first and second stages of the Kober reaction when ferrous sulphate was added to the first stage of colour production with oestrone and oestriol (50 µg.) was investigated.

The first stage was performed by adding the ferrous sulphate solution and water and sulphuric acid (3 ml.) to the oestrogen (50 µg.) and heating 12 min. The second stage was performed by adding water (the total volume added in both stages is shown in the table) and heating 4 min. The final colour was diluted to 15 ml. with 30% (v+v) sulphuric acid.

Total water added (ml.).

First stage	Second stage	Oestrone D_{603}	Oestriol D_{603}
0.8	3.0	.439	.343
	2.5	.465	.360
	2.0	.470	.382
	1.5	.465	.380
1.0	2.4	.470	.372
	2.2	.483	.382
	2.0	.480	.385
	1.8	.475	.375
1.6	3.0	.438	.340
	2.5	.455	.350
	2.0	.452	.353
	1.6	.425	.350

Evidently in the range 0.8 to 1.6 ml. of water in the first stage a total of 2.0 ml. in the second stage was optimal but not critical. Using this amount in the second stage the effect of water in the first stage was investigated more closely and in a similar manner.

Water (ml.)	0.4	0.6	0.8	1.0	1.2	1.4	1.6
D_{603} oestrone 50 µg.	.493	.488	.472	.463	.450	.452	.456
D_{603} oestriol 50 µg.			.385	.385	.382	.382	.343

Using a total of 2 ml. of water and heating 4 min. in the second stage, and varying the amount of water in the first stage, the effect of varying the heating time of the first stage was studied.

First stage.

Heating time (min.)		0	2	4	6	8	12	16	20
	Water ml.								
Oestrone 50 µg.	0.4	.460	.493	.495	.495	.503	.500	.493	
	1.0	.460	.507	.495	.495	.490	.483		
	1.6	.460	.475	.463	.464	.464	.452		
Oestriol 50 µg.	0.4	.090	.220	.294	.303	.312	.334	.325	
	1.0	.090	.310	.349	.370	.385	.385	.373	.360
	1.6	.090		.220		.289	.345	.350	.343

Using 1.0 ml. of water and 3 ml. of sulphuric acid and heating 10 min. for the first stage, and a total of 2.0 ml. of water in the second stage, the effect of varying the heating time of the second stage was studied.

Second stage.

Heating time (min.)	0	1	2	4	6	8
Oestrone 50 µg.	.357	.450	.480	.483	.470	.473
Oestriol 50 µg.	.341	.383	.375	.385	.388	

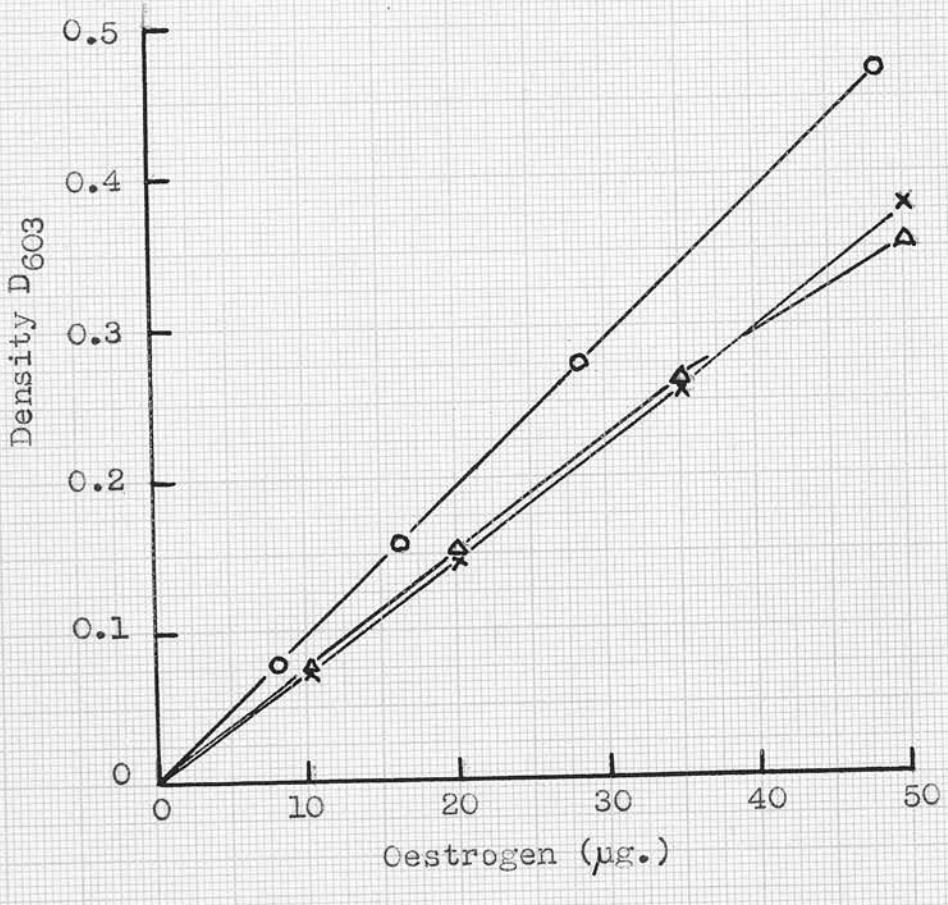


Fig. 4. The relationship between concentration of oestrogen and colour density using the ferrous sulphate colour method. x—x oestriol; O—O oestrone; Δ—Δ oestradiol.

The conditions for obtaining maximum colour densities with oestrone and oestriol were as follows:-

The first stage of colour production was performed by adding to the oestrogen, first ferrous sulphate (50 mg.) dissolved in water (1.0 ml.) slightly acidified with sulphuric acid and then concentrated sulphuric acid (3.0 ml.), and heating 10 min. After cooling, the second stage was performed by adding further water (1.0 ml.) and heating 4 min. The reaction products were cooled and diluted to 15 ml. with 30% (v+v) sulphuric acid. Colour densities were measured using Ilford No. 603 green filters and were stable. No part of the colour method was critical. Fig. 4 shows the relationship between colour density produced by this method and oestrogen concentration.

Under these conditions oestradiol-17β behaved erratically and sometimes produced no red colour. A considerable amount of work was performed in which the amounts of water added in the first and second stages and the heating times and temperatures were varied. The effect of adding ethanol and traces of ferric sulphate to the system was also investigated. Best results were obtained by omitting the first stage of the colour reaction but even then there were inconsistencies. Colour densities seemed to depend upon the mode of mixing the water, ferrous sulphate, sulphuric acid and oestradiol.

For instance, when the sulphuric acid was added carefully and with cooling to oestradiol (20 μg.) no red colour formed on heating (D_{603}=0.105). However, good colours (up to D_{603}=0.174) were obtained when the sulphuric acid was added quickly without cooling to the water, ferrous sulphate and oestradiol (20 μg.) and the mixture was allowed to stand a minute or two before heating in the water bath. It seemed that

the initial spontaneous heating caused by adding the sulphuric acid was a necessary factor but this could not be satisfactorily controlled.

The effect of ether residues upon the colour densities produced by this method was studied by adding oestrogens (20 µg.) to "peroxide free" diethyl ether (270 ml.) and distilling off the ether. The residues were transferred with ethanol to Kober tubes, the ethanol was removed and colours were developed with the ferrous sulphate method using the optimum conditions described above.

Oestrogen	D_{604} Standard from ethanol (0.4 ml.)		Standard from ether (270 ml.)	
Oestrone	.195	.195	.172	.178
Oestradiol-17β	.152		.127	.131
Oestriol	.148	.148	.123	.127

Contact of the oestrogens with ether caused a considerable depression in the colour densities formed with the ferrous sulphate method. Even though this method can produce higher colour densities than any other yet recorded, the depression by ether and the variable results obtained make it an unsatisfactory colour method for estimating oestrogens.

PHENOL AND p-CRESOL IN THE KOBER REACTION.

Dr. Clayton, in this laboratory, used the Kober colour method described by Venning et al. (1937) in which the reagent was a mixture of concentrated sulphuric acid and phenol. The method was performed as follows.

The reagent was prepared by adding slowly with careful mixing and mild cooling sulphuric acid (60 ml.) to melted, recently distilled

phenol (40 g.). The mixture was kept in the dark and used after 24 hrs.

Oestrogens, dissolved in ethanol, were distributed in 6 x $\frac{3}{4}$ in. test tubes, the ethanol was removed in a stream of air with the tubes in a boiling water bath, and the tubes and their contents were desiccated over anhydrous calcium chloride for at least 2 hrs.

The reagent was warmed to approx. 37°C and, using a large bore pipette, 3 ml. was measured into each tube at minute intervals. Each tube was heated for 20 min. without mixing and then cooled in an ice-salt freezing mixture for 9 min. Water (3 ml.) was added to each tube, the contents were stirred with a glass rod and kept in the freezing mixture a further 1 min., when they were stirred again. The tubes were heated another 3 min. with frequent stirring, and were then returned to the freezing mixture and kept there until all the tubes had been similarly treated. The contents of each tube (taken three at a time) were diluted to 15 ml. with 10% (v+v) sulphuric acid, stirred well, and the colours were measured immediately in the Spekker absorptiometer using 7 ml. of solution and Ilford No. 604 light filters. The remaining 8 ml. of solution was heated $1\frac{1}{2}$ hr. to fade the oestrogen colour*, volumes were corrected to 8 ml. with distilled water and the densities due to non Kober chromogens measured.

Using this method Dr. Clayton was unable to obtain 100% recoveries of oestrogens added to diethyl ether. Typical recovery results are given in the table. Oestrogens, in standard ethanol solutions, were merely added to 270 ml. of "peroxide free" diethyl ether, the ether was removed by distillation and the residues transferred with ethanol to Kober tubes. After removing the ethanol, colours were developed and were compared with those obtained by evaporating standard ethanol solutions.

*Correction method of Stevenson & Marrian (1947).

Oestrogen	D_{604} standard from ethanol.	D_{604} standard from ether (270 ml.)
Oestrone 20 µg.	.097	.112
" 50 µg.	.237	.272
Oestriol 20 µg.	.079	.092
" 50 µg.	.187	.212

Dr. Clayton showed that similar results were obtained when the oestrogens were added to the residue obtained when essentially peroxide free ether was evaporated. Careful purification of the ether to remove peroxides and distillation in complete darkness failed to remove the substance responsible for these results. This whole search for improved colour methods was made on the assumption that the substances left after evaporating ether did not destroy the oestrogens but only interfered in the critical stages of this colour method.

The colour method used by Dr. Clayton was the starting point for the investigation of reagents containing phenol or p-cresol and sulphuric acid. Preliminary experiments with phenol and p-cresol in the Kober reaction showed that their effects were similar and appeared to differ only in the tendancy of p-cresol to crystallize from concentrated mixtures when these were cooled in ice water. Because this crystallization was troublesome and p-cresol possessed no obvious advantage over phenol, a detailed investigation of reagents containing p-cresol was not performed. The work recorded below deals only with reagents containing phenol.

The phenol-sulphuric acid reagent of Cohen & Marrian.

Cohen & Marrian (1934) recommended the proportions of phenol and sulphuric acid which were later adopted by Venning et al. and used by

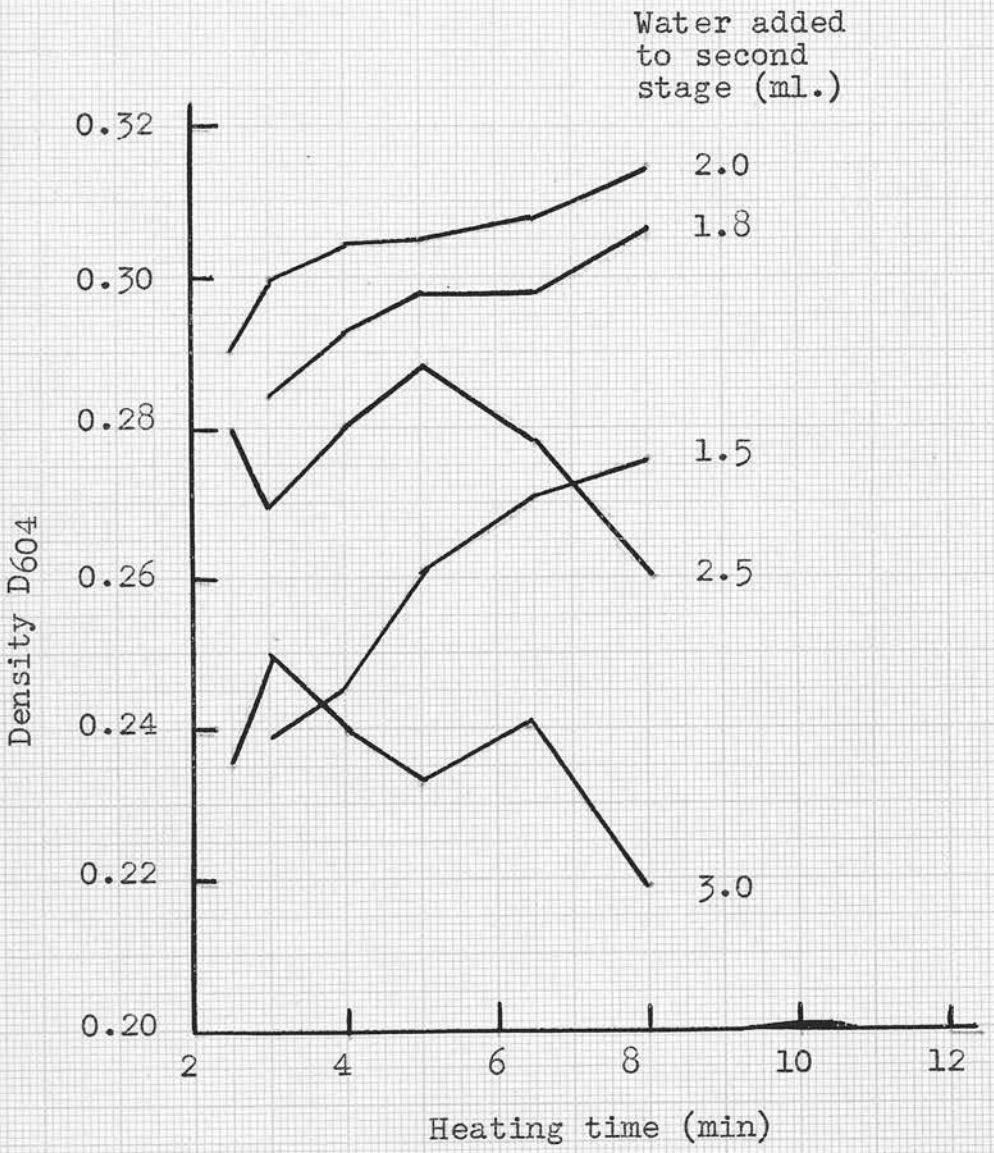


Fig. 5. The influence of the amount of water and the time of heating in the second stage of colour development upon the intensities of colours produced by the phenol sulphonic acid reagent and oestrone, 50 µg.

Dr. Clayton. The reagent and colour procedure used in this investigation were essentially as described above in Dr. Clayton's method. Variables investigated were the effect of water and heating times in the first and second stages of the Kober reaction.

The effect of varying the water added for the second stage of colour production was studied by heating oestrogen (50 µg.) with phenol-sulphuric acid reagent (3 ml.) for 20 min., cooling, and then adding various amounts of water and heating for a further 5 min. The resulting colours were diluted to 15 ml. with 10% (v+v) sulphuric acid and measured in the colorimeter using Ilford green /no. 604 light filters.

Volume of water (ml.)	D_{604} Oestrone	Oestradiol	Oestriol
3	.234	.141	.210
2.5	.288	.143	.227
2	.304	.145	.230
1.8	.298	.148	.226
1.5	.261	.128	.210

The effect of both heating time and water added for the second stage of colour production with oestrone (50 µg.) was examined in a similar manner, and is illustrated in Fig. 5.

It is seen that when the conditions recommended by Venning et al. were used (i.e. heating 3 min. with 3 ml. of water in the above experiment), a sharp maximum in colour intensity was obtained and this was followed by rapid fading. However, when 2.0 ml. of water was used, the colour intensity was not only increased but, once the maximum had been reached, it was less affected by continued heating. A similar stability to heating was observed with oestriol and oestradiol when colours were developed by adding 2.0 ml. of water for the second stage. This is shown in the next table.

Minutes - second heating	D_{604} Oestrone	Oestradiol	Oestriol
3	.300	.147	.219
4	.304	.153	.237
5	.304	.153	.233
6	.308	.137	.231
8	.314	.151	.231

The above experiments clearly indicated that with this phenol-sulphuric acid reagent the optimum procedure for the second stage was to add 2.0 ml. of water to the products of the first stage and to heat for 5 min..

Using these conditions for the second stage the effect of the time of heating in the first stage was examined. Phenol-sulphuric acid reagent (3 ml.) and 50 µg. of oestrogens were used as usual.

Minutes - first heating	D_{604} Oestrone	Oestradiol	Oestriol
6	.280	.150	.178
10	.284	.154	.222
15	.274	.150	.224
20	.272	.149	.220
30	.277	.152	.200

Evidently the duration of heating at the first stage was not critical, and the time (20 min.) recommended by Venning *et al.* was satisfactory.

The effect of adding water to the first stage was next investigated. Oestrogens (50 µg.), reagent (3 ml.), water and 20 min. heating were used in the first stage. Water was added for the second stage so that the total water added to the system was 2.0 ml., and the second heating time was 5 min. The results are shown in the table.

Water added to first stage (ml.)	D_{604} Oestrone	Oestradiol	Oestriol
0	.276	.153	.223
.05	.278	-	.204
.1	.289	.153	.176
.25	.291	.166	.170
.5	.270	.150	.171
.75		.157	
1.0		.154	
1.5		.151	

In the case of oestrone and oestradiol, water added to the first stage caused a small increase in colour densities but in the case of oestriol even traces of water caused a marked decrease in colour. These findings agreed with those of Dr. Clayton who had concluded that the day to day variations in the density of colours produced by the phenol-sulphuric acid reagent, especially with oestriol, were due to slight changes in the water activity of the reagent. She attempted to minimize these variations by desiccating tubes and contents before colour production and preventing entrance of water vapour to tubes during colour development.

A slight modification of Venning's colour method in which 2.0 ml. instead of 3.0 ml. of water and 5 min. instead of 3 min. heating was used in the second stage, appeared to be a distinct improvement on her original method. A summary of the modified method follows, the best technical details being the same as those used by Dr. Clayton and described at the beginning of this section. However, the care in observing precise heating times was no longer necessary.

Phenol-sulphuric acid reagent: concentrated sulphuric acid (60 ml.) plus phenol (40 g.).

Method: The oestrogen was heated 20 min. with phenol-sulphuric acid

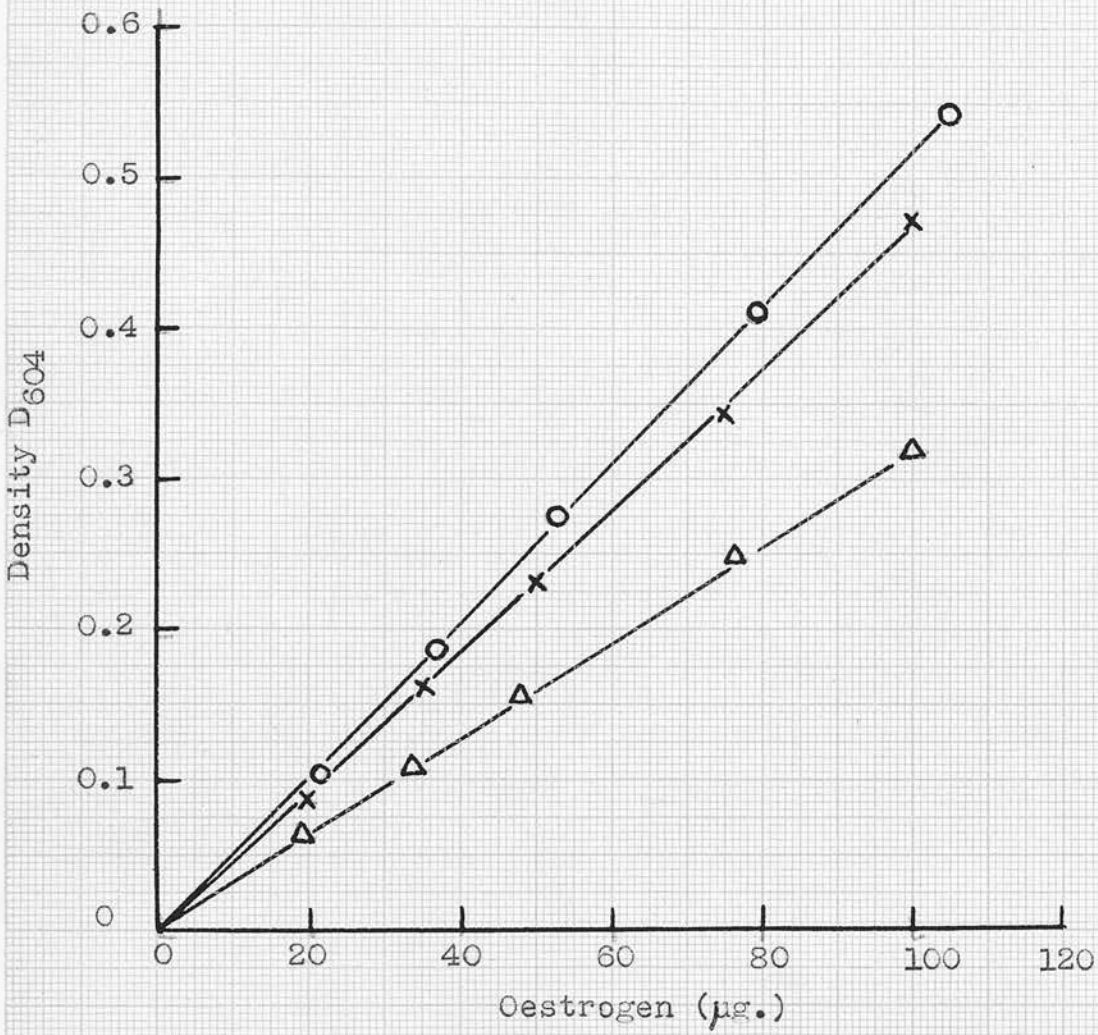


Fig. 6. The relationship between concentration of oestrogen and colour density using the modified phenol sulphonic acid colour method.
x—x oestriol; O—O oestrone; Δ—Δ oestradiol.

reagent (3 ml.). After cooling, water (2.0 ml.) was added and the mixture was heated 5 min., cooled and diluted to 15 ml. with 10% (v+v) sulphuric acid. Colour densities were measured using light filters transmitting maximally at 620 mμ.

This procedure is the "MODIFIED PHENOL-SULPHONIC ACID METHOD" used in later work.

Using this method the relationship between colour intensity and oestrogen concentration was investigated and is shown in Fig.6. Beer's Law was closely obeyed.

The effect of ether residues upon the colour densities developed by this method was studied by adding oestrogens to diethyl ether (270 ml.), and distilling off the ether either immediately or after the solution had been standing at room temperature for some time. The residues were transferred with ethanol to Kober tubes, the ethanol was removed in a stream of air with heating, and colours were developed.

a) Using essentially peroxide free ether which had been treated with ferrous sulphate solution and distilled under subdued light.

1. Using oestrogen (50 μg.)

D_{604}

	Standards (50 μg.)	Ether distilled after standing		
		Immediately	2 hours	4 hours
Oestriol	.228 .226	.228 .232	.229 .232	.232 .226
Oestrone	.275 .280	.276 .280	.272 .274	.278 .280
Oestradiol	.160 .156 .162	.156 .162 .156	.154 .162 .160	

2. Using various concentrations of oestrogens.

D_{604}

Ether distilled after standing 2 hours

	20 µg. standard from ether		35 µg. standard from ether		50 µg. standard from ether	
Oestriol	.084 .082	.084 .082	.148 .143	.143 .147	.221 .217	.220 .216
Oestrone	.099 .102	.102 .102	.184 .184	.181 .181	.271 .280	.274 .280

b) Using diethyl ether which contained peroxides.

Three grades of ether were used, (a) A.R. ether from the manufacturer (J.F. Macfarlan) without further purification, (b) a sample which had been stored 6 months and which gave a strong test for peroxides by the iodide-starch method, (c) purified ether to which a "trace" of hydrogen peroxide had been added.

The colour densities obtained using oestriol (50 µg.) and these grades of ether are shown in the table.

D_{604}

Standard reading	A.R. ether (Macfarlan)	Stored ether containing peroxides	Ether with added hydrogen peroxide
.230 .222	.222 .226	.194 .106	.214 .215

Apparently the modified phenol sulphonic acid method was not affected by ether residues when the ether was essentially peroxide free although depression of colour did occur when considerable amounts of peroxides were present.

As satisfactory recoveries of oestrogens from ether solutions were obtained by this colour method, it was considered suitable for further development of the oestrogen method, and was used in the preliminary work on the partition of oestrogens between immiscible solvent pairs.

Other phenol-sulphuric acid reagents.

The experiment recorded in the previous section in which water was added to the phenol-sulphuric acid reagent of Cohen & Marrian indicated that the sulphuric acid concentration of this reagent was not optimal for any one of the three oestrogens. Results obtained, even by the modified phenol-sulphonic acid method, were variable from one batch of reagent to another, and it was felt that an investigation into the effect of changing the sulphuric acid concentration of the reagent might lead to an even more stable colour reaction. The proportions of phenol and sulphuric acid recommended by Cohen & Marrian gave a reagent which was probably the best compromise between the conflicting requirements of the three oestrogens. On the basis of results obtained with solutions of sulphuric acid, water and hydroquinone it appeared that the phenol-sulphuric acid reagent had properties similar to those of a 75% sulphuric acid-2% hydroquinone reagent. It was therefore decided to assign to the phenol (40 g.)-sulphuric acid (60 ml.) solution an "active sulphuric acid concentration" equivalent of 75%, i.e. that of the 75% sulphuric acid hydroquinone reagent. Reagents with other sulphuric acid concentrations were prepared from the standard phenol (40 g.)-sulphuric acid (60 ml.) reagent by adding the amounts of sulphuric acid or water shown below.

"Active sulphuric acid concentration"	Added to 40 ml. of phenol-sulphuric acid reagent.	
	Sulphuric acid (ml.)	Water (ml.)
80%	10	-
75%	-	-
70%	-	3
66%	-	5
60%	-	10
55%	-	15

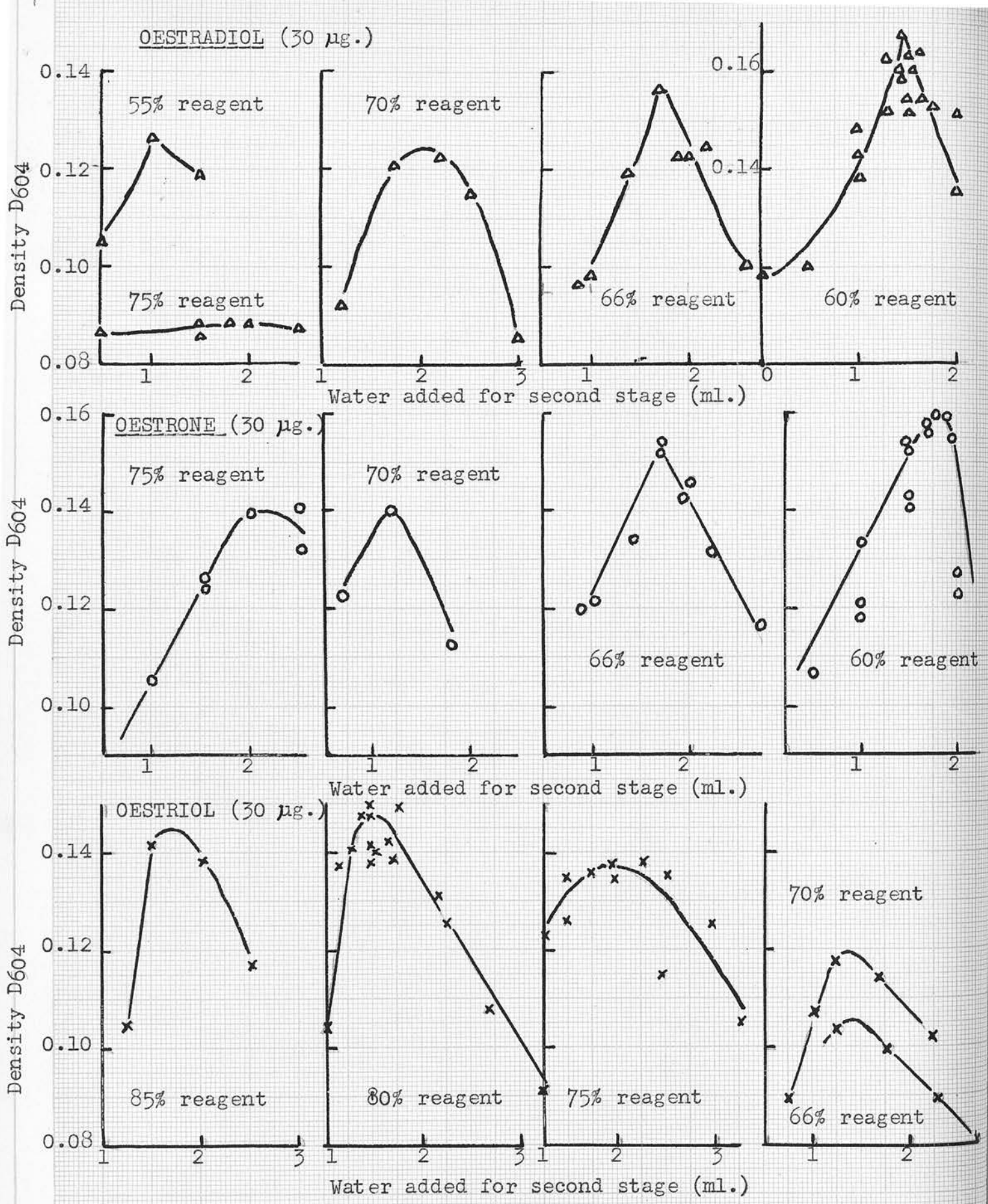


Fig. 7. The influence of water in the second stage on densities of colour developed with various phenol sulphonic reagents.

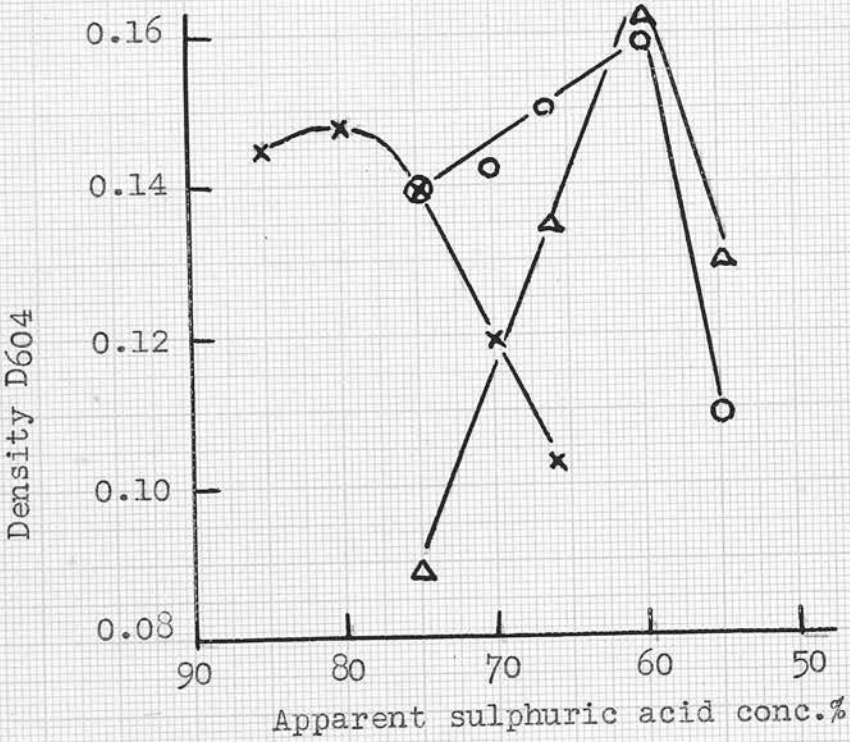


Fig. 8. The influence of the "apparent sulphuric acid-water ratio" in the first stage of the Kober reaction upon the density of colour using phenol sulphonic acid reagents. x—x, oestriol, 30 µg.; 0—0, oestrone, 30 µg.; Δ—Δ, oestradiol, 30 µg.

The effect of varying the water added for the second stage of colour production was studied by heating oestriol, oestrone or oestradiol-17β (30 µg.) with reagent (3 ml.) for 20 min. cooling and then adding various amounts of water and heating for a further 5 min. The resulting colours were diluted to 15 ml. with 30% (v+v) sulphuric acid and measured in the colorimeter using Ilford green No. 604 light filters.

The results are summarized in the **Figure 7.**

A considerable number of the experiments were repeated on different days, sometimes with fresh reagents and an extraordinary number of these gave results which did not agree closely with other replicates. The amount of water required for maximum production of colour was very critical and seemed to vary from day to day and between batches of reagent. The cause for this critical behaviour seemed to be an intrinsic part of the phenol-sulphuric acid reagents themselves.

The next figure (Fig. 8) relates the maximum colours obtained with each reagent, with the "active sulphuric acid concentration" of the reagent, and is very similar to the one obtained with the hydroquinone-sulphuric acid reagents (see page 40) except that the acid concentration again is more critical. The "active sulphuric acid concentration" which produced maximum colour densities was 80% for oestriol, 60% for oestrone and 60% for oestradiol-17β. Using these sulphuric acid concentrations (i.e. 80% for oestriol, 66% for oestrone and 60% for oestradiol-17β) and the optimum amounts of water for the second stage of colour development, the effect of varying the time of heating in the first stage was studied. The second stage was performed by adding water (1.5 ml. to both oestriol and oestradiol and 1.8 ml. to oestrone) to the products of the first stage and heating for 5 min.

Oestrogen 30 µg.	Reagent (3 ml.)	Water added for second stage (ml.)	D_{604} Minutes heating in first stage 5	10	20	30
Oestriol	"80%"	1.5		.162	.148	.130
Oestrone	"66%"	1.8	.110	.153	.163	.153
Oestradiol	"60%"	1.5	.117	.146	.154	.154

The effect of varying the time of heating in the second stage was studied by heating the oestrogens (30 µg.) with their appropriate reagent for 20 min., cooling, adding water and heating for various intervals of time. Colours were diluted and measured in the usual manner. In the case of oestriol three levels of water added for the second stage were studied.

Second stage	Oestriol "80%" reagent			Oestrone "66%" reagent	Oestradiol "60%" reagent
Water added (ml.)	1.2	1.5	1.8	1.7	1.4
Heating time	D_{604}				
2½ min.	.123	.143	.146	.140	.150
5 min.	.138	.142	.152	.153	.160
8 min.	.132	.141	.141	.151	.157
12 min.	.137	.140	-	.150	.150

Evidently the procedure for developing maximum colours with these phenol-sulphuric acid reagents was as follows.

Reagents (3 ml.)	Oestriol "80%" reagent Oestrone "66%" " Oestradiol "60%" "
Heating times:	20 min. for the first stage 5 min. for the second stage
Water added for the second stage	Oestriol 1.5 ml. Oestrone 1.8 ml. Oestradiol 1.5 ml.

An examination of the above results shows that colour densities produced by these procedures were not reproducible from experiment to experiment. In an attempt to find more stable conditions which would give more reproducible results the effect of various diluents was examined, since these were found to influence final colours obtained with the hydroquinone-sulphuric acid reagents. Two concentrations of sulphuric acid (10% v+v and 30% v+v) were examined and these were used to dilute the products of the second stage of colour development to 15 ml. Colour densities were measured at time intervals after dilution.

D_{604}

Time after dilution (min.)	Oestriol 30 µg.		Oestrone 30 µg.		Oestradiol 30 µg.	
	Sulphuric acid diluent (%v+v)					
	30%	10%	30%	10%	30%	10%
0	.148	.140	.157	.162	.159	.149
10	.152	.143	.158	.163	.158	.149
30		.140		.161		.141
60		.137				
120	.161		.165	.161	.158	.141

Colour densities were affected by the sulphuric acid concentration of the diluent but were fairly constant after dilution.

Further work using these modified phenol-sulphuric acid reagents failed to give more reproducible results. In this case achievement of maximum colour had not been accompanied by increased stability in the method and reproducibility of the final colour density. These more sensitive methods using phenol-sulphuric acid reagents were therefore abandoned.

HYDROQUINONE AND PYROCATECHOL IN THE KOBER REACTION.

Preliminary work showed that the behaviours of hydroquinone and pyrocatechol were similar in the Kober colour reaction. Only hydroquinone was selected for complete investigation because it was the cheaper chemical and very readily available.

Reagents were prepared by dissolving hydroquinone (B.D.H. reagent) in mixtures of sulphuric acid and water. A concentration of 2% w/v hydroquinone was selected because it was the highest concentration which did not crystallize at the lowest sulphuric acid concentration studied. Solution of hydroquinone in the sulphuric acid mixtures was usually aided by warming, and the reagents were kept at least overnight before use.

Reagents with sulphuric acid concentrations between 80% v/v and 64% v/v were studied.

The first table shows the effect of different amounts of water added for the second stage of colour development. The first stage was performed by heating the oestrogen (20 µg.) for 20 min. with 4 ml. of reagent. Water was then added and heating was continued for 4 min. The reaction mixture was cooled and diluted to 15 ml. with 30% (v+v) sulphuric acid. Colour densities were measured using both Ilford green No. 603 and No. 604 light filters. Densities were slightly higher with the No. 604 filters and only these are recorded in the table.

% H_2SO_4 in reagent	ml. H_2O added for 2nd stage	D_{604} Oestrone	Oestradiol	Oestriol
80	.8	.167	.076	.127
"	1.2	.159	.078	.125
"	1.6	.157	.080	.127
"	2.0	.149	.080	.126
76	1.0	.162	.098	.136
"	1.4	.162	.090	.135
"	1.8	.155	.092	.127
72	.4	.180	.108	.132
"	.8	.176	.109	.132
"	1.2	.175	.113	.134
"	1.6	.180	.115	.125
68	-	.178	.129	-
"	.4	.180	.131	-
"	.8	.175	.134	-
"	1.2	.175	.135	-
64	-	.173	.146	.068
"	.4	.174	.146	.063
"	.8	.179	.147	.062
"	1.2	.166	.139	.061

Evidently with these hydroquinone reagents the water requirements in the second stage of colour development were not at all critical. A sulphuric acid concentration of about 60% in the second stage appeared to be as satisfactory as any.

By adding water to the products of the first stage so that the acid concentration in the second was approximately 60% and heating 4 min., the effect of varying the time of heating at the first stage was studied. Reagent (4 ml.) and oestrogen (20 µg.) were used as before.

% H_2SO_4 in reagent	ml. H_2O for 2nd. stage	Heating time 1st stage (min.)	D_{604} Oestrone	Oestradiol	Oestriol
80	1.2	40	-	-	.123
"	"	20	.154	.077	.125
"	"	15	.161	.079	.131
"	"	10	.159	.078	.128
"	"	5	.161	.077	.092
"	"	$2\frac{1}{2}$	.163	.078	-
76	1.0	30	-	-	.136
"	"	20	-	-	.136
"	"	15	-	-	.134
"	"	10	-	-	.122
"	"	5	-	-	.081
72	0.8	40	-	-	.122
"	"	20	.179	.107	.132
"	"	15	.183	.112	.100
"	"	10	.176	.109	.073
"	"	5	.178	.110	.043
"	"	$2\frac{1}{2}$	.152	.110	-
68	.6	20	.181	.132	-
"	"	15	.181	.134	-
"	"	10	.181	.135	-
"	"	5	.162	.128	-
"	"	$2\frac{1}{2}$	.129	.123	-
64	0	40		-	.102
"	"	20	.188	.149	.068
"	"	15	.179	.139	.043
"	"	10	.173	.145	.027
"	"	5	.155	.134	.014
"	"	$2\frac{1}{2}$	.120	.127	

The higher the sulphuric acid concentration was in the first stage, the shorter was the optimum heating time. The best reagents required 20 min. heating in the first stage for maximum colour production.

Using the optimum heating times indicated in the previous experiment for the first stage and a concentration of 60% sulphuric acid in the second, the effect of varying the time of heating at the second stage was studied, with the results shown in the next table.

% H_2SO_4 in reagent	ml. H_2O for 2nd. stage	Heating time 2nd. stage	D_{604} Oestrone	Oestradiol	Oestriol
80	1.2	-	.125	.063	.127
"	"	2	.151	.073	.135
"	"	4	.159	.078	.130
"	"	6	.160	.082	.137
"	"	9	.161	.082	.136
76	1.0	-	-	-	.121
"	"	3	-	-	.138
"	"	4	-	-	.139
"	"	6	-	-	.138
"	"	9	-	-	.135
72	.8	-	.177	.098	.130
"	"	2	.183	.098	.133
"	"	4	.179	.109	.132
"	"	6	.182	.103	.136
"	"	9	.187	.111	.136
68	.6	-	.172	.131	-
"	"	3	.177	.131	-
"	"	4	.180	.132	-
"	"	6	.180	.138	-
"	"	9	.180	.138	-

Evidently the heating time for the second stage was not at all critical.

From the above work the following reagents containing hydroquinone were selected, 76% sulphuric acid for oestriol and 65% sulphuric acid for oestrone and oestradiol-17β.

A method for the colorimetric determination of oestriol, oestrone and oestradiol-17β based on hydroquinone reagents.

Preparation of reagents.

Oestriol reagent: Hydroquinone (2 g.) was dissolved with warming in 100 ml. of 76% (v/v) sulphuric acid, prepared by diluting with cooling, 76 ml. of pure sulphuric acid to 100 ml. with water.

Oestrone and oestradiol-17β reagent: Hydroquinone (2 g.) was dissolved similarly in 100 ml. of 65% (v/v) sulphuric acid.

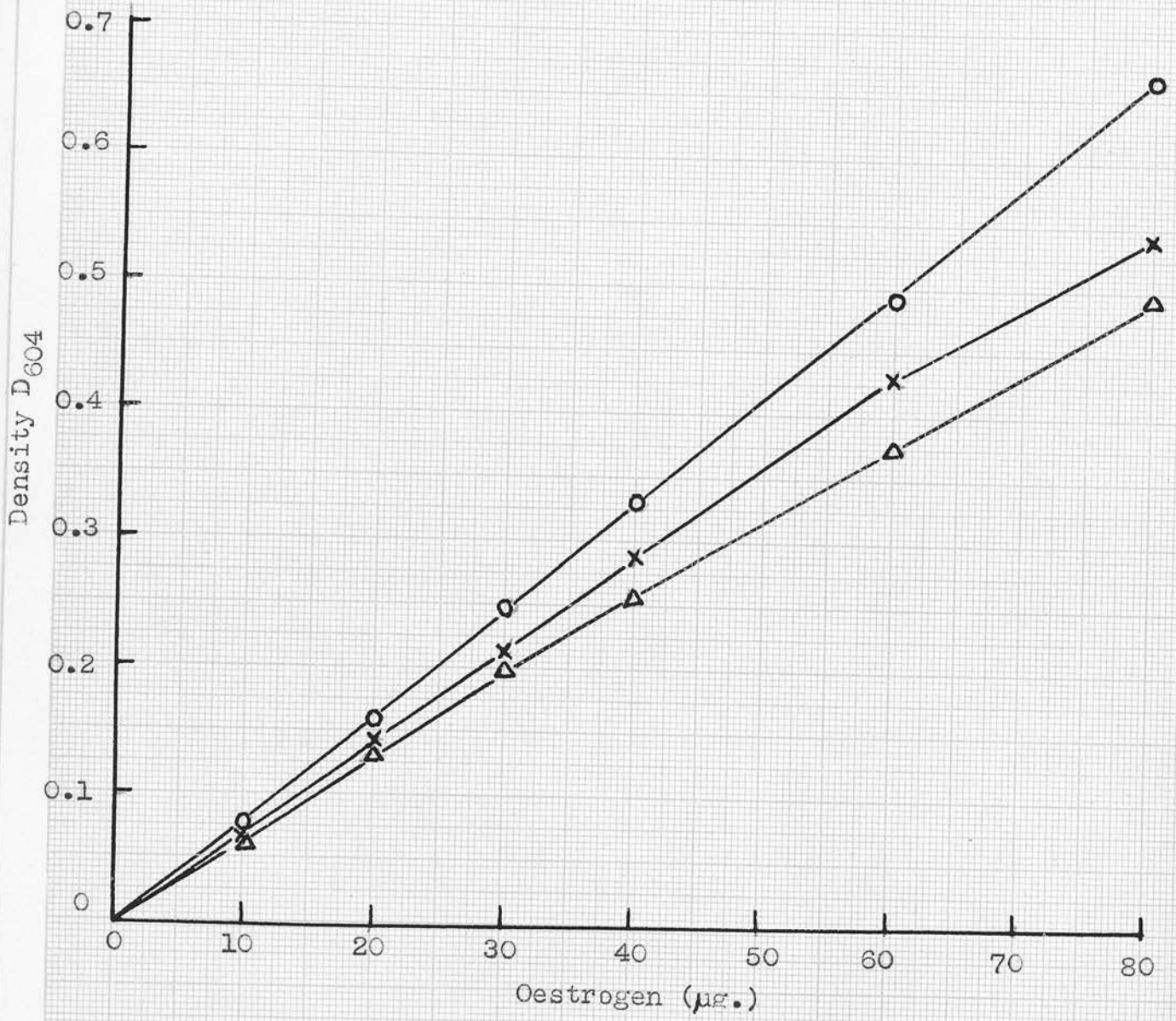


Fig. 9. The relationship between concentration of oestrogen and colour density using the hydroquinone-sulphuric acid reagents. x—x oestriol; O—O oestrone; Δ—Δ oestradiol.

These reagents kept well at room temperature. A certain maturing took place in the first week during which the solutions darkened to a light brown colour. After this the reagents were stable and colour densities formed by them were reproducible.

Sulphuric acid 30% (v+v): 30 ml. of concentrated sulphuric acid was added to 70 ml. of water.

Colour development.

4 ml. of the appropriate reagent was added to the oestrogens in tubes and heated for 20 min. in a boiling water bath. The tubes were shaken once during the first 5 min. (notes 1 and 2). After heating, the tubes were cooled in ice water for about 5 min. (note 3). 1 ml. of water was added to the oestriol tubes and 0.5 ml. of water to the oestrone and oestradiol tubes (note 4). After mixing, the tubes were heated 5 min. (note 2) and then cooled in ice water about 5 min. (note 3). The contents were diluted to 15 ml. with 30% (v+v) sulphuric acid. The tubes were allowed to stand at room temperature at least 5 min. and were read in the colorimeter within 45 min. of dilution. Colours absorbed light maximally at wavelengths between 515 and 520 mμ.

Notes on the hydroquinone colour method.

1. Small amounts of water did not interfere so that, during heating, no precautions were required to prevent entrance of water vapour.

2. The heating times were not critical. For oestriol the first heating time could be varied between 15 and 45 min. and for oestrone and oestradiol-17β, between 10 and 30 min. without appreciable change in colour density.

3. At these stages reaction products were stable almost indefinitely.

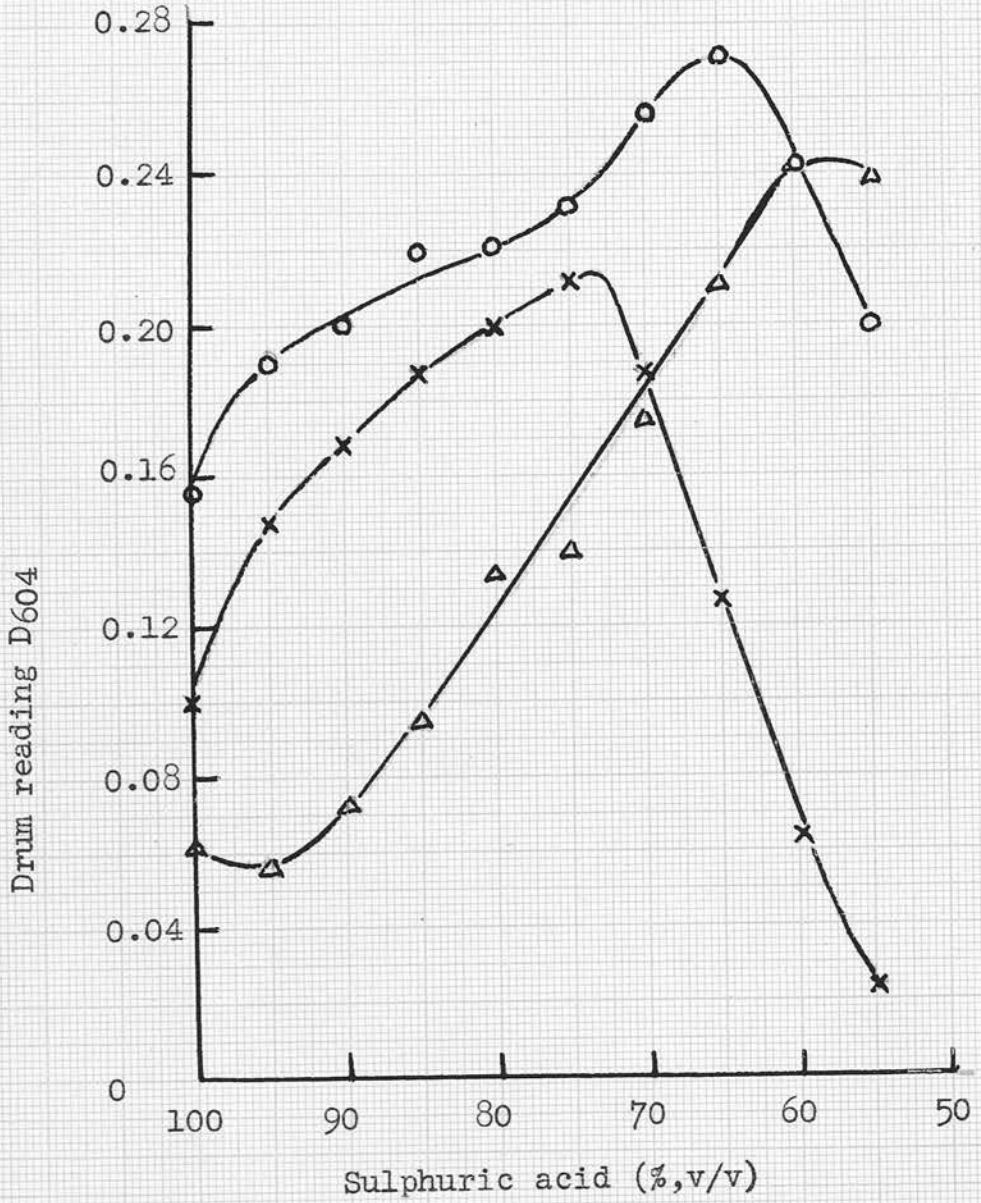


Fig. 10. The influence of the sulphuric acid-water ratio in the first stage of the Kober reaction upon the intensity of colour. using hydroquinone reagents. x—x oestriol, 30 µg.; 0—0 oestrone, 30 µg.; Δ—Δ oestradiol, 30 µg.

4. The water required for the maximum production of colour was not critical. For oestriol the water added could be varied between 0.5 and 1.5 ml. and for oestrone and oestradiol-17β the addition of water and second heating could be omitted without significant change in colour densities.

The method gave good proportionality between the amount of oestrogen present and the density of colour produced, as shown in Fig. 9. Later, when a method was developed which could separate oestradiol from oestrone in mixtures, a third reagent containing 60% sulphuric acid 2% hydroquinone was introduced for oestradiol and the sulphuric acid concentration of the oestrone reagent was increased to 66%. The methods for developing colours with these reagents were the same as before except that, in the case of the 60% sulphuric acid reagent, the addition of water and heating for the second stage of colour production was omitted.

Experiments with the hydroquinone colour method.

The effect of sulphuric acid concentration in the first stage of the Kober colour reaction using hydroquinone as the reducing agent was studied in more detail than previously.

Hydroquinone reagents containing 2% w/v hydroquinone in varying concentrations of sulphuric acid and water, were prepared and stored 24 hrs. before use. The first stage of colour development was performed by adding reagent (4 ml.) to oestrogen (30 µg.) and heating 20 min. The second stage was performed by adding water so that the total sulphuric acid concentration was 60% and heating 4 min. The reaction mixture was cooled and diluted to 15 ml. with 30% (v+v) sulphuric acid and after standing at least 5 min. at room temperature, colours were measured against their reagent blank, (Fig.10). The optimum sulphuric acid concentration in the

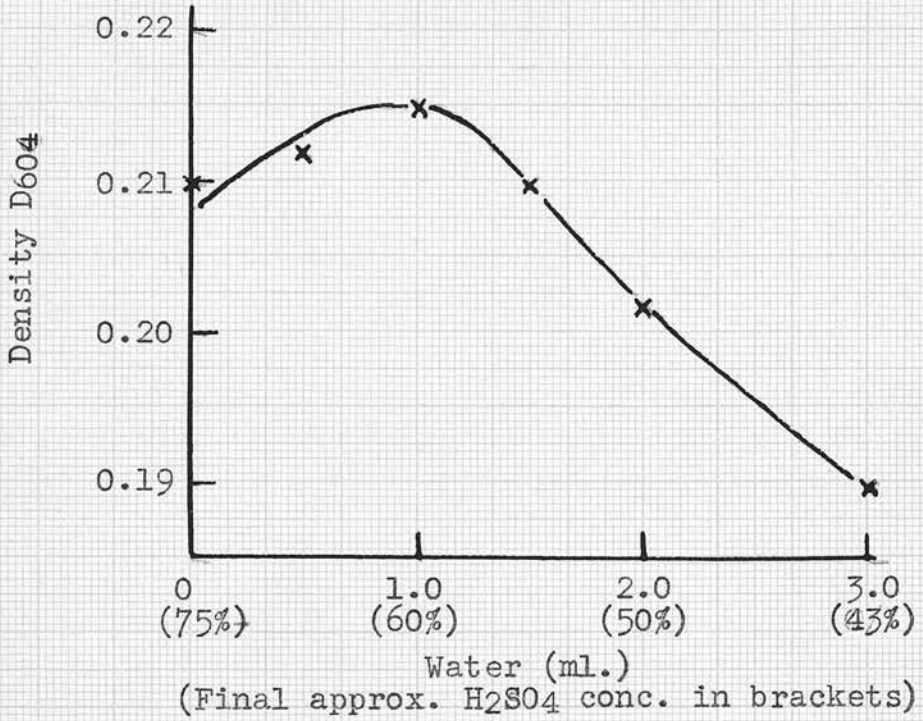


Fig. 11. The effect of varying the water in the second stage on the colour produced by oestriol (30 µg.) and the sulphuric acid 76%-hydroquinone 2% reagent.

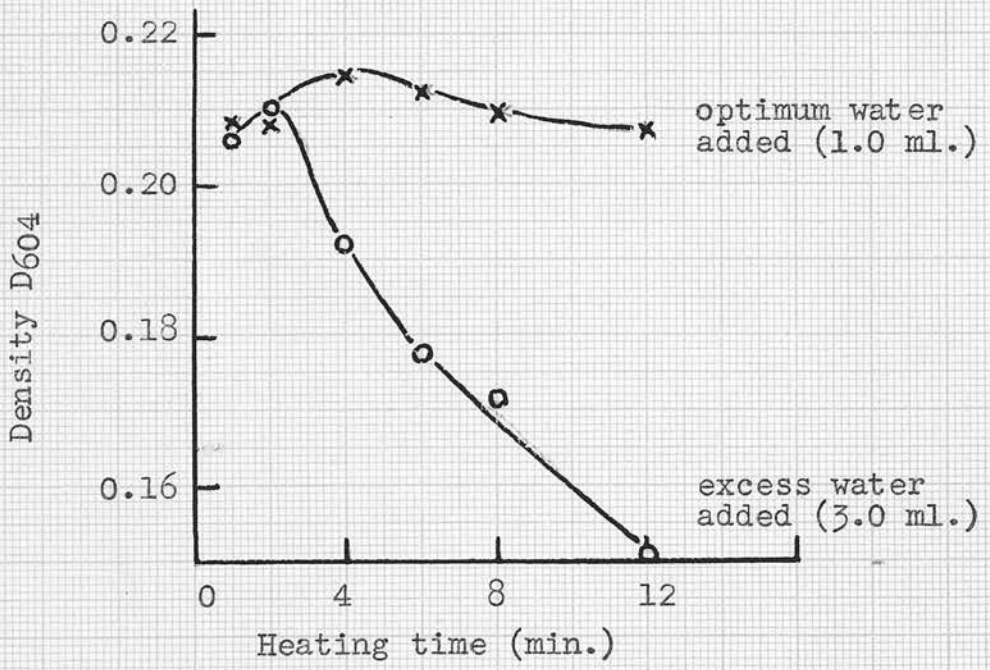


Fig. 12. The effect of varying the heating time and the amount of water added in the second stage of colour production with oestriol (30 µg.) and the 76% sulphuric acid-2% hydroquinone reagent.

first stage was different for each oestrogen, and was approximately 76% (v/v) acid for oestriol, 65% acid for oestrone and 60% acid for oestradiol-17β. When the sulphuric acid concentration was decreased below 73% the intensity of red colour produced with oestriol decreased rapidly, while when the sulphuric acid concentration was greater than 76% oestradiol formed no visible red Kober colour in the second stage of colour development. These differences in response to the sulphuric concentration of the reagent are probably the main cause for the different properties of the various Kober reagents which have been described in the past. It is obvious that no one reagent is likely to be suitable for estimating all three oestrogens.

The effect of water in the second stage using the 76% sulphuric acid-2% hydroquinone reagent (4 ml.), oestriol (30 µg.) and 20 min. heating in the first stage was also studied in more detail. The results are shown in Fig.11. Evidently the amount of water (1.0 ml.) selected for the above colour method was optimal.

The influence of the second heating time upon colour production was repeated using oestriol (30 µg.) and the 76% sulphuric acid-2% hydroquinone reagent. The first stage was performed by heating 20 min. with 4 ml. of reagent and the second stage by adding (a) 1.0 ml. (optimal) and (b) 3.0 ml. (excess) water and heating again (Fig.12).

The results show the stability of the red colour to heating when the optimal sulphuric acid concentration was used in the second stage of colour development, and instability when excess water was used.

The effect of time between diluting the Kober colour and reading its absorption in the colorimeter.

The technical work on the development of the hydroquinone colour method was performed by J.B.B. However, another worker, H.A.F.B. using the method

obtained consistently lower colour densities than J.B.B., especially with oestriol or oestriol methyl ether. This was not due to the use of different reagents or to the use of reagents of different ages. The following experiment supported previous findings in this respect.

Colour densities produced by oestriol (20 µg.) with two 76% sulphuric acid 2% hydroquinone reagents, one prepared 24 hrs. previously by H.A.F.B. and the other prepared 3 months previously by J.B.B. were compared by both workers.

	D_{604}	
	J.B.B.	H.A.F.B.
New reagent	.135	.110
Old reagent	.135	.115

A careful comparison between workers showed that H.A.F.B. still followed the phenolsulphuric acid method in the care taken to chill the products of the second stage, dilute them singly and to measure the colours immediately while J.B.B. diluted the colours batchwise and therefore allowed the diluted colours to stand for some time before measuring their density. The cause of the differences in colour densities might therefore be due to differences in temperature of the solutions at the time of colour measurement or to a change in the density on standing after dilution.

The effect of the temperature of the diluted colour on its density was studied by developing colour with oestriol (20 µg.) and measuring the density after dilution at various temperatures.

Approx. temperature of the diluted colour °C.	32	25	21	20	15
Colour density D_{604}	.137	.133	.136	.135	.135

Evidently temperature had no effect on the colour density.

The effect of time between diluting the colour and measuring its absorption was studied with oestriol (20 µg.) by cooling the products of the second stage—(a) rapidly to 30°C., diluting to 15 ml. with 30% sulphuric acid and measuring the colour immediately and at time intervals thereafter.

Time after dilution (min.)	0	2	5	8	30	40	100
D_{604}	.117	.127	.131	.134	.134	.136	.132

(b) in ice water for 10 min. (temperature then 8°C.), diluting to 15 ml. with 30% sulphuric acid (temperature then 18°C.) and measuring the colour immediately and at time intervals thereafter.

Time after dilution (min.)	1	3	8	13	20	60
D_{604}	.125	.130	.137	.141	.140	.141

Evidently the discrepancy between the figures obtained by the two workers was due to the fact that after dilution the colour densities increased and reached a maximum in about 6 min. The density then remained constant for more than an hour. When this was appreciated, all diluted colours were allowed to stand at least 5 min. at room temperature before measuring their densities. Results obtained by both workers were then identical.

A similar increase in colour after dilution was noted when the modified phenol sulphonic acid colour method was used.

The effect of ether residues upon the colour densities produced by the hydroquinone-sulphuric acid colour method was studied by adding oestrogen in standard alcoholic solution to "peroxide free" diethyl ether (300 ml.) allowing this to stand 2 hrs. at room temperature and distilling off the ether. The residues were transferred with ethanol to Kober tubes. The

ethanol was removed by heating in a stream of air, and colours were developed with the hydroquinone-sulphuric acid colour method described above.

Oestrogen	Standard from ethanol only	Standard from ether (300 ml.)
Oestrone (25 µg.)	.211	.213
Oestradiol (20 µg.)	.125	.129
Oestriol (20 µg.)	.120	.121

The hydroquinone colour method therefore gave reliable results in the presence of ether residues. This was further confirmed before the method was used to replace the modified phenol sulphonic acid method as the routine procedure for estimating oestrogens. (See sections on partition coefficients and recovery experiments).

THE DEVELOPMENT OF THE KOBER COLOUR WITH OESTROGEN METHYL ETHERS.

Marlow (1950) showed that oestrone methyl ether produced with a modified Kober reagent containing β-naphthol and sulphuric acid, colours which had densities greater than those obtained from equivalent weights of the free oestrogen.

Using the hydroquinone and the modified phenol sulphonic acid colour methods, the density of colours produced by various concentrations of pure oestrogen methyl ethers (see appendix) was studied. The results shown were obtained with standard ethanol solutions evaporated by heating in a stream of air, and the colour densities were measured immediately after dilution.

Methyl ethers	Hydroquinone reagent							
	10 µg.		20 µg.		35 µg.		50 µg.	
Oestriol	.057	.059	.120	.122	.199	.204	.290	.283
Oestrone	.074	.078	.154	.150	.235	.238	.363	.369
Oestradiol	.054	.056	.108	.118	.179	.176	.243	.250

Methyl ethers	Phenol sulphonic acid							
	10 µg.		20 µg.		35 µg.		50 µg.	
Oestriol	.040	.039	.082	.079	.148	.143	.210	.212
Oestrone	.050	.046	.099	.094	.169	.174	.250	.254
Oestradiol	.028	.031	.062	.059	.112	.110	.156	.150

The results obtained were very similar to those for the free oestrogens. However, the conditions which were optimal for the free oestrogens could not be assumed to be optimal for the methyl ethers without further investigation.

The effect of the sulphuric acid concentration of the reagent and the time of the heating in the first stage of colour development was studied.

Hydroquinone reagents containing 2% w/v hydroquinone in varying

concentrations of sulphuric acid and water, were prepared and stored at least 24 hrs. before use. The first stage of colour development was performed by adding reagent (4 ml.) to oestrogen methyl ethers (20 µg. measured in standard ethanolic solutions and the ethanol evaporated in a stream of air with heating), and heating for varying periods of time. The second stage was performed by adding water so that the total sulphuric acid concentration was 60% and heating 4 min. The reaction mixture was cooled and diluted to 15 ml. with 30% (v+v) sulphuric acid and the colours were measured against their reagent blank without considering the time between dilution and measurement.

Oestriol methyl ether.

Using sulphuric acid 76%-hydroquinone 2% reagent (4 ml.) only:-

Minutes heating	10	20	30	40
D_{604}	.097	.125	.129	.129

Changing the sulphuric acid concentration of the reagent, but heating 30 min. for the first stage of colour development.

Sulphuric acid conc. (%v/v)	80	76	74	72	68
D_{604}	.123	.126	.130	.127	.113

Oestrone and oestradiol methyl ethers.

H_2SO_4 conc. in reagent. (% v/v)	Water for 2nd. stage (ml.)	Heating time 1st. stage (min.)	D_{604} Oestrone methyl ether (20 µg.)			Oestradiol methyl ether (20 µg.)
72	0.8	20		.139		.121
		15		.146		.105
		10		.148		.116
		5		.147		.102
66	0.5	20	.149	.185	.182	.137
		15	.170	.181	.172	.132
		10	.143	.173		.137
		5	.130	.153		.132
60	0	20		.172		.148
	2nd. stage	15		.160		.144
	omitted	10		.137		.148
		5		.098		.122

The response of the methyl ethers to sulphuric acid concentration and heating times in the first stage was therefore similar to that of the free oestrogens.

The effect of water in the second stage using oestrone and oestradiol methyl ethers (20 µg.) and hydroquinone reagents (4 ml.) containing 72%, 66% and 60% sulphuric acid and 15 min. heating in the first stage was studied in a similar manner.

H_2SO_4 conc. in reagent % v/v	Water for 2nd. stage (ml.)	D_{604} Oestrone methyl ether			Oestradiol methyl ether
72	1.2		.163		.112
	0.8		.163		.100
	0.4		.173		.111
66	1.0	.179	.157	.180	.130
	0.5	.176	.170	.175	.115
	0	.178	.180	.169	.120
60	0.4		.159		.128
	0		.159		.143

The influence of the heating time in the second stage of colour development was studied using oestrone methyl ether (20 µg.) and the 66% sulphuric acid-2% hydroquinone reagent. The first stage was performed by heating 20 min. with reagent (4 ml.) and the second stage by adding water (0.5 ml.) and heating for various periods of time.

Heating time (min.)	0		2		8	
D_{604}	.170	.187	.180	.176	.164	.185

The conditions for developing maximum colours with the oestrogen methyl ethers and the hydroquinone-sulphuric acid reagents were essentially the same as for the free oestrogens. However, colour densities developed, especially by oestrone methyl ether, were not entirely reproducible and poor agreement between replicates was observed.

The cause for these inconsistencies was further investigated by applying the following strict method of colour development and varying some of the minor details of procedure.

Oestrone methyl ether (20 µg.) in 0.4 ml. of standard ethanol solution (5 mgm.%) was measured into tubes and the ethanol removed in a stream of air with heating. Sulphuric acid 66%-hydroquinone 2% reagent (4 ml.) was added and the tubes immersed in boiling water for 20 min. The contents were carefully mixed after heating 1½ and 5 min. After heating 20 min. the tubes were cooled 3½ min. in ice water, water (0.6 ml.) was added and mixed immediately. Tubes were heated 4 min. in boiling water and cooled in ice water 5 min. The contents were diluted to 15 ml. with 30% (v+v) sulphuric acid with immediate mixing and the colour densities were measured at least 5 min. after dilution.

By this procedure the colour density D_{604} = 0.172; when the reagent was added to the oestrone methyl ether and left 70 min. before heating in the water bath for the first stage of colour development D_{604} = 0.170; when water was added for the second stage but was not mixed immediately but was allowed to stand for 5 min. before mixing D_{604} = 0.175; when the final colour was diluted to 15 ml. but was allowed to stand unmixed for 5 min. D_{604} = 0.173.

Standard oestrone methyl ether from which the alcohol had been removed 24 hrs. before (a) was compared with that from which the alcohol had just been removed (b).

(a) D_{604} = 0.175
(b) D_{604} = 0.175

The effect of different mixing procedures during the first stage of colour development was studied.

(a)	Mixing at 5 min. only.	D_{604} = 0.178
(b)	Mixing at 10 min. only.	0.175
(c)	No mixing whatever.	0.175

Therefore none of these details was responsible for the inconsistent results noted before.

Even worse results were obtained when oestrone and oestradiol methyl ethers were added to petroleum ether (B.P. 40-60 C.°), or were partitioned between petroleum ether and water or N.sodium hydroxide.

Typical results are shown in the table. The methyl ethers (30 µg.) in ethanol solution (0.6 ml.) were added to petroleum ether (100 ml.) which was evaporated to dryness (a) directly, (b) after extraction with

water (100 ml.) and (c) after extraction with N.sodium hydroxide (100 ml.) and then with water to remove alkali.

The residues were transferred with ethanol to Kober tubes, the ethanol was removed by heating in a stream of air and colours were developed with the above strict procedure.

	D_{604}					
	Oestrone methyl ether (30 μg.)			Oestradiol methyl ether (30 μg.)		
From ethanol (0.6 ml.)	.267	.229	.250	.165	.193	.193
	.261	.248	.230			
(a) From pet.ether (100 ml.)	.249	.231	.301*	.190	.194	.187
	.220	.205	.232			
(b) Pet.ether/water (100 ml.)	.225	.220	.221	.178	.175	.192
(c) Pet.ether/N.NaOH (100 ml.)	.222	.238	.253	.191	.176	.182

*By mistake, this sample evaporated to complete dryness without the aid of the jet of air normally used to assist evaporation and prevent bumping. This experiment was repeated with a similar result. It appeared then that the jet of air might have caused destruction by oxidation during evaporation of the ethanol. Without the jet of air, the column of vapour above the evaporating ethanol would displace the air and effectively prevent atmospheric oxidation until all the solvent had evaporated.

The validity of the oxidation theory depended upon whether the destruction occurred when air was excluded by other means.

In the first experiment, oestrone methyl ether (30 μg.) was added to four flasks containing petroleum ether (50 ml.), the petroleum ether was distilled until no more than 1-2 ml. remained. The flasks were allowed to cool, and the pools of petroleum ether were transferred to Kober tubes with the aid of 4 ml. of ethanol each. Oestrone methyl ether

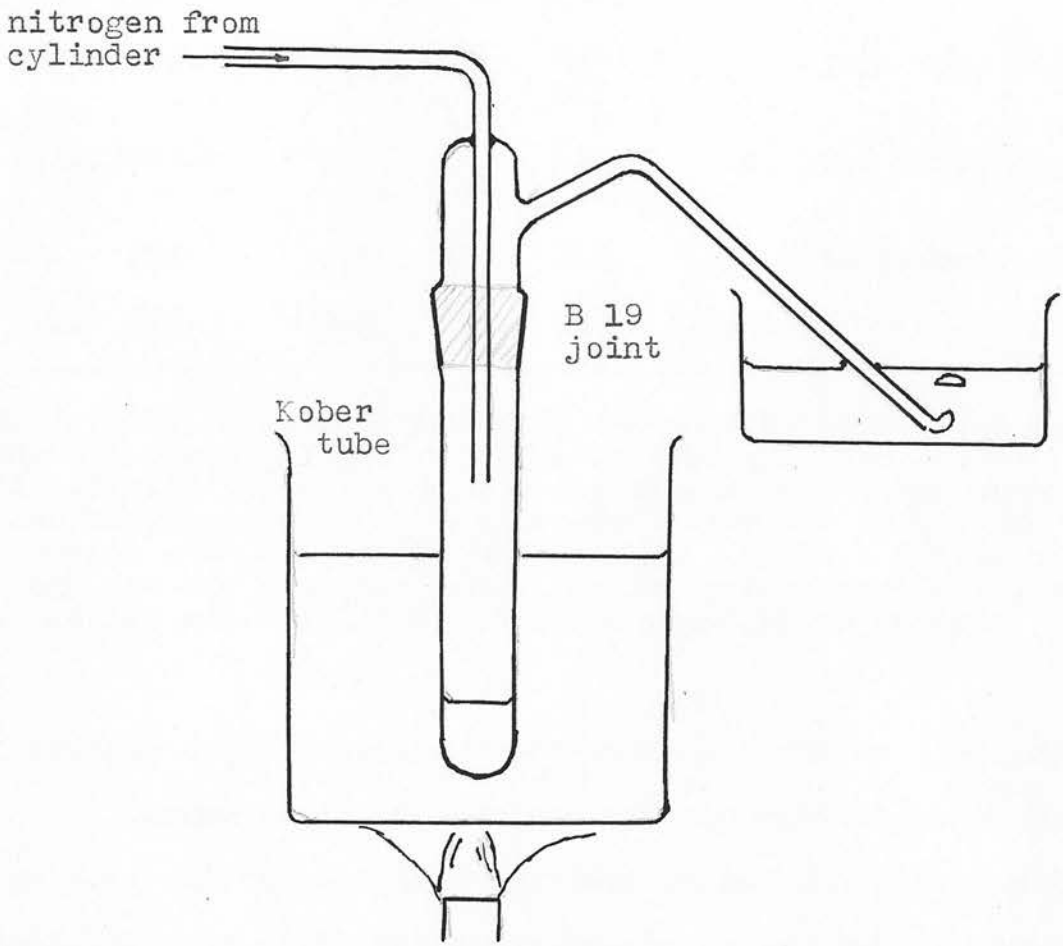


Diagram 1. The apparatus for distilling solvents from Kober tubes in a stream of nitrogen.

(30 µg.) in ethanol solution (0.6 ml.) was added to another four Kober tubes. Two of each were allowed to evaporate to dryness in a boiling water bath, the last traces of solvent being removed with a jet of air when the tubes were somewhat cool, the others were evaporated under a jet of nitrogen and heating.

	D_{604}			
	Standard from ethanol		From petroleum ether	
Simple evaporation by heating.	.273	.275	.279	.270
Evaporation under a jet of nitrogen and heating.	.264	.272	.233	.200

A jet of nitrogen therefore was no better than a jet of air. However, the conditions used could not be considered anaerobic as the nitrogen jet probably sucked air with it in the manner of an injection pump.

Better anaerobic conditions were obtained in the second experiment by a completely closed system from which the air was displaced by passing through a rapid stream of nitrogen. The solvent was then evaporated by heating in a boiling water bath in a slow stream of nitrogen. The apparatus is illustrated in **Diag.1**, and using this instead of the jet of nitrogen, the previous experiment was repeated.

	D_{604}			
	Standard from ethanol		From petroleum ether	
Simple evaporation by heating	.267	.272	.287	.280
Evaporation under nitrogen and heating	.270	.273	.260	.267

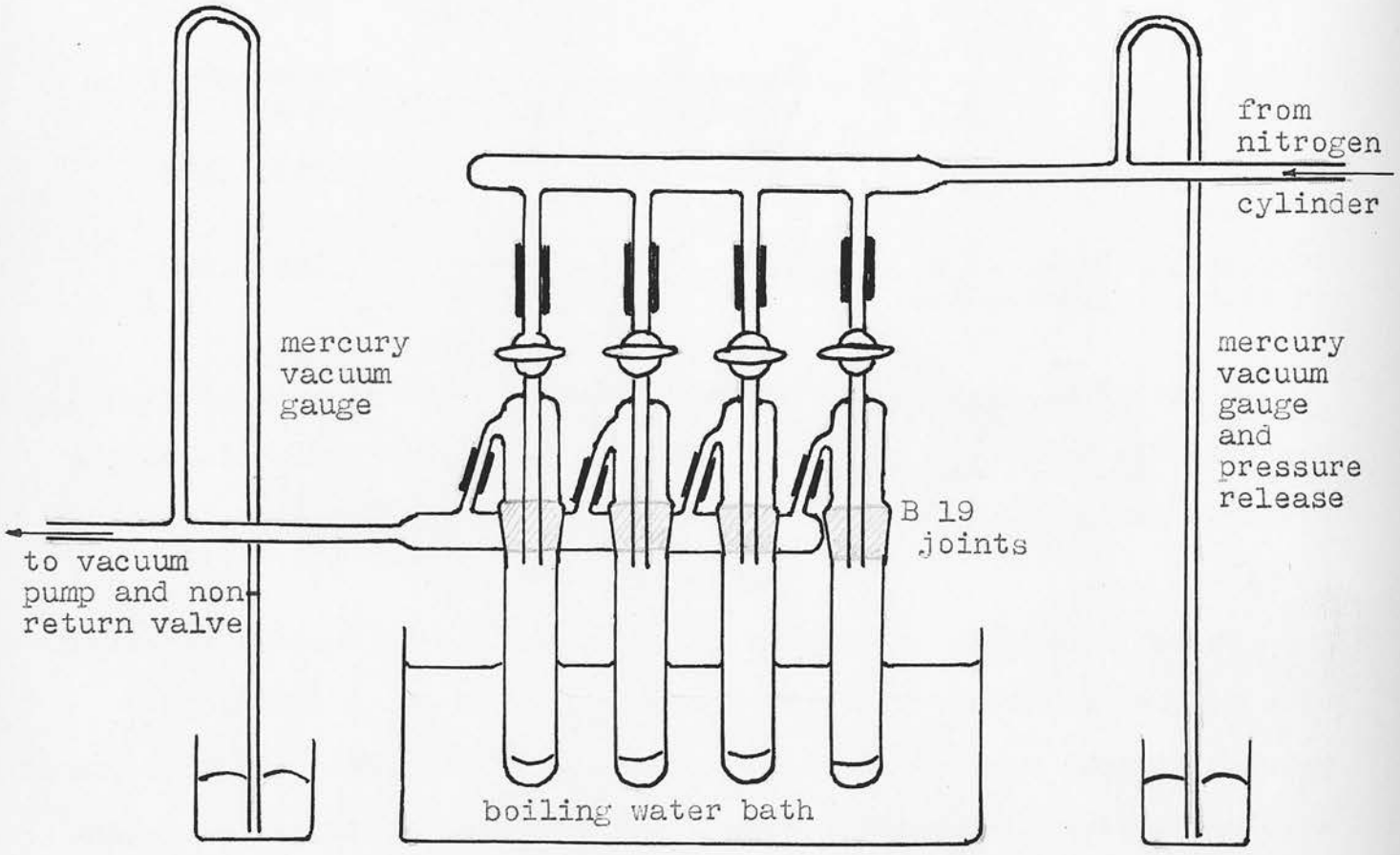


Diagram 2. Apparatus for the distillation of solvents from Kober tubes by heating under nitrogen and reduced pressure.

These were the required results and further work has confirmed them. It seems that the oestrogen methyl ethers should not be heated unnecessarily in the presence of oxygen.

To obtain the best conditions for removing solvents from Kober tubes the apparatus shown in Diag.2 was designed.

With this apparatus and Kober tubes with standard ground glass fittings, best results were obtained in the following manner.

With the taps open the apparatus was evacuated to about 650 mm. of mercury and was filled with nitrogen. This was repeated 3 to 4 times to remove all air. The taps were closed and a partial vacuum of about 300 mm. of mercury was applied to the Kober tube side of the system. The tubes were immersed cautiously in boiling water and the rate of evaporation was controlled by the vacuum applied. Benzene and ethanol boiled quietly under these conditions but care was required with low boiling solvents such as diethyl ether and petroleum ether. Bumping was not a serious factor, for even though the contents boiled into the upper part of the distilling assembly, later condensation of solvent returned the non volatile material to the Kober tube. This was demonstrated every time in the innumerable cases where vigorous boiling occurred during recovery experiments. When all the solvent had evaporated the vacuum was increased to between 650 and 700 mm., the tubes were removed from the water bath, the taps were opened and a stream of nitrogen was passed through until the tubes had cooled somewhat, when they were removed from the apparatus. The usual amount of solvent evaporated from each tube was about 4 ml. but quantities as great as 12 ml. could be evaporated from 6 x $\frac{3}{4}$ in. tubes if a piece of porous pot was present. Evaporation was usually rapid and the time saved compared

with the old air jet method was considerable.

The above procedure gave better results than those obtained by passing a slow stream of nitrogen through the apparatus during evaporation of the solvent. Apparently commercial nitrogen is not absolutely oxygen free.

Anaerobic evaporation of oestrogen solutions was conveniently applied to larger volumes by distilling until 1-2 ml. of solvent remained in the distilling flask and never evaporating this completely except in the above apparatus. Solvents with low boiling points such as diethyl ether or petroleum ether were distilled at atmospheric pressure while higher boiling solvents such as benzene and ethanol were distilled under partially reduced pressure with porous pot to prevent bumping. Benzene is a particularly convenient solvent as it does not easily superheat and boils quietly under these conditions.

With the introduction of this evaporation procedure the anomalies noted above completely disappeared. The procedure has been completely justified by later work (see sections on partition coefficients of the methyl ethers and recovery experiments). For instance, using the anaerobic evaporation procedure, 30 µg. oestrogen methyl ether standards and hydroquinone reagents, the following replicate colour densities were obtained.

Methyl ether	D_{604}			
Oestriol	.182	.180	.175	.190
Oestrone	.251	.254	.250	.252
Oestradiol	.208	.210	.208	.212

PROCEDURES FOR CORRECTING FOR SUBSTANCES CHROMOGENIC IN THE KOBER COLOUR METHODS BUT WHICH DO NOT GIVE THE TRUE KOBER RED COLOUR.

Few substances other than oestriol, oestrone and the oestradiols form a red colour in the Kober reaction. However, in urine extracts other chromogenic substances occur which produce brown colours in the Kober reaction. These brown colours interfere in the colorimetric measurement of the oestrogen red colour when Kober colour methods are applied to the estimation of natural oestrogens in urine. In fact, the extent to which these colours interfere is the limiting factor in any method which finally measures the oestrogens colorimetrically by applying the Kober reaction. Two general methods have been developed to correct for this interference. One depends upon the preferential destruction of the Kober red colour, so that the difference between densities determined before and after discharge of the red colour is a measure of the oestrogen content of the extract. The other depends upon spectroscopic differences between the red and brown colours which allow the application of a spectrophotometric correction. Both these procedures have been investigated.

Fading the red Kober colour.

Kober (1931) showed that the Kober red colour was destroyed by the addition of hydrogen peroxide or by neutralization. Cohen & Marrian (1934) used hydrogen peroxide to discharge the red colour and thus measured the light absorption of the brown component. Later, Kober (1938) used acetone for this purpose. However, Venning et al. (1937) and Bachman & Pettit (1941) claimed that the brown colour also faded somewhat under these conditions. Stevenson & Marrian (1947) described a procedure in which the diluted Kober red colour obtained by the phenol

sulphonic acid method of Venning et al. was faded merely by heating for 1½ hours. They claimed that other chromogenic material was little affected by this procedure. Dr. Clayton, in this laboratory, used this fading technique, the details of which are described in the section on the phenol-sulphonic acid reagent (Page 24). However, in this laboratory, heating for 1½ hours did not always fade the red colour completely, and even with pure oestrogens faded values of D_{604} = 0.020 were often obtained with 50 µg. of oestrogen. It was found that these faded values could be reduced by prolonging the heating time.

Colours developed by the modified phenol-sulphonic acid method were even more resistant to the fading procedure. Faded values also depended upon the oestrogen concentration and the time of heating as shown in the table.

Oestrone (µg.)	10	20	35	50	80	100	150
D_{604} faded value 2 hour heating.	0	.002	.007	.012	.017	.030	.050
3 hour heating						.019	.034

The sulphuric acid concentration of the diluted colour obtained by the modified phenol-sulphonic acid method would be higher than that obtained by the Venning colour method as less water was added for the second stage of colour development. This, together with other evidence, indicated that the rate of fading of the oestrogen colour was dependent upon the sulphuric acid concentration. Water was always lost during fading and this had to be replaced before colorimetry. Therefore, it seemed that a lower initial sulphuric acid concentration could be obtained by adding this water before fading. This was tested in the following experiment in which oestrone (50 µg.) was added to diethyl

ether (270 ml.), the ether was allowed to stand for various periods of time and was then evaporated. The residues were transferred to Kober tubes and colours were developed with the modified phenol sulphonic acid colour method. Water (2 ml.) was added to those 8 ml. of colour solution marked with an asterisk, before fading with heating. The volumes were adjusted to 8 ml. before colorimetry.

	Standard (50 µg.) from ethanol		Ether evaporated - Immediately		After 2 hrs.		After 6 hrs.	
D_{604}	.275	.280	.276	.280	.272	.274	.278	.280
Faded D_{604}	.013	.006*	.013	.007*	.013	.006*	.012	.006*

Lower faded values were obtained by adding water before fading. However, when the modified phenol sulphonic acid colour method was applied to the phenolic fractions from acid hydrolysed male urine (100 ml.) the brown colour obtained also faded considerably when the more vigorous fading conditions were used. For instance, using two samples of male urine, the colours produced by the "oestriol" fractions decreased from D_{604} = .190 to .124, and .216 to .147, and the "oestrone" fractions from D_{604} = .135 to .100, and .170 to .141. These figures represented approximately a 2/3 reduction of density which could not be due to oestrogen. Stevenson & Marrian observed a less marked fading of the chromogenic material from most of the male urines they studied. It was felt that this fading was a serious disadvantage of the method.

The conditions for discharging the red Kober colour obtained with the hydroquinone-sulphuric acid colour method were next studied. Using oestriol (30 µg.) colours were developed by heating 20 min. with the 76% sulphuric acid-2% hydroquinone reagent (4 ml.) and then adding

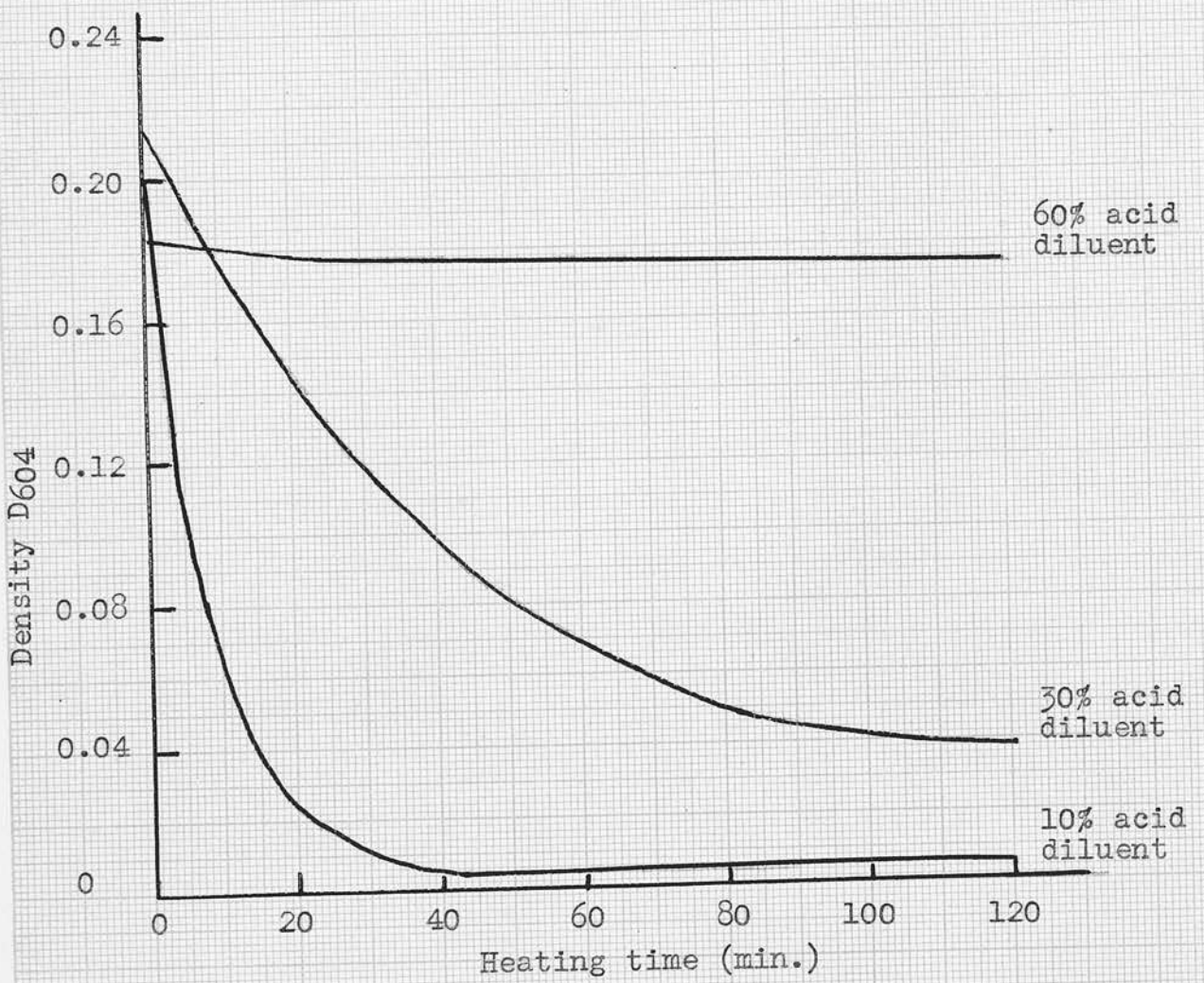


Fig. 13. The influence of the final diluent upon the fading of colour at 100°C. Colour was produced with oestriol (30 µg.) and the 76% acid-2% hydroquinone reagent.

water (1.0 ml.) and heating another 5 min. After cooling, the colours were diluted to 15 ml. with (a) 60% (v+v) sulphuric acid, (b) 30% (v+v) sulphuric acid and (c) 10% (v+v) sulphuric acid and heated at 100°C. Colours were measured after various periods of time (see Fig.13). Volumes were readjusted by adding water before each density measurement.

Colours were stable to heating when the diluent was 60% sulphuric acid, they faded considerably in 2 hrs. when the diluent was 30% sulphuric acid, and they faded completely in 40 min. when the diluent was 10% sulphuric acid.

The effect of the acid concentration of the diluent on the intensity of colour before and after fading was studied. Colours were developed by heating oestriol, oestrone and oestradiol (30 µg.) with 76%, 66% and 60% sulphuric acid-2% hydroquinone reagents in the usual manner, and were diluted to 15 ml. with 10% and 30% (v+v) sulphuric acid diluents. Colours were measured after standing for various periods of time at room temperature and after adding water (1.0 ml.) to colour solutions (8 ml.) and heating 2 hours. When colours were diluted with 10% sulphuric acid, heat was evolved which necessitated cooling in ice water.

10% sulphuric acid diluent.

D_{604}

Oestrogen (30 µg.)	%H_2SO_4 in reagent	Time (min.) after dilution 5		15		30		45		Faded value	
Oestriol	76	.192	.180	.188	.192	.178	.178	.180	.180	0	0
Oestrone	66	.220	.210	.222	.220	.220	.210	.218	.210	0	0
Oestradiol	60	.190	.195	.180	.190	.180	.183	.183	.183	0	0

30% sulphuric acid diluent.

D_{604}

Oestrogen (30 µg.)	$\%H_2SO_4$ in reagent	Time (min.) after dilution 5		15		30		45		Faded value	
Oestriol	76	.194	.192	.194	.192	.204	.202	.204	.200	0	0
Oestrone	66	.250	.256	.250	.256	.252	.260	.246	.250	0	0
Oestradiol	60	.212	.212	.208	.206	.208	.206	.210	.210	0	0

The sulphuric acid concentration of the diluent determined the densities of the diluted colour, which in the case of oestrone and oestradiol were higher with the 30% acid diluent than with the 10% diluent. The 30% acid diluent was selected for further work as by this time confidence had been gained in it as a diluent. Heating 8 ml. of colour solution with 1 ml. of water for 1½-2 hours and readjusting the volume was a satisfactory method for discharging the red Kober colour developed with these hydroquinone reagents. This fading technique was applied to colours developed with partly purified extracts of male urine and with pure oestrogen methyl ethers.

Male urine extract No.	1	2	3	4	5	6	7
D_{604} before fading	.033	.061	.071	.010	.076	.034	.139
D_{604} after fading	.037	.067	.092	.015	.090	.048	.180

Male urine extract	"Oestriol" fraction				"Oestrone" fraction		"Oestradiol" fraction	
D_{604} before fading	.037	.193	.023	.230	.053	.063	.066	.031
D_{604} after fading	.026	.160	.021	.197	.033	.053	.053	.024

Oestrogen methyl ether (approx. 30 µg.)	Oestriol		Oestrone		Oestradiol	
D_{604} before fading	.175	.172	.241	.238	.183	.187
D_{604} after fading	.011	.011	.010	.009	.008	.015

Using the fading method, the results with both the modified phenol sulphonic acid and hydroquinone-sulphuric acid colour methods were disappointing and therefore other methods of correcting for interfering chromogenic material were investigated.

Spectrophotometric correction for interfering chromogenic material.

Venning et al. (1937), using their phenol sulphonic acid colour method, showed that the interfering brown colour from urine extracts absorbed light strongly at 420 mµ. and that the oestrogen red colour, which showed an absorption maximum at about 520 mµ., was nearly transparent at this wavelength. By assuming that the spectrophotometric characteristics of the non oestrogen brown colour were constant for different urines, they were able to calculate from the measured absorption at 420 mµ. the absorption at 520 mµ. due to this brown colour. This correction method was further developed by Stimmel (1946b). However, the validity of the basic assumption that the spectrophotometric characteristics of the brown colour were constant for different urines was questioned by Bachman & Pettit (1941), and indeed an analysis of the figures reported by Stimmel shows that considerable error in the correction method could arise through lack of constancy in this respect.

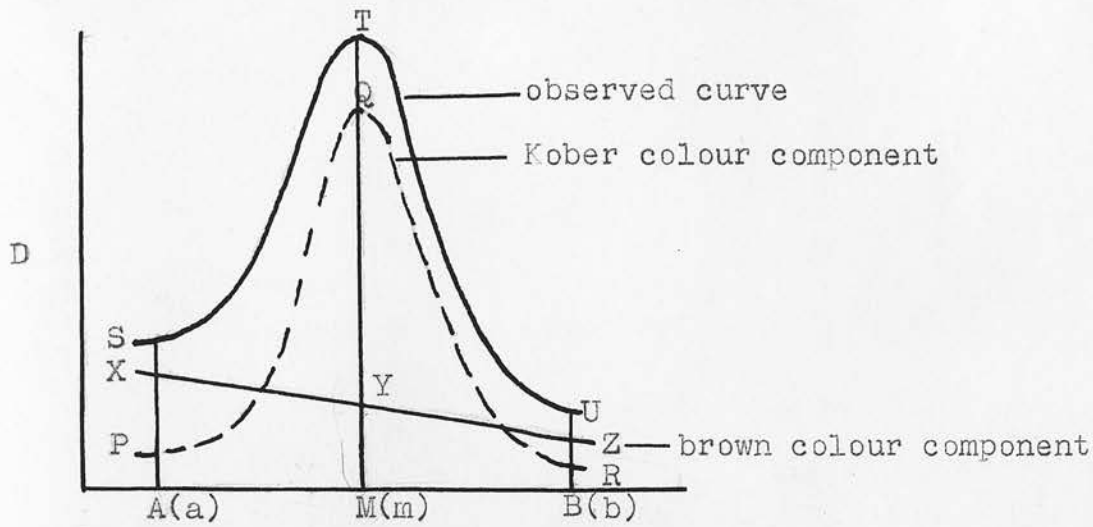
Allen (1950) described a spectrophotometric correction method for the oestrogen Kober colour based on measuring the light absorption at three different wavelengths, similar in derivation to the spectrophoto-

metric correction method described by Morton & Stubbs (1946) for the analysis of mixtures and Vitamin A oils. These correction methods were based on the assumption that if density measurements were made at three wavelengths, one of which was the absorption maximum for the pure compound being measured, and if the three wavelengths were sufficiently close to one another, the absorption curve due to the interfering brown colour would be a straight line between the three wavelengths. Allen showed that this was true with his modification of the Kober colour method. By applying a formula calculated by simple geometry to density measurements at the three wavelengths, the extent to which the brown colour absorbed at the absorption maximum of the pure colour could be calculated and subtracted from the observed density at that wavelength. Both Allen and Morton & Stubbs used devices to simplify the correction formula.

One wavelength used for density measurement is always at the absorption maximum for the pure substance being measured. Morton & Stubbs selected the other two wavelengths, so that for the pure substance the ratios between the density at the absorption maximum and the densities at these two wavelengths were the same. Allen selected two wavelengths which were equidistant from the maximum. By keeping the three wavelengths close together a straight line absorption could be assumed for the interfering chromogenic material.

These devices can only be used with spectrophotometers giving essentially monochromatic light and are impossible with the Spekker filter type absorptiometer used in this laboratory. However, with this instrument the basic method could still be used in the following manner.

The general correction formula for measurements at three wavelengths was derived in a manner similar to that of Allen and Morton & Stubbs as follows.



Da, Dm and Db are the observed densities at wavelengths a, m and b; **m being** the absorption maximum of the component being estimated.

TM - YM = QM = corrected Dm, the density of the pure component at wavelength m.

$$TM = Dm$$

$$SA = Da$$

$$UB = Db$$

$$SP = XA$$

$$UR = ZB$$

$$\text{constant ka} = \frac{PA}{QM} \qquad \text{constant kb} = \frac{RB}{QM}$$

Where ka and kb are the **ratios** between the absorption of the pure component at wavelengths a/m and b/m.

From similar triangles:-

$$YM.AB = XA.MB + ZB.AM = MB(SA - PA) + AM(UB - RB)$$

$$= MB.SA - MB.QM.ka + AM.UB - AM.QM.kb$$

$$= MB.SA + AM.UB. - QM(MB.ka + AM.kb)$$

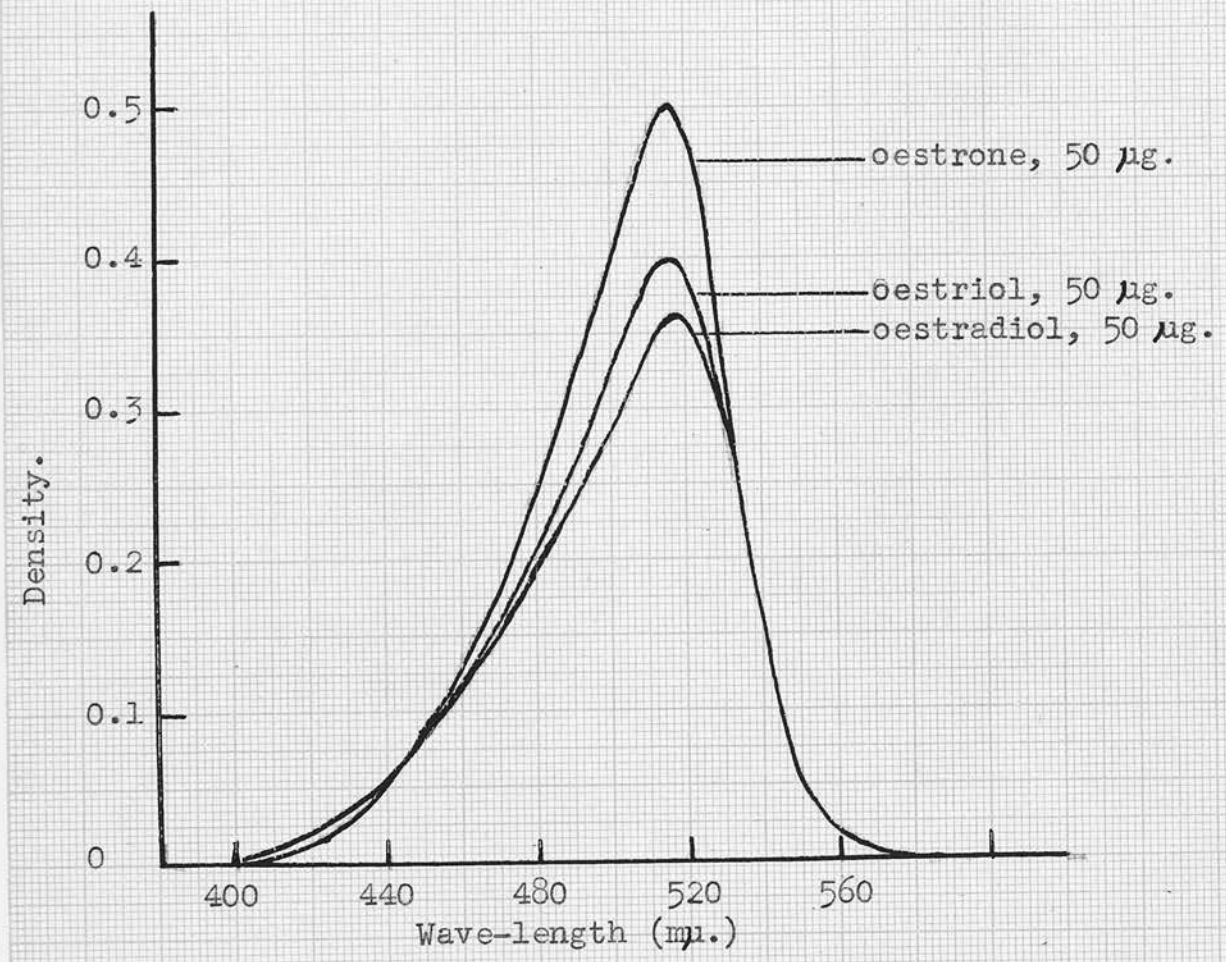


Fig. 14. The spectral characteristics of colours produced with oestriol, oestrone and oestradiol and the hydroquinone reagents.

$$QM = TM - YM$$

$$AB.QM = AB.TB - MB.SA - AM.UB + QM(MB.ka + AM.kb)$$

$$QM(AB - MB.ka - AM.kb) = AB.TM - MB.SA - AM.UB$$

$$\therefore QM = \frac{AB.TM - MB.SA - AM.UB}{AB - MB.ka - AM.kb}$$

$$\text{or Dm corr.} = \frac{ab.Dm - mb.Da - ma.Db}{ab - mb.ka - am.kb}$$

where ab, mb, and am, are the constant distances between the wavelengths a, b and m.

Allen simplified the general equation by making am = mb, and Morton & Stubbs made ka = kb.

The spectral characteristics of the colours produced with oestriol, oestrone and oestradiol-17β and 76%, 66% and 60% sulphuric acid-2% hydroquinone reagents respectively were measured in a Unicam U.V. spectrophotometer using 1 cm. cuvettes and are shown in Fig.14.

The colours absorbed maximally at 515 mμ., and not at all at 400 mμ. and 600 mμ. The no. 604 Ilford green filter transmitted light corresponding to the wavelength of maximum absorption. In selecting the other two filters, closeness to the absorption maximum was sacrificed for filters which transmitted light minimally absorbed by the Kober red colour. The reason for this was that, at the minimum, slight changes in spectroscopic properties of the filters or of the coloured solution would not lead to the errors inherent in measurements taken on steep slopes of the absorption curve. The ease with which the absorption maximum of the Kober colour could be altered by small differences in reagents or technique was ample justification for this choice.

Filters selected were Ilford violet no. 601 and Ilford yellow no. 606. With these filters the distances ab, mb and am, in the above formula, could not be calculated from the available data, but could only be obtained by trial and error to find the best figures to fit the observed facts. This method of trial and error was not a serious handicap, because whatever formula was used, measurements on a number of oestrogen free urines would be necessary to establish that the wavelength - absorption curves for the interfering brown colours from different urines were linear over the wavelength range used. In this case, the wavelengths selected would be too far apart to maintain a linear wavelength - absorption curve for the impurities, but the final correction formula based, not on theory but on actual results, would allow for this.

Colour densities developed with the hydroquinone-sulphuric acid colour method and pure oestrogens or extracts from hydrolysed male urines were measured with the three filters. A large number of these measurements were made over a period of time and are collected in the section on recovery experiments (Pages 180-9). A survey of the results showed that colours developed with pure oestrogens did not absorb the yellow light transmitted by the no. 606 filter so that $D_{606}/D_{601} = 0$ The ratio D_{601}/D_{604} varied slightly about a median value of 0.2

The above correction formula then became:-

The density due to the oestrogen Kober colour,

$$D_{604} \text{ corrected} = \frac{abD_{604} - mbD_{601} - maD_{606}}{ab - 0.2\ mb}$$

when a = filter no. 601

b = filter no. 606

m = filter no. 604

constant ka = D_{601}/D_{604} = 0.2

constant kb = D_{606}/D_{604} = 0

The constants ma and mb were next found by applying this formula to density measurements on chromogenic material obtained from oestrogen free urine where D_{604} corrected was zero. Male urine was assumed to have a negligible oestrogen content at the levels studied and **was used** for this purpose. The data obtained from the recovery experiments gave by trial and error the following best values for these constants.

am = 3, mb = 1

and therefore ab = 4

The formula then became

$$D_{604}\ \text{corr.} = \frac{4D_{604} - D_{601} - 3D_{606}}{3.8}$$

Over a period of time the validity of this formula has been adequately demonstrated by applying it to the recovery of oestrogens added to pure solutions (Pages 151-156) and to male urine (Pages 181-189), and in metabolic experiments performed on post-menopausal women.

THE INTERFERENCE OF URINARY MATERIAL IN THE DEVELOPMENT OF KOBER COLOUR WITH OESTRIOL AND HYDROQUINONE REAGENTS.

When the hydroquinone colour method was applied to the recovery of oestriol added to hydrolysed urine, colour densities lower than expected were obtained, especially when the urine blank was considerable.

This is illustrated in the following examples in which extracts from acid hydrolysed male urine were partly purified by methylation and chromatography and, using the 76% sulphuric acid-2% hydroquinone reagent, and equivalent urine fractions colours were developed with

(a) the urine fraction (blank),

(b) urine fraction plus added oestriol (30 µg.) (blank + standard),

(c) pure oestriol (30 µg.) (standard),

with the following results:-

D_{604}			
(a) Urine blank	(c) Standard	(b) Blank + Standard (found)	Standard calc. from (b) - (a)
.093	.190	.246	.153
.068	.190	.218	.150

Colours developed with oestrone and oestradiol were not similarly affected as shown in the following example in which the densities were measured with the three filters.

	Oestrone 33 µg. 66% reagent			Oestradiol 32 µg. 60% reagent		
	D_{604}	D_{606}	D_{601}	D_{604}	D_{606}	D_{601}
(a) Urine blank	.076	.038	.131	.064	.033	.120
(c) Standard methyl ether	.293	.004	.051	.213	.010	.048
(b) Blank + Standard (found)	.371	.046	.181	.283	.046	.159
Standard calc. from (b) - (a)	.295			.219		
Standard calc. from (b) by the correction formula	.306			.220		

Small changes in the hydroquinone colour method were studied in an effort to overcome the depressing effect of urine extracts. The oestriol fraction from hydrolysed male urine (800 ml.) was methylated and divided into 16 equal fractions in ethanol solution. Oestriol methyl ether (30 µg.) was added to some of the fractions in Kober tubes before removing the solvent by heating under nitrogen and reduced pressure.

The effect of varying the heating times in the first and second stages of colour development was studied. Colours were measured with the Ilford no. 604 and 606 filters.

				D_{604}				
				Urine fraction plus added oestriol methyl ether.				
Oestriol methyl ether standard 30 ug.	Urine blank	Calc. sum		2nd. heating time (min.) with 20 min. first.			1st heating time (min.) with 4 min. second.	
				4	8	12	40	60
D_{604} .192	.131	.323		.292	.287	.289	.315	.340
D_{606} .003	.082	.085		.085	.084	.089	.095	.110

The increase in density with increase in the first heating time was more due to increase in the urine blank than to a real increase in oestrogen colour. The depressing effect was not altered by increasing the heating times in either the first or second stages of colour development.

The effect of ethanol on colour development with oestriol, urine extracts and hydroquinone reagents.

Dr. W.S. Bauld, working in the Edinburgh University Biochemistry Department discovered that, if the oestriol and urine extract were dissolved in ethanol (0.25 ml.) before adding the hydroquinone reagent (4 ml.) and developing the colour, no depression occurred and the colour density found was the same as that calculated from the sum of the separate components.

When Dr. Bauld's discovery was investigated in this laboratory, a sample of our "purified" ethanol gave high reagent blanks and low colour densities when colours were developed with pure oestriol. However, a sample of ethanol kindly supplied by Dr. Bauld did not

affect the reagent blank or the colour density produced by pure oestriol and was very effective in preventing the depressing effect of urine extracts on the oestriol colour.

The optimum conditions for developing colour with oestriol, ethanol and hydroquinone reagents were studied. Dr. Bauld's ethanol was used in these experiments.

The addition of ethanol would be expected to alter the sulphuric acid concentrations required in the first and second stages of colour development. Using hydroquinone reagents the optimum amount of water required in the second stage of colour development was shown not to be at all critical and in the section dealing with ethanol in the Kober reaction, ethanol was shown to replace water volume for volume.

On this basis, the volumes of water added in the second stage of colour development were calculated for the various combinations of hydroquinone, sulphuric acid and ethanol.

The effect of the sulphuric acid content of reagents (4 ml.) containing 2% hydroquinone and ethanol (0.25 ml./4 ml. reagent) and 5% hydroquinone on colours produced with oestriol (30 µg.) was studied. The usual technique for developing colour was used.

	D_{604}	
Sulphuric acid concentration in reagent %	2% hydroquinone reagent plus ethanol (0.25 ml./4 ml.)	5% hydroquinone reagent
74	.195	.169
77	.199	.191
80	.195	.189
Reagent blank against normal reagent.	.005	0

Using the optimum sulphuric acid concentrations indicated in the last experiment, the effect of various combinations of hydroquinone and ethanol on colours produced by oestriol in the presence of urine extracts was studied.

Acid hydrolysed male urine (500 ml.) was extracted with ether and the acid and polyhydroxy phenol fractions were removed. The "oestriol" fraction obtained by benzene-water partition followed by ether extraction, was taken up in ethanol and fractions were distributed into Kober tubes. Oestriol (30 µg.) in ethanol was added to some of these and the ethanol was removed by distilling under nitrogen and reduced pressure. Hydroquinone-sulphuric acid reagents were prepared at least 24 hours before use. When ethanol was added to the colour reaction, the oestriol and urine extracts were dissolved in it before adding the reagent (4 ml.) and the whole was mixed well before heating (20 min.). Water (1.0 ml., calculated to be the optimum amount in all cases) was then added and heating was continued 5 minutes. Colours were diluted and measured with the three filters in the usual manner.

Reagents containing hydroquinone 2%.

	H_2SO_4 76% no ethanol				H_2SO_4 77% ethanol 0.1 ml.			
	D_{604}	D_{606}	D_{601}	corr. D_{604}	D_{604}	D_{606}	D_{601}	corr. D_{604}
Oestriol std.	.201	.003	.047	.198	.201	.004	.042	.198
Oestriol + urine	.202	.037	.158	.142	.239	.033	.178	.179
Urine blank	.060	.036	.118	.004	.066	.037	.128	.007
Difference	.139				.173			
Reagent blank against normal reagent.	0				0			

	H_2SO_4 79% ethanol 0.25 ml.			
	D_{604}	D_{606}	D_{601}	corr. D_{604}
Oestriol std.	.201	.007	.040	.196
Oestriol + urine	.248	.032	.173	.191
Urine blank	.068	.037	.139	.008
Difference	.180			
Reagent blank against normal reagent.	.004			

Reagents containing hydroquinone 5%.

	H_2SO_4 77% no ethanol				H_2SO_4 78% ethanol 0.1 ml.			
	D_{604}	D_{606}	D_{601}	corr. D_{604}	D_{604}	D_{606}	D_{601}	corr. D_{604}
Oestriol std.	.211	.004	.037	.208	.211	.004	.034	.210
Oestriol + urine	.215	.039	.160	.153	.253	.031	.161	.200
Urine blank	.062	.039	.119	.003	.064	.037	.126	.005
Difference	.153				.189			
Reagent blank against normal reagent.	0				0			

	H_2SO_4 80% ethanol 0.25 ml.			
	D_{604}	D_{606}	D_{601}	corr. D_{604}
Oestriol std.	.209	.005	.036	.207
Oestriol + urine	.260	.033	.176	.202
Urine blank	.073	.039	.148	.007
Difference	.187			
Reagent blank against normal reagent.	0			

Evidently reagents containing 5% hydroquinone were less affected by urine material than those containing 2% hydroquinone, and the addition of ethanol was effective in minimising the depression of colour.

The best oestriol colour method based on this data follows:-

Reagent: Hydroquinone (5 g.) was dissolved with warming in 79% (v/v) sulphuric acid (100 ml.) and stored at least 24 hours before use.

Colour method: Pure ethanol (0.2 ml.) was added to oestriol in Kober tubes so that the oestriol was dissolved. Hydroquinone reagent (4 ml.) was added to this solution, the two were mixed thoroughly and heated 20 minutes in a boiling water bath. The tubes were shaken once during the first 5 minutes. After heating, the tubes were cooled in ice water. Water (1.0 ml.) was added and mixed, the tubes were heated 5 minutes and then cooled in ice water. The contents were diluted to 15 ml. with 30% (v+v) sulphuric acid and allowed to stand at least 10 minutes at room temperature before colorimetry. Colours were identical with those developed by the 76% sulphuric acid-2% hydroquinone colour method, and precautions during colour development were similar.

Using this method the effect of time between diluting the colour produced by oestriol methyl ether (30 µg.) and measuring its density was studied. The products of the second stage of colour development, after being cooled, were diluted to 15 ml. with 30% (v+v) sulphuric acid and colour densities were measured immediately and at time intervals thereafter.

Time after dilution (min.)	0	5	10	15	20	40	60	80
D_{604}		.168	.183	.192	.191	.191	.192	.189	.182

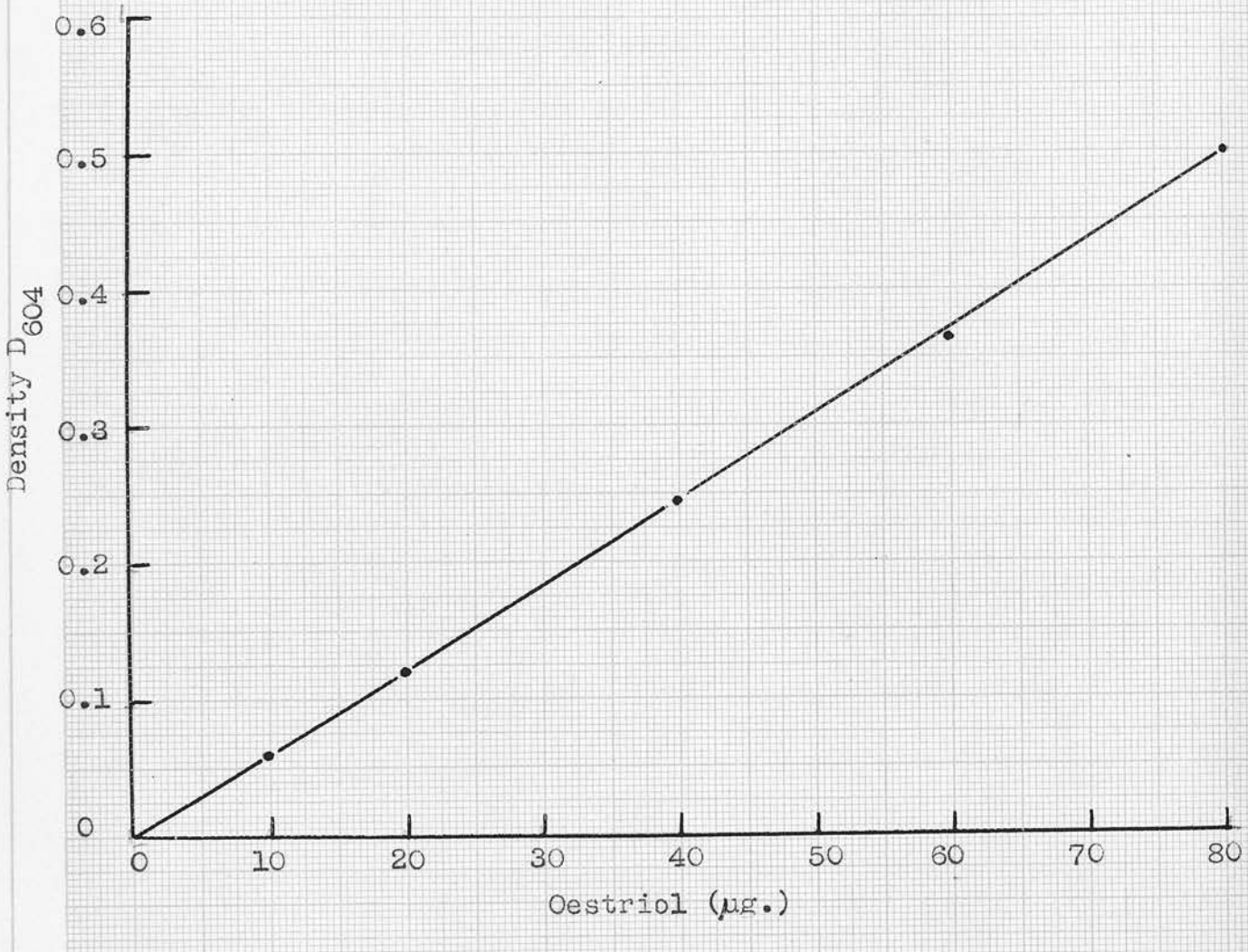


Fig. 15. The relationship between concentration of oestriol and colour density using the ethanol-5% hydroquinone-79% sulphuric acid reagent.

The colour reached a maximum 10 minutes after dilution and was then stable up to 60 minutes after dilution.

The method gave excellent proportionality between the amount of oestriol present and the density of colour produced as shown in Fig. 15. Pure oestriol methyl ether was used in this experiment.

The method applied to the recovery of oestriol methyl ether added directly to an "oestriol" fraction obtained from hydrolysed male urine (24 hr. specimen) and purified by methylation and chromatography, gave very satisfactory results as shown.

	D_{604}	D_{606}	D_{601}	D_{604} corr.
(a) Urine blank	.026	.014	.049	.003
(b) Urine blank plus added oestriol methyl ether (30 µg.)	.225	.017	.091	.198
D_{604} corr. by subtraction (b) - (a)	.199			
Standard oestriol methyl ether (30 µg.)	.201	.000	.042	.201

Applying this colour method to the recovery of oestriol added to acid hydrolysed male urine gave better and more consistent yields than when the 76% sulphuric acid-2% hydroquinone reagent was used. Recoveries from pure solutions were also improved.

The purification of ethanol for use in the Kober reaction.

Only certain samples of ethanol were suitable for use in the above modification of the Kober reaction. For instance, a sample of ethanol which had been purified by refluxing with sodium hydroxide followed by two distillations depressed the colour produced by oestriol (30 µg.)

to D_{604} = 0.116 and gave yellow blanks of D_{604} = 0.013 against a blank prepared without ethanol. Further work showed that the depression of colour was directly related to the yellow colour of the blank, and that samples of ethanol giving practically zero blanks were suitable for use in the new colour method. The substances responsible for the high blank values were volatile since they did not interfere when the ethanol was removed by evaporation before colour development and were not separable from ethanol by fractional distillation. Dr. Bauld showed that treatment of some samples of ethanol with m-phenylene diamine hydrochloride to remove aldehydes, followed by repeated distillation, also removed the troublesome substances. However, this treatment was ineffectual with many samples of ethanol.

Another sample of ethanol was treated in this laboratory by distillation through an efficient fractionating column, with m-phenylene diamine hydrochloride or 2.4 dinitrophenyl hydrazine with and without acidification followed by fractional distillation, by reduction with sodium, and by oxidation with permanganate, without improving its colour properties. Occasionally a batch of suitable ethanol occurs among laboratory grades and it seems best to examine these until a suitable batch is found and to store this for future use. Fortunately, only a small volume of special ethanol is required for each oestriol determination so that, once a suitable batch is found, it will last a long time. Suitable ethanol seems to keep indefinitely. For instance, Dr. Bauld's original ethanol was still suitable after standing stoppered at room temperature for nine months.

SUMMARY OF SECTION I.

The two stage Kober reaction with oestriol, oestrone and oestradiol-17β has been investigated starting with the simple sulphuric acid method of Cohen & Bates. The ethanol used in the method by these workers was shown to be important, and this was due to its reducing activity. Other reducing agents were successfully employed in the Kober reaction and ferrous sulphate, phenol and hydroquinone were fully investigated in the search for improved colour methods. A method using reagents containing hydroquinone and sulphuric acid was found to be the best.

The proportions of sulphuric acid and water were found to be important in both the first and second stages of the Kober reaction. In the second stage, maximum intensity and stability of the red colour were usually obtained in the presence of 50 to 60% sulphuric acid and water. Under these conditions the second heating time was not critical. In the first stage, optimal sulphuric acid-water ratios differed for the three oestrogens and were approximately 76% for oestriol, 66% for oestrone and 60% sulphuric acid for oestradiol. The phenol-sulphuric acid reagent used by many workers appears to owe its efficacy to the action of the phenol which acts partly as a reducing agent and partly as a diluent of the sulphuric acid.

A satisfactory method using the hydroquinone-sulphuric acid reagents is described. With this method, the oestrogen methyl ethers behaved in exactly the same manner as the free oestrogens. When evaporating solutions of the oestrogen methyl ethers, anaerobic conditions were found to be necessary to prevent their partial destruction which occurred when air was present. With this colour method urinary material was

found to depress the colour developed with oestriol. This depression was prevented by dissolving the oestriol in a small volume of ethanol before adding the colour reagent.

Methods for correcting for the brown colours produced with Kober reagents by urinary material were investigated, and a spectrophotometric method which measures colour densities at three different wavelengths of light, was found to be the best.

The procedures of evaporation of solvents, colour development and correcting for interfering urinary material are fully described in a later section describing the complete oestrogen method.

PART II

METHODS FOR THE EXTRACTION AND PURIFICATION OF OESTRIOL, OESTRONE AND OESTRADIOL FROM HYDROLYSED URINE.

INTRODUCTION.

Theoretically it should be possible to estimate conjugated oestriol, oestrone and oestradiol as they occur in urine by applying the Kober colour method. However, the only known general method of separating and estimating oestrogens from urine is to hydrolyse their conjugates and extract the free oestrogens with organic solvents. This general method was first devised by Cohen & Marrian (1934) and no fundamental departure from their procedure has since been devised. The method is based on the fact that the oestrogens are phenols. They are therefore extracted from acid urine by certain fat solvents, and from these fat solvents by alkali. This alkali extract also contains the organic acid fraction which is separated from the phenol fraction by differential solubility in carbonate solutions. Phenols are weak acids and are displaced from their alkali salts by carbonic acid, so that, when the alkali extract is neutralized with carbon dioxide and extracted with organic solvent, the phenols go into the organic phase and the acids remain in the aqueous phase. This latter procedure can also be performed by extracting the organic phase with carbonate solutions which preferentially remove the acid fraction.

The success of using these extraction techniques in quantitative methods depends upon precise information concerning the partition of the oestrogens between organic solvents and aqueous solutions. A number of workers have attempted to measure the partition coefficients, but there has not always been agreement between results obtained by different laboratories. For instance, while all workers agree that

oestrone and oestradiol are completely extracted from water or dilute acid solution by shaking with one equal volume of ether, they do not agree about the exact behaviour of oestriol when partitioned between these solvents. Mather (1942) using bioassay methods found only 86% of the total oestriol in the ether phase. Bachman & Pettit (1941), using the Kober colour method, found 96% of the oestriol in the ether phase. Engel, Slaunwhite, Carter & Nathanson (1950), using a fluorimetric method or absorption of U.V. light at 280 mμ., found 98% of the oestriol in the ether phase but showed no confidence in this figure because they still extracted hydrolysed urine four times with quarter volumes of ether to get quantitative recoveries of oestriol. This typical confusion was probably due to errors inherent in the methods used for estimating the oestrogens. Consequently, before the best extraction and purification procedure could be devised, more reliable information was required concerning the partition of oestrogens between relevant immiscible solvent pairs. The modified phenolsulphonic acid and later the hydroquinone-sulphuric acid colour methods, which were shown in the previous section to be reliable for this purpose, were used. Experiments were designed to check as many factors as possible. For instance, solutions of oestrogens in organic solvent were merely evaporated without partition to make sure that solvent residues did not affect the final oestrogen colour. The amount of oestrogen in both aqueous and organic phases was estimated and correlated with the total added before partition. The oestrogen content of the aqueous phase was usually estimated, either by partitioning a second time with the same organic solvent, as in the case of the ether/aqueous partitions, or with a better

organic solvent, as in the case of the benzene/aqueous partitions where acidification and ether extraction were used. By taking care to avoid possible causes of error and checking results where possible, sufficiently accurate data for designing an extraction procedure is claimed.

Solvent pairs were selected from those used by other workers. The basic method chosen was that used by Clayton in this department. In her method, the oestrogens were extracted from hydrolysed urine with ether, the acid fraction was removed by extracting the ether solution with saturated aqueous sodium bicarbonate solution, and the ether was distilled. The residue was taken up in benzene with the aid of a little ethanol, oestriol was extracted from the benzene with water, and the oestrone and oestradiol with sodium hydroxide. The aqueous extracts were neutralized and the oestrogens were extracted with ether. Many other workers (e.g. Cohen & Marrian, 1934; Venning, Evelyn, Harkness & Browne, 1937; Salter, Humm & Oesterling, 1948; Engel et al., 1950) used ether for the preliminary extraction from hydrolysed urine. However, Bachman & Pettit (1941) showed that although oestriol was not easily extracted from water with benzene, it could be extracted with benzene when the water was saturated with sodium chloride. They claimed that although extraction with ether gave better recoveries of oestrogens, benzene extracted much less contaminating urinary chromogenic material. However, benzene, because of its relatively poor solvent power for oestriol, is not as versatile a solvent as ether. Cohen & Marrian (1934) showed that oestrone is not easily extracted from ether solutions with alkali, but is readily extracted from toluene. Later workers (Bachman & Pettit, 1941;

Mather, 1942) have confirmed this and have shown that benzene is as good as toluene for this purpose. Therefore, to satisfy the conflicting partition requirements of the three oestrogens, ether is the better solvent when the oestrogens are required to remain in the organic solvent phase, i.e. extraction from urine and the removal of the acid fraction with carbonate solutions, and benzene or toluene are better when the oestrogens are wanted in the aqueous phase, i.e. when they are being separated from the neutral fraction by extraction with alkali. Ethyl acetate is an even better solvent than ether for oestriol and this was also investigated in the partition experiments.

The greater solubility of oestriol in aqueous solutions has been used to separate it from the less water soluble oestrone and oestradiol. At first this difference was thought to be due to oestriol being a stronger phenol than the other two. Sodium carbonate solutions were therefore used to extract oestriol preferentially from benzene solutions. However, Bachman & Pettit (1941) showed that distilled water was equally good for this purpose and it has been used by many workers since. Friedgood, Garst & Haagen-Smit (1948) claimed that 0.2M disodium phosphate was even better than water, but their finding was not confirmed by Clayton in this department, although on the assumption that oestriol is a strong phenol it seemed reasonable to expect that its extraction from benzene by aqueous solutions would be improved by increasing the pH of the aqueous phase. The failure of 0.2M disodium phosphate to achieve this might be due to salting out effects counterbalancing the pH effect. Consequently the partition of the oestrogens between benzene and a series of very dilute borate buffer solutions with pHs between 7 and 10 was investigated. In this pH

range, the partition of oestriol was found to be surprisingly independent of pH. This finding led to the investigation of the partition of oestrogens between ether and concentrated alkaline carbonate solutions and to an improved procedure for removing acid and polyhydroxy phenols from urine extracts without simultaneous loss of oestriol.

Large volumes of urine have to be processed to obtain measurable amounts of oestrogens when these are present in small amounts. The larger the volume of urine processed, the more efficient the purification procedure needs to be to remove the interfering chromogenic material which contaminates the final oestrogen fraction. The phenolic fraction obtained by the best use of simple partitions in separating funnels was not sufficiently free from interfering material to enable these low concentrations to be estimated as desired. Therefore, methods for the further purification of the phenolic fraction were required.

Stimmel (1946a) described a chromatographic method using alumina columns which not only removed a considerable amount of interfering urinary material, but also conveniently separated oestriol, oestrone and oestradiol from each other. However, a considerable loss of oestriol occurred in his method owing to a diffuse trailing boundary during elution from the column and this could not be corrected by increasing the eluting power of the solvent because this also eluted large amounts of urinary impurities. The method received widespread criticism especially from Engel (1950), who stated that-"the application of adsorption chromatography as a quantitative procedure has certain theoretical and technical drawbacks. Successful use of the method

depends upon precise standardization of equipment, reagents and adsorbent. Slight variations in the properties of the alumina may have profound effects upon the behaviour of the adsorbed compounds. The shape of the adsorption isotherm is such that the front is usually sharp but trails off in such a manner that unless the peaks are widely separated, overlapping may occur. This trailing also makes it difficult to obtain quantitative recovery. Furthermore, although a series of adsorbed compounds will always be eluted in the same order with the same solvent sequence, the presence of impurities will influence the retention volume of a given component as shown by Dobriner (1948) in the chromatographic separation of ketosteroids". Further, certain labile steroids can undergo decomposition on alumina columns (Dasler, 1948) and great caution is necessary when interpreting results by this method.

Engel et al. (1950) described a method for purifying urine extracts and fractionating the oestrogens by a 24 transfer Craig counter-current distribution machine. This method was able to prove the identity of an oestrogen fraction by relating its behaviour during distribution in the machine to its theoretical behaviour calculated from its partition coefficient. Engel (1952) has been using this method successfully for a number of years and has improved it considerably by using a 50 transfer machine. The method does not completely purify the oestrogen fractions, and urinary background material still interferes when large volumes of urine are processed. However, it is the best method yet published for estimating low concentrations of oestrogen in urine. The expensive Craig machine is the limiting factor to its routine use because one machine can process but one urine extract a day. Therefore, the method is unlikely to be widely used.

More recently, Braunsberg, Stern & Swyer (1952) and Bauld (1952), working independently, have described methods in which purification and separation of the oestrogens is achieved by using partition chromatography on "celite" columns. In theory, partition chromatography does not suffer from some of the disadvantages of adsorption chromatography; for instance, decomposition of labile substances on the column does not occur, and trailing boundaries are not expected. However, in practice, partition chromatography is more difficult. Temperature has to be regulated carefully when partition coefficients are affected by temperature and rates of flow of solvents through columns have to be very slow so that equilibrium is reached at each interface. These slow rates of solvent flow allow diffusion of components in the column so that boundaries are not as sharp as expected and in practice are often no better than those obtained by partition chromatography where equilibrium is quickly reached and solvent flows can be rapid and temperature effects are not important.

With three other laboratories investigating partition methods, it seemed better to concentrate on overcoming the disadvantages of adsorption chromatography and thus improve upon Stimmel's method.

The free oestrogens are not ideally suited to adsorption chromatography on polar adsorbents because, being phenols, they themselves are highly polar. It would be expected that a modification of the phenol group to make it less polar would produce compounds better suited to this type of chromatography. Modification to improve chromatographic properties is not new. For instance, Brooks, Klyne & Miller (1951) successfully used steroid benzoates for the separation

of isomeric pairs on alumina columns. Preliminary experiments in which oestrogens and equivalent urine extracts were methylated or benzoylated indicated that the mono-methyl ethers might be suitable for this purpose. These were found to behave like the free oestrogens in the Kober colour methods, and their only disadvantage was their biological inactivity which would make it impossible to check low oestrogen levels in purified urine extracts by biological assay. Before the methyl ethers could be used in a method, information was required concerning their solubilities in organic solvents, partition between relevant immiscible solvent pairs and their quantitative formation from the free oestrogens. The first methylating agent chosen for investigation was dimethyl sulphate because, except for its poisonous nature, it is the ideal methylating agent for phenols in alkaline solution and would therefore fit into the general oestrogen method where the phenolic fraction is extracted into alkali. Also dimethyl sulphate is a cheap and readily available chemical. These investigations are described in the following sections. The methyl ethers behaved ideally on alumina columns and this work is described fully in the section on chromatography.

When the methylation and extraction procedures without chromatography were applied to phenolic fractions from acid hydrolysed urine, considerable purification was found to occur. Apparently, the "phenolic" fraction still contained substances which were not true phenols. About 40% of the methylated material was alkali saponifiable and therefore removable from the true methoxy-phenols, but no method was found to do this without simultaneous loss of oestrone methyl ether.

PARTITION EXPERIMENTS.

EXPERIMENTAL.

The pure oestrogens or their methyl ethers (see appendix) were dissolved in redistilled 95% ethanol (5 mg./100 ml.) and appropriate volumes of these standard solutions were added, without removing the ethanol, to the solvent pairs before equilibration. Equilibration between immiscible solvent pairs was performed in separating funnels by shaking 100 times. (Preliminary experiments showed that equilibrium was reached in 30 shakes).

THE PARTITION OF OESTRIOL, OESTRONE AND OESTRADIOL BETWEEN RELEVANT IMMISCIBLE SOLVENT PAIRS.

Partition between equal volumes of di-ethyl ether and water.

The oestrogen (200 µg.) in ethanol solution was added to ether (100 ml.) and equilibrated with water (100 ml.). The two phases were separated and the aqueous layer was again equilibrated with an equal volume of ether. The two ether extracts were separately evaporated and a quarter fraction of the first and all of the second were transferred with ethanol to Kober tubes, the ethanol was removed and colours were developed using the modified phenol-sulphonic acid technique. Recoveries were calculated by comparing these colours with those produced by standards from ethanol. The % oestrogen in the second ether phase was calculated by comparing the amount in this phase with the amount remaining in the aqueous phase after the first partition. This figure could not be as accurate as that obtained from the first partition. Each experiment

was performed in triplicate.

	D_{604} Standard 50 µg.	D_{604} Ether phases		Total µg. in ether phases		% of oestrogen in ether phases	
		First	Second	First	Second	First	Second
Oestriol	.216	.189	.092	175	21.3	87.5	87.7
	.217	.178	.094	165	21.3	82.6	60.7
		.192	.108	178	24.3	87.8	100
Oestrone	.260	.255	0	200	0	100	-
	.255	.270	.003	208	0	104	-
		.262	0	202	0	101	-
Oestradiol	.155	.157	0	202	0	101	-
	.150	.155	0	200	0	100	-
		.152	0	200	0	100	-

When the oestrogens were partitioned between equal volumes of di-ethyl ether and water, approximately 85% of the oestriol, and 100% of the oestrone and oestradiol were recovered in the ether phase.

Partition between equal volumes of di-ethyl ether and dilute hydrochloric acid (conc. acid 15 ml. + water 100 ml.).

This experiment was performed in the same manner as the partition between ether and water.

	D_{604} Standard 50 µg.	D_{604} Ether phases		Total µg. in ether phases		% oestrogen in ether phases	
		First	Second	First	Second	First	Second
Oestriol	.216	.178	.126	166	29	83	83
	.217	.194	.130	179	29	90	139
		.189	.121	175	30	87	110
Oestrone	.260	.286	.003	222	0	111	-
	.255	.278	.004	214	0	107	-
		.267	.004	208	0	104	-

	D_{604} Standard 50 µg.	D_{604} Ether phases		Total µg. in ether phases		% oestrogen in ether phases	
		First	Second	First	Second	First	Second
Oestradiol	.155	.173	0	214	0	107	-
	.150	.162	0	210	0	105	-
		.160	0	206	0	103	-

When the oestrogens were partitioned between equal volumes of di-ethyl ether and dilute hydrochloric acid, approximately 85% of the oestriol and 100% of the oestrone and oestradiol were recovered from the ether phase.

Partition between equal volumes of di-ethyl ether and 5% sodium bicarbonate solution.

Oestrogen (200 µg.) in ethanol solution was added to each of three separating funnels which contained ether (100 ml.). The content of one funnel was extracted once, another twice, and the other three times with equal volumes of 5% sodium bicarbonate solution; the ether was distilled and quarter fractions of the residues were transferred with ethanol to Kober tubes. Kober colours were developed with the modified phenol-sulphonic acid technique. Experiments were performed in duplicate. The oestriol recoveries were corrected for losses of oestriol during the water extraction of the ether solutions.

	D_{604}	D_{604} ether phase No. of bicarb. extns.			Corrected % recovery in ether No. of bicarb. extns.		
	Standard 50 µg.	One	Two	Three	One	Two	Three
Oestriol	.223	.200	.194	.193	95	91	90.6
	.230	.210	.199	.192	99	92	91

	D_{604}	D_{604} ether phase No. of bicarb. extns.			Corrected % recovery in ether No. of bicarb. extns.		
	Standard 50 µg.	One	Two	Three	One	Two	Three
Oestrone	.270	.290	.300	.294	100	100	100
	.272	.287	.294	.296	100	100	100
Oestradiol	.152	.158	.159	.160	100	100	100
	.157	.158	.159	.163	100	100	100

Each equal volume of 5% sodium bicarbonate solution removed on an average 3% of oestriol but no oestrone or oestradiol from ether solution.

Oestrone and oestradiol gave increased colour densities when their ether solutions had been in contact with sodium bicarbonate. This increase was caused by a slight non-specific turbidity which appeared during colour development.

The partition of oestriol between equal volumes of ethyl acetate and water.

Oestriol (200 µg.) in ethanol solution was added to ethyl acetate (100 ml.) and extracted with water (100 ml.). The water phase was extracted again with ethyl acetate (100 ml.). The ethyl acetate extracts were separately evaporated to near dryness. A quarter fraction of the first and all of the second ethyl acetate extract residues were transferred to Kober tubes with ethanol. Kober colour was developed with the modified phenol-sulphonic acid method.

Standard 50 µg.	First ethyl acetate extract			Second ethyl acetate extract	
D_{604}	D_{604} ¼ fraction	Oestriol (µg.)	% present	D_{604} complete fraction	Oestriol (µg.)
.243	.238	192	97	.018	4
.250	.230	186	93	.022	5
	.245	200	100	.028	6

These favourable figures indicated that ethyl acetate should be an excellent solvent for extracting oestriol from aqueous solutions. However, its use was limited because acid and alkaline solutions caused it to hydrolyse slightly at room temperatures to substances which interfered in the Kober colour reaction.

Partition between equal volumes of benzene and water.

Oestriol (200 µg.) in ethanol solution was added to benzene (100 ml.) and partitioned twice with water (100 ml.). The water phases were separately extracted once with ether (100 ml.). The ether was evaporated and appropriate fractions of the residues were transferred with ethanol to Kober tubes. Oestriol was estimated by developing Kober colour with the modified phenol-sulphonic acid method. To correct for the oestriol lost in the ether/water extraction, results were multiplied by the factor 1/0.85.

Standard 50 µg.	First water extract.			Second water extract.		
D604	D604 ¼ fraction	Oestriol (µg.)	% present	D604 complete fraction	Oestriol (µg.)	% present
.218	.154	173	87	.077	22	81
.210	.158	173	87	.079	22	83
	.150	170	85	.081	23	76

Oestrone and oestradiol (200 µg.) were added to benzene (100 ml.) and partitioned with water (100 ml.) and the water was extracted once with ether (100 ml.). The benzene and ether solutions were evaporated, and appropriate fractions of the residues were transferred with ethanol to Kober tubes. Oestrogens were estimated using the modified phenol-sulphonic acid colour method. Oestrone was also estimated in quarter

fractions of the benzene phase by the new hydroquinone-68% sulphuric acid colour method.

To eliminate the possibility that mere evaporation of oestrogens with benzene might interfere with colour development, benzene (100 ml.) containing oestrone (200 µg.) was evaporated without water partition and the oestrone was estimated in quarter fractions of the residue with both the modified phenol-sulphonic acid and the hydroquinone-68% sulphuric acid colour methods.

Standard 50 µg. reagent.		Control standard in benzene ¼ fraction.		Benzene phase ¼ fraction.		Water phase complete.	% in benzene.
D_{604} phenol	D_{604} hydroquinone.	D_{604} phenol	D_{604} hydroquinone.	D_{604} phenol	D_{604} hydroquinone	D_{604} phenol	
Oestrone							
.274	.435	.273	.443	.273	.439	0	100
.270	.443	.270	.439	.279	.444	0	100
				.270	.445	0	100
Oestradiol							
.153				.152		.003	100
.149				.148		.006	100
				.148		.007	100

When oestriol, oestrone and oestradiol were partitioned between equal volumes of water and benzene 85% of the oestriol was found in the water phase and 100% of the oestrone and oestradiol were found in the benzene.

Partition between benzene and normal sodium hydroxide solution.

Benzene (100 ml.) containing oestrone or oestradiol (200 µg.) was shaken with two lots of normal sodium hydroxide solution. /(100 ml.) The sodium

hydroxide solutions were acidified with hydrochloric acid and extracted once with equal volumes of ether. The ether extracts were extracted with two lots of water (25 ml.), to remove acid, evaporated, and appropriate fractions were transferred to Kober tubes.

Control experiments to show that oestrone and oestradiol could be recovered quantitatively from sodium hydroxide solutions were performed by adding the oestrogens to normal sodium hydroxide, acidifying, and extracting with ether etc. as in the above experiment.

Oestrone and oestradiol were estimated with the modified phenol-sulphonic acid reagent. Oestrone was also estimated where possible with the new hydroquinone-68% sulphuric acid colour reagent.

By the modified phenol-sulphonic acid method:

D604 Standard 50 µg.	D604 Control from N.NaOH	First NaOH extract of benzene: D604 ¼ fraction	Oestrogen (µg.)	Recovery %	Second NaOH extract of benzene: D604 complete fraction	Oestrogen (µg.)	Recovery %
Oestrone							
.287	.280	.220	155	78	.140	25	56
.281	.281	.248	174	87	.131	24	92
	.283	.230	162	81	.146	26	69
Oestradiol							
.152	.144	.142	190	95	.023	8	80
.152	.147	.144	192	96	.022	7	88
.145	.147	.144	192	96	.032	7	88

With the hydroquinone reagent:

D_{604} Standard 51.4 µg.	Control from N.NaOH D_{604} ¼ fraction		First NaOH extract of benzene D_{604} ¼ fraction		Recovery %
Oestrone					
.451	.462	.463	.375	.371	82
.459	.462	.462	.379	.388	84
	.460	.472	.372	.377	82

When oestrone and oestradiol were partitioned between equal volumes of aqueous normal sodium hydroxide and benzene, approximately 82% of the oestrone and 96% of the oestradiol were found in the sodium hydroxide.

Since the Kober colour method using the hydroquinone-sulphuric acid reagent appeared to be as satisfactory for the estimation of oestrogens as that using the phenol sulphonic acid reagent and since it was the more convenient method, it was chosen for further work.

Partition between benzene and 0.2 N.sodium hydroxide, and between benzene and 0.4 N.sodium hydroxide.

Oestriol, oestrone and oestradiol (30 µg.) were added in ethanol solution (0.6 ml.) to benzene (100 ml.) which was shaken with an equal volume of aqueous sodium hydroxide solution. The oestrogens were recovered from the sodium hydroxide layer by slight acidification and extraction with an equal volume of ether. The ether was extracted three times with water (10 ml.) to remove acid and was evaporated to near dryness and the residues transferred to Kober tubes with ethanol.

~~XXXXXXXXXXX~~. The benzene layer in the case of oestrone was extracted a second time with sodium hydroxide. The benzene layers were then extracted with water until neutral, evaporated, and the residues transferred to Kober tubes with ethanol.

In the case of oestriol, the water washes of the benzene were also slightly acidified and extracted with ether, and the ether was examined for the presence of oestriol.

Oestrogens were estimated by the hydroquinone-sulphuric acid colour method.

Oestriol:

	D_{604} Standard 30 µg.	D_{604} NaOH layer.	D_{604} benzene layer.	D_{604} water washes of benzene.	Recovered in NaOH corrected for calculated losses in ether extraction (i.e. x 100/81).
0.2 sodium hydroxide.	.199	.130	0	0	80%
	.199	.132	0	0	82%
0.4 N.sodium hydroxide	.199	.142	0	0	88%
	.199	.135	0	0	84%

Oestrone and oestradiol:

	D_{604} Standard 30 µg.	First NaOH extraction		Second NaOH extraction		Benzene layer D_{604}
		D_{604}	% oestrogen	D_{604}	% oestrogen	
Oestrone						
0.2 N.sodium hydroxide	.275	.175	70	.050	65	.027
	.275	.175	70	.048	64	.027
0.4 N.sodium hydroxide	.282	.218	80	.045	83	.009
	.280	.215	79	.048	100	.000
Oestradiol						
0.2 N.sodium hydroxide	.182	.179	96			.008
	.185	.182	96			.008
0.4 N.sodium hydroxide	.200	.192	97			.005
	.198	.185	96			.008

The low overall recovery of oestriol from the alkali-benzene partition was difficult to explain. Approximately 85% was accounted for in the sodium hydroxide solution, but none could be recovered either from the benzene layer or its water washes. Later work showed that this loss was not due to destruction, but to interference in the colour reaction. Since none could be detected in the benzene after partition, it was reasonable to assume that all the oestriol was extracted by the sodium hydroxide.

Therefore, when oestriol, oestrone and oestradiol were partitioned between equal volumes of benzene and aqueous sodium hydroxide solutions, all the oestriol, 70% of the oestrone and 96% of the oestradiol were found in the sodium hydroxide when this was 0.2 N., and all of the oestriol, 80% of the oestrone and 96% of the oestradiol were found in the sodium hydroxide when this was 0.4 N..

Partition of oestriol between benzene and buffer solutions, pH between 7 and 10.

The buffer solutions wer prepared according to the instructions of Britton (1932) by mixing stock solutions of -

boric acid 0.2 M (boric acid 1.24 g. dissolved in 100 ml. of aqueous solution),
sodium borax 0.05 M (sodium diborate 1.91 g./100 ml. of aqueous solution),
sodium hydroxide 0.1 N,

as follows:-

pH	Boric acid 0.2 M.	Borax 0.05 M.	NaOH 0.1 N.
7	9.4	0.6	-
8	7.0	3.0	-
9	2.0	8.0	-
10	-	6.0	4.0

These buffer solutions were diluted 1 in 10 for the partition experiments.

Oestriol (30 µg.) was partitioned between benzene (50 ml.) and two lots of borate buffer solution (50 ml.). The borate solutions were slightly acidified with hydrochloric acid and extracted once with one volume and twice with half volumes of ether. The ether extract was extracted three times with water (10 ml.) to remove acid, taken to near dryness and the residues transferred with ethanol to Kober tubes. Oestriol was estimated with the hydroquinone-76% sulphuric acid colour method.

Standard D_{604}	Buffer pH	First buffer extract		Second buffer extract	
		D_{604}	Recovery corrected for water wash of ether.	D_{604}	Recovery corrected for water wash of ether.
0.196	7.0	.134	71.5%	.016	29%
	8.0	.143	77%	.019	42%
	9.0	.142	76%	.020	44%
	10.0	.142	76%	.022	47%

When oestriol was partitioned between equal volumes of benzene and dilute buffer solutions, the buffer contained 72% of the oestriol when its pH was 7 and 76% when its pH was between 8 and 10. These figures are not as good as those given by distilled water alone.

Partition of oestrone and oestradiol between toluene and 0.4 N.sodium hydroxide.

Toluene (50 ml.) containing oestrone and oestradiol (30 µg.) in ethanol (0.6 ml.) was extracted three times with 0.4 N.sodium hydroxide (25 ml.). The soda extracts were acidified and extracted once with equal volumes of ether. The ether was extracted twice with water

(5 ml.), evaporated, and the residues transferred with ethanol to Kober tubes. Colours were developed with the hydroquinone-sulphuric acid reagents.

In the table, recoveries are calculated for equal volumes of toluene and alkali.

	Standard	1st 25 ml. NaOH		2nd 25 ml. NaOH		3rd 25 ml. NaOH	
	D_{604}	D_{604}	Calculated recovery	D_{604}	Calculated recovery	D_{604}	Calculated recovery
Oestrone	.270	.179	80%	.053	74%	.029	60%
Oestradiol	.189	.167	94%	.023	100%	.008	

When oestrone and oestradiol were partitioned between equal volumes of toluene and 0.4 N.sodium hydroxide approximately 80% of the oestrone and 94% of the oestradiol were recovered from the sodium hydroxide. These figures are very similar to those for partitions between benzene and 0.4 N.sodium hydroxide.

Partition of oestriol between ether and concentrated carbonate buffer solutions.

The preparation of carbonate buffer solutions with known pHs was first investigated.

Sodium bicarbonate solution, prepared by dissolving sodium bicarbonate (80 g.) in water and diluting to 1 litre, was titrated with 5 N.sodium hydroxide (sodium hydroxide pellets, 20 g. in 100 ml. of aqueous solution). The hydrogen ion concentrations of the mixtures were measured with a glass electrode pH meter.

ml. 5 N.sodium hydroxide added to 100 ml. sodium bicarbonate solution.	0	2	4	6	8	10	15
pH	8.3	9.0	9.35	9.6	9.82	10.03	10.52

Stock carbonate buffer solutions were therefore prepared as follows:-

(a) pH 9.0, by mixing 8% sodium bicarbonate solution (100 ml.) with 5 N.sodium hydroxide (2 ml.).

(b) pH 10.5, by mixing 8% sodium bicarbonate solution (100 ml.) with 5 N.sodium hydroxide (15 ml.).

(c) 10% sodium carbonate solution by dissolving anhydrous sodium carbonate (10 g.) in water and making up to 100 ml. of solution.

Ether (50 ml.) containing oestriol (30 µg.) was shaken with carbonate buffer solutions (50 ml.). The carbonate solutions were acidified with hydrochloric acid and extracted once with one volume and twice with a half volume of ether. These ether solutions were extracted twice with water (10 ml.) to remove acid, evaporated to small volumes of ether and these were transferred with ethanol to Kober tubes. The solvent was removed by heating under nitrogen and reduced pressure. Oestriol was estimated with the hydroquinone-76% sulphuric acid colour method. The results will be discussed in the section dealing with the removal of acids and poly-hydroxy phenols from urine extracts.

Carbonate solution	D_{604} Standard oestriol 30 µg. 0.212, 0.212 oestriol in the carbonate buffer solution.			
	D_{604}		% present	
pH 9.0	.012	.006	6	3
pH 10.5	.008	.006	4	3
10% sodium carbonate	.035	.026	17	12

THE PARTITION OF THE METHYL ETHERS OF OESTRIOL, OESTRONE AND OESTRADIOL BETWEEN A NUMBER OF IMMISCIBLE SOLVENT PAIRS.

Partition of the oestrogen methyl ethers between equal volumes of di-ethyl ether and water and of di-ethyl ether and N.sodium hydroxide solution.

Water or N.sodium hydroxide solution (100 ml.) containing oestrogen methyl ethers (60 µg.) was extracted two times with di-ethyl ether (100 ml.). The ethyl ether extracts of the sodium hydroxide solution were further extracted three times with water (25 ml.) to remove alkali. The ether layers were evaporated to dryness, half fractions were transferred with ethanol to Kober tubes and the ethanol was removed by heating in a current of air. Oestrogen methyl ethers were estimated with both the modified phenol-sulphonic acid and the hydroquinone-sulphuric acid colour methods.

Control experiments were performed, in which the oestrogen methyl ethers (60 µg.) were added directly to di-ethyl ether (100 ml.) and this was evaporated and the residues transferred.

	Phenol-sulphonic acid						Hydroquinone-sulphuric acid					
Methyl ethers of:	Oestriol		Oestrone		Oestradiol		Oestriol		Oestrone		Oestradiol	
Std. 30 µg. D_{604}	.138	.134	.153	.153	.086	.088	.160	.164	.225	.220	.128	.135
Std. 30 µg. in di-ethyl ether	.125	.128	.142	.139	.082	.084	.150	.159	.230	.222	.131	.135
Ether/water 1st extn. D_{604}	.124	.124	.148	.154	.088	.084	.140	.148	.186	.208	.128	.130
Ether/water 2nd extn. D_{604}	0	0	0	0	0	0	0	0	0	0	0	0
Ether/water % recovery in ether 1st extn.	100		100		100		100		100		100	

Methyl ethers of:	Phenol-sulphonic acid						Hydroquinone-sulphuric acid					
	Oestriol		Oestrone		Oestradiol		Oestriol		Oestrone		Oestradiol	
Std. 30 µg. D_{604}	.142	.141	.152	.150	.095	.100	.168	.170	.230	.225	.150	.154
Ether/NaOH 1st extn. D_{604}	.136	.141	.146	.150	.100	.097	.162	.162	.220	.222	.152	.157
Ether/NaOH 2nd extn. D_{604}	0	0	0	0	0	0	0	0	0	0	0	0
Ether/NaOH % recovery in ether 1st extn.	100		100		100		100		100		100	

Partition between equal volumes of petroleum ether (bp. 40 - 60°) and water, and of petroleum ether or benzene and N.sodium hydroxide solution.

This experiment was performed in a manner similar to the last, except that 50 ml. of solvent and 30 µg. of oestrogen methyl ethers were used. The oestrogen methyl ethers were estimated only by the hydroquinone-sulphuric acid colour methods.

	Oestriol			Oestrone			Oestradiol		
	D_{604}		% in organic phase	D_{604}		% in organic phase	D_{604}		% in organic phase
Standard 30 µg.	.202	.202		.232	.240		.174	.173	
Standard control in pet. ether	.192	.192		.250	.219		.173	.175	
Pet.ether/water 1st extraction	.030		16	.210	.270	100	.169		100
2nd extraction	.021		13	0	0		0		
Pet.ether/NaOH 1st extraction	.041	.040	22 21	.243	.189	100	.172	.133	100
2nd extraction	.031	.023	20 15	0	0		0	.007	

	Oestriol			Oestrone			Oestradiol		
	D_{604}		% in organic phase	D_{604}		% in organic phase	D_{604}		% in organic phase
Standard	.198	.198		.235	.235		.171	.177	
Standard control in benzene	.184	.192		.240	.268		.191	.192	
Benzene/NaOH 1st extraction	.180	.180	100	.302	.267	100	.195	.195	100
2nd extraction	.002	.002		0	0		0	0	

It was not until after this experiment that the following phenomena were discovered and controlled.

1. The colours produced by the hydroquinone-sulphuric acid colour method gradually increase after final dilution with 30% sulphuric acid and do not reach a stable maximum until 10 minutes after dilution.

2. The oestrogen methyl ethers, particularly that of oestrone, are susceptible to atmospheric oxidation when heated and therefore should be heated only under anaerobic conditions (see section on colorimetric methods).

Applying this information to experiments on the partition of oestriol methyl ether between equal volumes of benzene and N.sodium hydroxide and of oestrone and oestradiol methyl ethers between petroleum ether and water, petroleum ether and N.sodium hydroxide, and petroleum ether and 10% ethanol in water; the following results were obtained.

Oestriol methyl ether (30 µg.):

System	Standard 30 µg.		Benzene control		Benzene/ NaOH (50 ml.)	
D_{604}	.176	.178	.178	.176	.172	.182

Oestrone and oestradiol methyl ethers (30 µg.):

	Standards	Benzene control	Pet.ether control	Pet.ether/ water	Pet.ether/ NaOH	Pet.ether/ 10% ethanol in water
Oestrone D_{604}	.270 .265	.260 .260	.255 .250	.255 .255	.260 .250	.252 .252
Recovery in organic layer				100%	100%	100%
Oestradiol D_{604}	.220 .215	.213 .210	.210 .210	.213 .210	.212 .208	.210 .210
Recovery in organic layer				100%	100%	100%

The recovery figures were calculated by comparing the colour densities obtained from the organic layer after partition with those produced from the same organic solvent plus oestrogen without partition ("the control").

Evidently when the methyl ethers of oestriol, oestrone and oestradiol were partitioned between di-ethyl ether or benzene and water or alkali, all of the methyl ethers were recovered quantitatively from the organic solvent layer. However, when they were partitioned between equal volumes of petroleum ether and aqueous solutions only the methyl ethers of oestrone and oestradiol were recovered quantitatively from the petroleum ether; oestriol methyl ether was distributed between the two phases so that only 16% was in the petroleum ether when the aqueous phase was water and 21% when it was normal sodium hydroxide.

A summary of results obtained by these partition experiments.

System	% oestrogen in organic layer for equal volumes			Partition coefficient = concentration in organic / concentration in aqueous		
The free oestrogens	Oestriol	Oestrone	Oest-radiol	Oestriol	Oestrone	Oest-radiol
Ether-water	85	100	100	5.7	∞	∞
Ether-15% HCl	85	100	100	5.7	∞	∞
Ether-$NaHCO_3$ 5%	97	100	100	33	∞	∞
Ether-conc.carbonate pH9	96			24		
Ether-conc.carbonate 10.5	96			24		
Ether-sod.carbonate 10%	85			5.7		
Benzene-water	15	100	100	0.18	∞	∞
Benzene-dil.buffer pH 9	23	100	100	0.3	∞	∞
Benzene-dil.buffer pH 10	24	100	100	0.32	∞	∞
Benzene-0.2 N.NaOH	0	30	4	0	.43	.04
Benzene-0.4 N.NaOH	0	20	3	0	.25	.03
Benzene-N.NaOH	0	20	4	0	.25	.04
Toluene-water		100	100		∞	∞
Toluene-dil.buffer pH 9		100	100		∞	∞
Toluene-0.4 N.NaOH	0	20	4	0	.25	.04
Ethyl acetate-H_2O	95			19		
The oestrogen methyl ethers						
Di-ethyl ether-water	100	100	100	∞	∞	∞
Di-ethyl ether-N.NaOH	100	100	100	∞	∞	∞
Benzene-water	100	100	100	∞	∞	∞
Benzene-N.NaOH	100	100	100	∞	∞	∞
Petroleum ether-water	16	100	100	.19	∞	∞
Petroleum ether-N.NaOH	21	100	100	.27	∞	∞

THE REMOVAL OF ACIDS AND POLYHYDROXY PHENOLS FROM URINE EXTRACTS.

Mather (1942) found that when oestriol was partitioned between equal volumes of diethyl ether and 0.3 M.sodium carbonate solution, 65% was recovered in the ether phase. Using 0.9 M.sodium carbonate, Bachman & Pettit (1941) found 80% of the oestriol in the ether phase. Cohen & Marrian (1934) neutralized the strongly alkaline extract containing the urinary phenolic fraction with gaseous carbon dioxide until it was no longer alkaline to phenolphthalein (pH 9.0), and extracted the phenols with ether. Cleaner urine fractions were obtained in this early method than in later methods where neutralization was more conveniently achieved by adding mineral acids. Engel, Slaunwhite, Carter & Nathanson (1950) confirmed these findings and re-introduced the extraction with ether of the urinary phenols from their alkaline solutions adjusted to pH 9 to 9.5 with mineral acid and potassium carbonate (the "Engel procedure"). Engel (1950) also suggested the use of 0.85 M.sodium carbonate instead of saturated sodium bicarbonate solution for the preliminary removal of the urinary acid fraction but does not seem to have developed this further.

As indicated by Engel, the solubility of oestriol in aqueous solvents is not due to a "strong" phenolic character since its dissociation constant does not differ greatly from that of oestrone (pK 9.11 compared with 9.36). Its solubility in water is less dependent upon pH than was at first thought, and must therefore depend upon the presence of the glycol grouping as well as the phenolic hydroxyl. For instance, experiments described in the section on partition coefficients showed that the partition coefficient of oestriol between ether and hydrochloric acid is the same as that between ether and water and its partition coefficient between benzene and water pH 7.0 is the same as between benzene and water pH 10. A further property of oestriol

which does not seem to have been fully appreciated, is that although its solubility in water is practically independent of pH between 1 and 10, it is easily salted out from aqueous solution as shown by its much higher partition coefficient (33) between ether and saturated sodium bicarbonate solution than between ether and water (5.6).

It was hoped that the application of this knowledge might lead to improved procedures for removing the acid fraction from urine extracts.

The "Engel procedure" also removes polyhydroxy phenols which oxidize readily in strongly alkaline solution and are then not re-extracted by ether at pH 9. There was every reason to expect that the removal of these polyhydroxy phenols could be similarly achieved by shaking ether solutions with strong alkali, adding sodium bicarbonate to decrease alkalinity and salt out the oestriol, and shaking again before separating the two phases.

Preliminary work recorded in the section on the partition of the oestrogens between immiscible solvent pairs described the preparation of concentrated carbonate buffer solutions, and gave the following figures for the partition of oestriol between them and ether.

System	% oestriol in ether for equal volumes	Partition coefficient
ether - $NaHCO_3$ 5%	97	33
ether - conc. carbonate pH 9	96	24
ether - conc. carbonate pH 10.5	96	24
ether - sod. carbonate 10%	85	5.7

The purification of ether extracts by carbonate solutions of various pHs.

Pooled male urine (600 ml.) was acid hydrolysed and extracted with a total of 1200 ml. of ether. Fractions (50 ml.) of this ether extract were used in the following experiments.

Fractions (50 ml.) were extracted once with equal volumes of (a) 5% sodium bicarbonate solution (b) carbonate buffer pH 10.5 (c) 10% sodium carbonate solution, each in duplicate, and the ether was evaporated. One of each duplicate was transferred to a Kober tube, the other was dissolved in ethanol (1 ml.) and benzene (25 ml.) and extracted with 0.4 N.sodium hydroxide (1 x 1, 2 x ½ vol.). The benzene layers containing the neutral fractions were extracted once with water, the benzene was distilled and the residues transferred to Kober tubes. The soda extracts were first adjusted with hydrochloric acid and potassium carbonate to approximately pH 9 and extracted with ether (1 x 1, 2 x ½ vol.) (Engel's fraction) and then acidified with hydrochloric acid and extracted again with ether (1 x 1, 2 x ½ vol.). The ether extracts were evaporated and transferred to Kober tubes. The amounts of urinary chromogens in the various fractions were estimated by applying the colour reaction using the 2% hydroquinone-76% sulphuric acid reagent.

D_{604}

	Total ether extractable	phenolic + neutral	neutral	phenolic		Total phenolic
	Ether before carbonate extraction	ether after carbonate extraction	benzene fraction	ether from NaOH pH 9	ether from pH 9 acidified	+ neutral
$NaHCO_3$ 5%	1.05	.480	.161	.120	.156	.437
$NaHCO_3$ pH 10.5	1.05	.375	.157	.097	.103	.357
Na_2CO_3 10%	1.05	.312	.158	.088	.058	.304

The effect of various carbonate and alkali extractions on the recovery of oestriol and purification of urine extracts.

Fractions (50 ml.) of the above ether extract of hydrolysed urine and ether solutions (50 ml.) of oestriol (30 µg.) were extracted in the following manners:

1. Twice with 5% sodium bicarbonate solution (2 x 10 ml.).

2. Twice with sodium carbonate buffer pH 10.5 (2 x 10 ml.).

3. Once with 5% sodium bicarbonate (10 ml.), once with 10% sodium carbonate (10 ml.), and again with 5% sodium bicarbonate (10 ml.).

4. Once with carbonate buffer pH 9.0 (10 ml.), and shaken with N.sodium hydroxide (2 ml.) then with 8% sodium bicarbonate solution (8 ml.) to neutralize the alkali to pH 9.0, and extracted again with sodium carbonate pH 9 (10 ml.).

5. Once with carbonate buffer pH 10.5 (10 ml.), and shaken with 2 N.sodium hydroxide (3 ml.) and then 8% sodium bicarbonate (8 ml.) to neutralize the alkali to pH 10.5, and extracted again with carbonate buffer pH 10.5 (10 ml.) and then with 5% sodium bicarbonate (10 ml.).

The ether solutions without water washes were evaporated, the residues were transferred to Kober tubes and colours developed using the 2% hydroquinone-76% sulphuric acid reagent

	$NaHCO_3$ 5%	CO_3^{II} pH 10.5	$NaHCO_3$ 5% Na_2CO_3 10% $NaHCO_3$ 5%	CO_3^{II} pH 9 NaOH+$NaCO_3^H$ CO_3^{II} pH 9	CO_3^{II} pH 10.5 NaOH+$NaHCO_3$ CO_3^{II} pH 10.5 $NaHCO_3$ 5%
Oestriol 30 µg. D_{604}	.198 .195	.188	.198	.194 .190	.192 .196
Standard 0.212 Recovery %	93	89	93	91	92
Urine extract D_{604}	.480 .470	.376	.354	.304 .296	.260 .258
Less 0.160 neutral fraction (calculated).	.315	.216	.194	.140	.099

The figure, 0.099, obtained by the last procedure agrees closely with the figure, 0.097, in the previous experiment by applying the Engel purification step after extraction with carbonate buffer pH 10.5 and is better than the figure, 0.120, which represents the value obtained by his complete procedure. Evidently, then, the application of carbonate buffer and alkali washes to ether extracts from urine is a convenient method of removing acids and polyhydroxy phenols without loss of oestriol and is superior to the original "Engel" procedure.

To show whether the alkali - carbonate buffer extraction removes material which would otherwise pass through the methylation step.

Pooled male urine (800 ml.) was acid hydrolysed and extracted with ether (1 x 1, 2 x ½ vol.).

Fractions (100 ml.) of ether solution were extracted either twice with fifth volumes of 5% sodium bicarbonate solution or with alkali and carbonate in the following manner. The ether solution was shaken with

carbonate buffer solution pH 10.5 (20 ml.) which was discarded, and then with 2 N.sodium hydroxide (6 ml.) to oxidize the polyhydroxy phenols. Sodium bicarbonate solution (16 ml. of 8%) was shaken with the ether and alkali and the combined aqueous solution was separated and discarded. The ether was extracted again with carbonate buffer pH 10.5 (20 ml.) (which removed no visible pigment) and then with 5% sodium bicarbonate solution (20 ml.).

The ether solutions were evaporated and the residues dissolved in ethanol (1 ml.) and benzene (25 ml.) and extracted with water (1 x 1, 2 x ½ vol.) ("oestriol" fraction) and then with 0.4 N.sodium hydroxide (1 x 1, 2 x ½ vol.) ("oestrone" fraction). The fractions were methylated at 37°C with dimethyl sulphate (2 x 1 ml.) in the presence of boric acid (0.8 g.). The methylation products were extracted with benzene or petroleum ether and the solvents were removed. Urinary chromogens in the residues were estimated with the appropriate hydroquinone-sulphuric acid colour method.

	D_{604}			
	"oestriol" fraction		"oestrone" fraction	
5% sodium bicarbonate extraction	.106	.098	.060	.056
Alkali and carbonate buffer extraction	.035	.028	.038	.035

The alkali - carbonate procedure.

The final procedure which was adopted follows.

The ether extract of hydrolysed urine was shaken with 1/5th. volume of carbonate buffer pH 10.5. The aqueous layer was discarded. The ether extract was then shaken at least a hundred times with 1/20th. volume of 2 N.sodium hydroxide, a 1/5th. volume of 8% sodium bicarbonate solution was added and the whole shaken again. The aqueous layer was discarded. The ether was extracted with 1/20th. volume of 8% sodium bicarbonate and then with 1/40th. volume of water.

The carbonate buffer solution pH 10.5 was prepared by mixing 8% sodium bicarbonate solution (100 ml.) with 5 N.sodium hydroxide (15 ml.).

THE QUANTITATIVE METHYLATION OF OESTRIOL, OESTRONE AND OESTRADIOL WITH DIMETHYL SULPHATE.

The methylation of phenols with dimethyl sulphate is generally performed (see Gatterman, 1934; Houben, 1943) by shaking a solution of the phenol in excess sodium hydroxide with slightly more than the theoretical amount of dimethyl sulphate. Reaction is completed and excess dimethyl sulphate is destroyed by heating on a water bath. The methylated phenol is separated from the still alkaline reaction mixture, usually by extraction with ether. Methylation is more rapid at higher pHs and sodium hydroxide is better than potassium hydroxide for methylating in aqueous solution. When the phenol is difficult to methylate, methylation is forced by adding a large excess of dimethyl sulphate and its equivalent amount of alkali in batches and with heating. Difficult substances, like the sugars, which are also destroyed by strong alkalis, are methylated in weakly alkaline solutions by adding a large excess of dimethyl sulphate continuously with vigorous stirring and just enough sodium hydroxide to keep the reaction mixture alkaline. No information could be found concerning the rate of hydrolysis of dimethyl sulphate in aqueous solutions or whether hydrolysis is complete when all the dimethyl sulphate is dissolved.

Before quantitative methylation of the oestrogens could be studied, a method was required for the quantitative separation and estimation of the methyl ethers from alkaline solutions, and also their separation from unmethylated oestrogens. In the section on the partition of oestrogen methyl ethers between immiscible solvent pairs, petroleum ether was shown to be a good extracting solvent for oestrone and oestradiol methyl ethers from aqueous solutions, and benzene to be a good solvent for oestriol methyl ether. These were further tested in

the following manner.

Thirty microgrammes of the free oestrogens and of their methyl ethers were added to aqueous normal sodium hydroxide (50 ml.) and kept at room temperature overnight. Solutions containing oestriol and oestriol methyl ether were extracted with benzene (50 ml.) and those containing oestrone and oestradiol and their methyl ethers with petroleum ether (50 ml.). The benzene and petroleum ether extracts were extracted three times with water (10 ml.). In the case of the methyl ethers, the combined aqueous extracts were re-extracted with benzene or petroleum ether. Solvents were completely evaporated, the residues were transferred with ethanol to Kober tubes and the ethanol was removed by heating in a current of air. Colours were developed with the hydroquinone-sulphuric acid reagents.

System	Oestrogen	D_{604} organic phase	Standard D_{604}
Benzene-NaOH	Oestriol	.000	
" "	Oestriol methyl ether (first extract)	.190	.190
" "	Oestriol methyl ether (second extract)	.000	
Pet.ether-NaOH	Oestrone	.000	
" " "	Oestrone methyl ether (first extract)	.197	.245
" " "	Oestrone methyl ether (second extract)	.005	
" " "	Oestradiol	.000	
" " "	Oestradiol methyl ether (first extract)	.153	.187
" " "	Oestradiol methyl ether (second extract)	.002	

The low D_{604} readings obtained when oestrone and oestradiol methyl ethers were recovered from petroleum ether by aerobic evaporation, have been discussed elsewhere. This experiment showed that oestriol methyl ether could be separated quantitatively from free oestriol by one partition between equal volumes of benzene and normal sodium hydroxide, and oestrone and oestradiol methyl ethers from the free oestrogens by one partition between equal volumes of petroleum ether and normal sodium hydroxide; in each case the methyl ether was recovered quantitatively from the organic phase.

Preliminary experiments were performed in which oestriol (30 µg.) in sodium hydroxide solution (50 ml.) was methylated by shaking with dimethyl sulphate (1 ml.). Methylated oestriol was extracted with benzene from the alkaline reaction mixtures. Heating the final solution (30 min.) in the conventional manner gave low recoveries, even when pure oestriol methyl ether itself was subjected to the methylation procedure. Apparently oestriol methyl ether was partly destroyed when heated with strong alkali. Methylation in normal sodium hydroxide with subsequent heating gave poor recoveries (25%) of the methyl ether; these were increased (to 66%) by adding more dimethyl sulphate (3 x 1 ml.). The best recovery (86%) was obtained by methylating, without subsequent heating, in 0.2 N.sodium hydroxide (50 ml.) with dimethyl sulphate (1 ml.) and adding alkali from time to time to keep the reaction just alkaline. As with sugars, methylation seemed to be slow relative to the rate of alkaline hydrolysis of the dimethyl sulphate and therefore dimethyl sulphate or sodium hydroxide had to be added to replace that lost by hydrolysis. These two processes should depend on the pH of the reaction mixtures. Therefore, the following

experiments were performed to determine the effect of pH on the hydrolysis of dimethyl sulphate and its methylation of the oestrogens, and whether heating to destroy excess dangerous dimethyl sulphate was really necessary.

The hydrolysis of dimethyl sulphate in aqueous solutions at various pHs.

At room temperature dimethyl sulphate is hydrolysed by sodium hydroxide solutions according to the following equation:

$$(CH_3O)2SO_2 + NaOH = CH_3OSO_2ONa + CH_3OH$$

The speed of this reaction at various pHs was measured by adding dimethyl sulphate to a large excess of alkali and determining, by back titration, the amount of alkali used up in the reaction. Methyl sulphuric acid is a "strong" acid and therefore methyl orange was used as the titration indicator.

The following solutions were prepared:

0.1 M trisodium phosphate (PO_4")
0.1 M sodium diborate ("borax") (equal volumes)
0.1 M sodium diborate + 0.1 N sodium hydroxide ("Na borate")
0.1 N sodium hydroxide
0.11 N Hydrochloric acid (by titration against oxalic acid)

These solutions were mixed as shown in the table below.

Dimethyl sulphate (0.25 ml. B.D.H. reagent distilled) was added to buffer solutions (50 ml.) at room temperature. Solution was rapid with shaking. Five ml. fractions were taken at time intervals and titrated immediately with 0.11 N hydrochloric acid using methyl orange as the indicator.

The figures given in the table are calculated as ml. of 0.11 N sodium hydroxide neutralized by the dimethyl sulphate which had

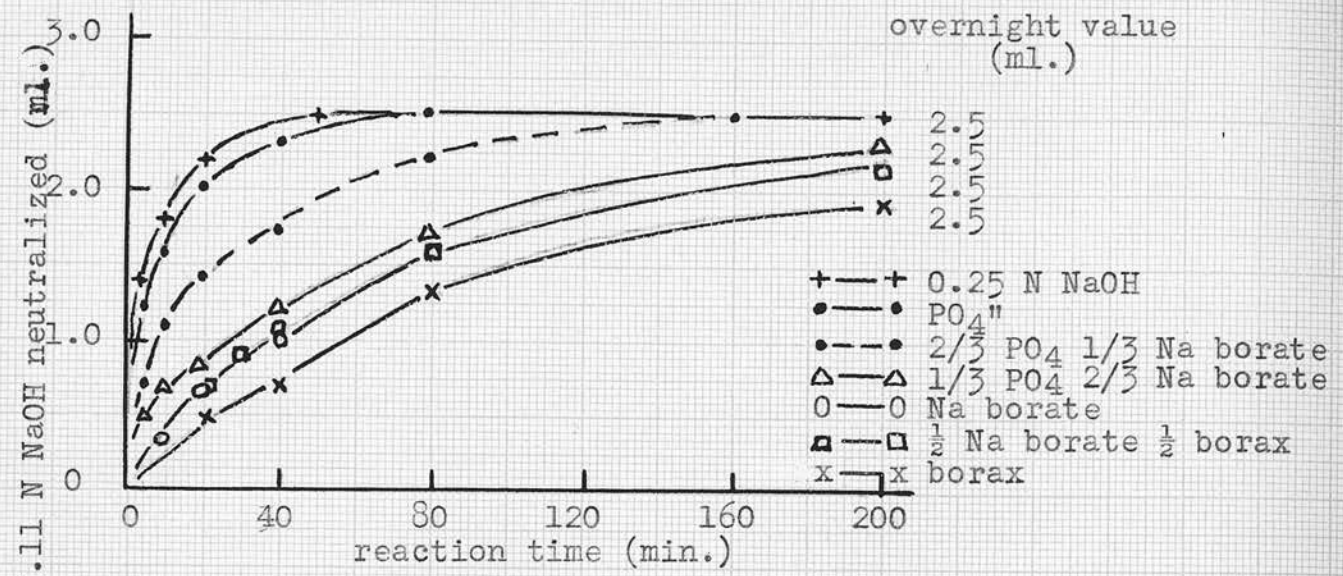


Fig. 16. The hydrolysis of dimethyl sulphate in alkaline buffer solutions at room temperature, showing the effect of alkalinity and time on the amount of NaOH neutralized by the hydrolysed dimethyl sulphate.

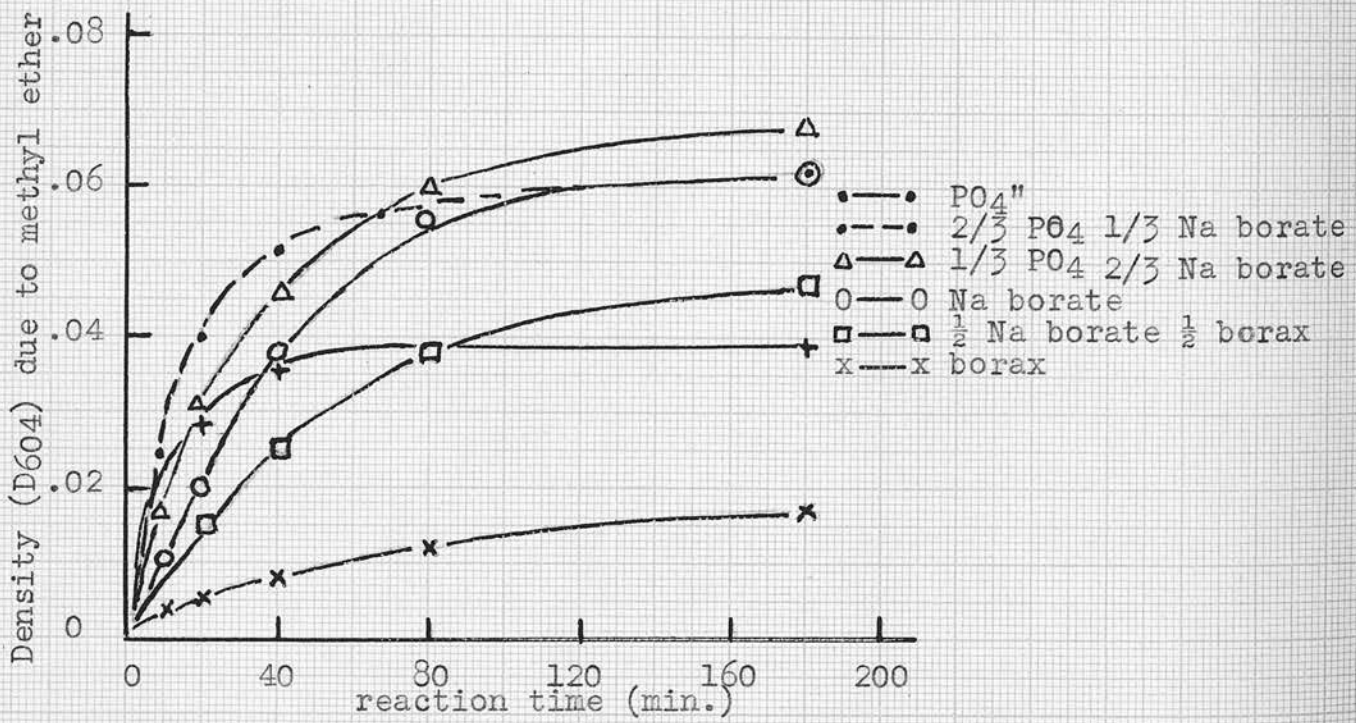


Fig. 17. The methylation of oestriol (30 µg.) in alkaline buffer solutions with dimethyl sulphate at room temperature, showing the effect of the reaction time and the alkalinity on the amount of oestriol methylated.

hydrolysed - (theoretically, when one methyl group hydrolyses, 0.025 ml. of dimethyl sulphate should neutralize 2.5 ml. of 0.11 N sodium hydroxide).

A glass electrode pH meter was used to measure pH.

Time (min.)	NaOH (0.25N)	PO_4''	2/3 PO_4'' 1/3 Na borate	1/3 PO_4'' 2/3 Na borate	Na borate	½Na borate ½ borax	Borax
2½	0.8	0.7	0.5	0.4	0.1		
5	1.4	1.2	0.7	0.5	0.3	0.3	0.2
10	1.8	1.6	1.1	0.7	-	0.4	0.3
20	2.2	2.1	1.4	0.8	0.7	0.7	0.5
40	2.4	2.3	1.7	1.2	1.0	1.1	0.7
80	2.5	2.5	2.2	1.7	1.6	1.6	1.3
200	2.5	2.5	2.6	2.3	2.1	2.2	1.9
Overnight	2.5	2.5	2.6	2.5	2.5	2.5	2.5
Initial pH	?	?	11.2	10.4	10.3	9.9	9.4
Final pH	?	11.4	10.2	9.9	9.8	9.5	8.9

See fig. 16 also.

Apparently dimethyl sulphate dissolves unchanged, at first, in alkaline solution so that the absence of undissolved dimethyl sulphate does not mean absence of active dimethyl sulphate. Therefore, when the dimethyl sulphate is completely dissolved further shaking is unnecessary. The rate of hydrolysis is more rapid the higher the pH, but is almost independent of pH between pH 10.4 and 9.4. Dimethyl sulphate in even slightly alkaline solution, is completely hydrolysed to sodium monomethyl sulphate on standing at room temperature overnight, and heating is then unnecessary.

The methylation of oestriol in buffer solutions.

Dimethyl sulphate (0.3 ml.) was dissolved with shaking in the above buffer solutions (60 ml.) containing oestriol (180 µg.). Ten ml.

fractions of solution were removed at time intervals, made normal with sodium hydroxide, and extracted immediately with benzene (12 ml.). The benzene extract was separated as quickly as possible, extracted with water to remove alkali and the benzene evaporated from Kober tubes by heating under nitrogen and reduced pressure. Oestriol methyl ether was estimated in the residue with the hydroquinone-sulphuric acid colour method.

Time (min.)	PO_4''	2/3 PO_4'' 1/3 Na borate	1/3 PO_4'' 2/3 Na borate	Na borate	½ Na borate ½ borax	Borax
			D_{604}			
5	.025					
10	.025	.025	.017	.011	.010	.005
20	.029	.041	.031	.021	.016	.006
40	.036	.052	.046	.038	.026	.009
80		.067	.060	.056	.038	.013
180		.062	.068	.062	.047	.018
Overnight	.039	.069	.079	.071	.054	.024
Initial pH	?	11.2	10.4	10.3	9.9	9.4
Final pH	11.4	10.2	9.9	9.8	9.5	8.9

See fig. 17 also.

Methylation was more rapid the higher the pH, as expected, but the maximum yield of oestriol methyl ether was obtained between pH 11 and 9.5.

The most vigorous methylating conditions would be those employing the most dimethyl sulphate and the most concentrated borate solutions to buffer its hydrolysis. The borate buffer solutions were the limiting factors because 0.1 M borax solution is nearly saturated at room temperature. By the ratio of molecular weights, 0.1 molar is equivalent to 1.25 g. of boric acid in 50 ml. of solution. This amount

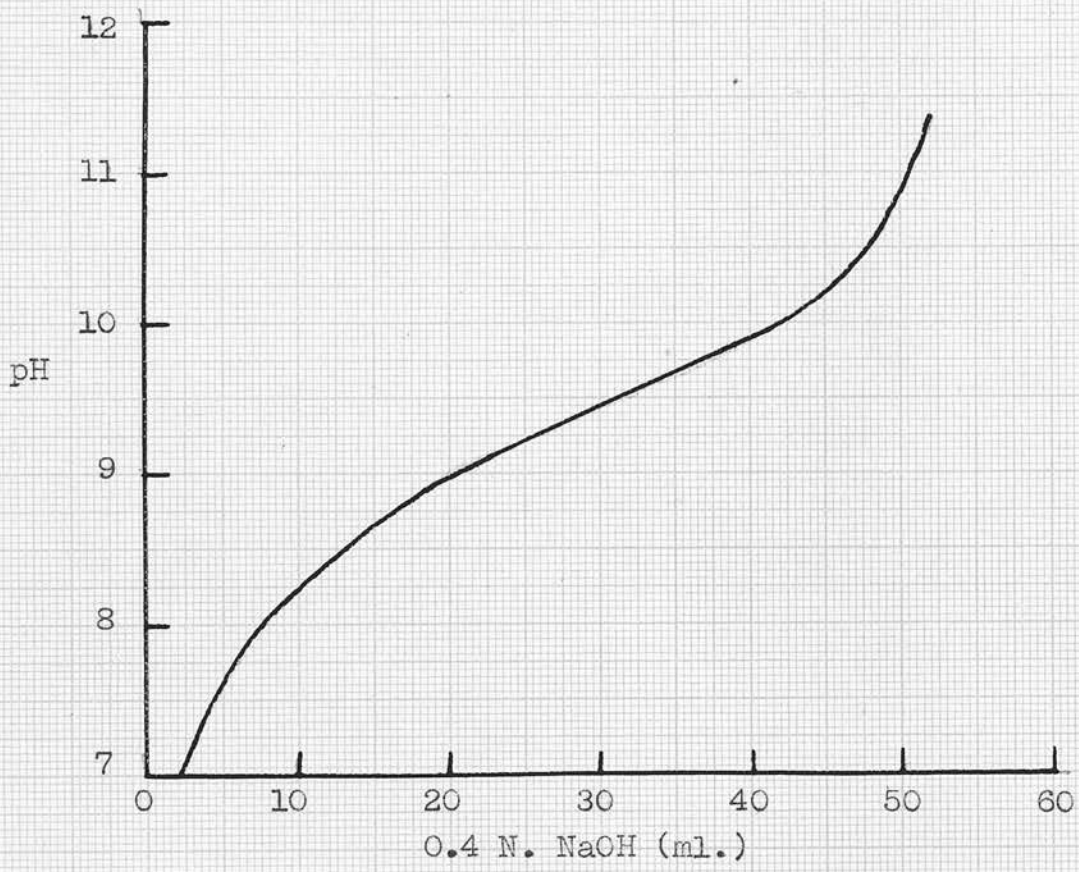


Fig. 18. The titration of boric acid (1.25 g.) with 0.4 N.NaOH, showing the relationship between NaOH (ml.) and pH.

of boric acid powder (B.P. grade) was titrated with 0.4 N.sodium hydroxide and the pH was determined with a glass electrode pH meter (see fig.18). The pH of a solution of 1.25 g. of boric acid in 50 ml. of 0.4 N.sodium hydroxide was 11, and the pH range 11 to 9.2 was equivalent to 25 ml. of 0.4 N.sodium hydroxide or the hydrolysis products of 0.96 ml. of dimethyl sulphate.

The following methylation conditions were derived from this data - (the "first methylation procedure").

Dimethyl sulphate (1.0 ml.) was dissolved with shaking in a solution of boric acid (1.25 g.), 0.4 N.sodium hydroxide (50 ml.) and oestrogen. Because methylation was almost complete in 60 min. at room temperature, a further 1 ml. of dimethyl sulphate could then be added together with 2 ml. of 5 N.sodium hydroxide to return the pH of the solution to its original value. This process could be repeated at hourly intervals. After standing overnight, 5 N.sodium hydroxide (10 ml.) was added to make the methylation mixture approximately normal to sodium hydroxide and the methylated oestrogens were extracted with benzene or petroleum ether.

The methylation of oestrogens using "the first methylation procedure".

When studying the formation of oestrogen methyl ethers from the free oestrogens, it is necessary to relate the colour produced by the methyl ether through its standard to the amount of free oestrogen started with. Using the hydroquinone-sulphuric colour method, a close molecular relationship was shown to exist between the colour densities produced by the free oestrogens and by their methyl ethers. Therefore, free oestrogen colour standards were usually used in experiments which started with the free oestrogen, even though the substance finally measured was the methyl ether.

Oestriol, oestrone and oestradiol were added to 0.4 N.sodium hydroxide (50 ml.) and methylated by the above procedure using two lots of 1 ml. of dimethyl sulphate. Oestriol methyl ether was extracted with benzene and oestrone and oestradiol methyl ethers with petroleum ether. These solutions were extracted with water until neutral, the solvent was evaporated and the residue transferred with ethanol to Kober tubes. The ethanol was removed in a stream of air with heating, and oestrogen methyl ethers in the residue estimated by the hydroquinone-sulphuric acid colour method.

Micrograms oestrogen added	Oestriol		Oestrone		Oestradiol	
	D_{604}	Recovery %	D_{604}	Recovery %	D_{604}	Recovery %
10	.046	71	.068	82	.053	92
20	.122	94	.170	103	.098	85
40	.232	89	.325	99	.209	91
80	.262 x 2	101	.290 x 2	88	.220 x 2	96
160	.272 x 2	105	.294 x 4	89	.211 x 4	92
Standards 40 µg.	.260		.329		.230	

Using anaerobic removal of solvent a similar experiment was performed using 30 µg. of oestrogen to show whether two separate additions of 1 ml. of dimethyl sulphate was superior to one addition of 1 ml.

	Standard 30 µg.	Dimethyl sulphate ml.	D_{604}	Recovery %
Oestriol	.190	1	.182	96
		2 x 1	.190	100
Oestrone	.260	1	.239	92
		2 x 1	.250	96
Oestradiol	.200	1	.187	93
		2 x 1	.199	100

Apparently, two additions of 1 ml. of dimethyl sulphate with an

hour between each, was sufficient to cause quantitative methylation. The method was considered to be satisfactory and was used for some time to develop the complete oestrogen method. However, in cold weather solution of dimethyl sulphate in aqueous solutions was slow and this, with the hour wait between the two additions of dimethyl sulphate, was time consuming. Also, some low recoveries of oestrogens from urines containing large amounts of interfering chromogenic materials, were suspected, but not proved, to be due to interference in a methylation procedure which was not vigorous enough. The following experiments were performed to find a procedure which would be more vigorous and less tedious.

The methylation of oestrone with dimethyl sulphate and borate buffers at 37°C.

Dimethyl sulphate (1 ml.) was dissolved with shaking in solutions maintained at 37°C of oestrone (100 µg.) and varying amounts of boric acid in 0.4 N.sodium hydroxide (50 ml.). Ten ml. fractions were withdrawn at time intervals, added to 5 N.sodium hydroxide (2 ml.), and extracted immediately with petroleum ether (10 ml.). The petroleum ether extract was washed with water to remove alkali, and evaporated under nitrogen with heating and reduced pressure. Oestrone was estimated in the residue by the hydroquinone-sulphuric acid colour method. Results are given in the table in terms of drum readings with Ilford no. 604 filters (D_{604}).

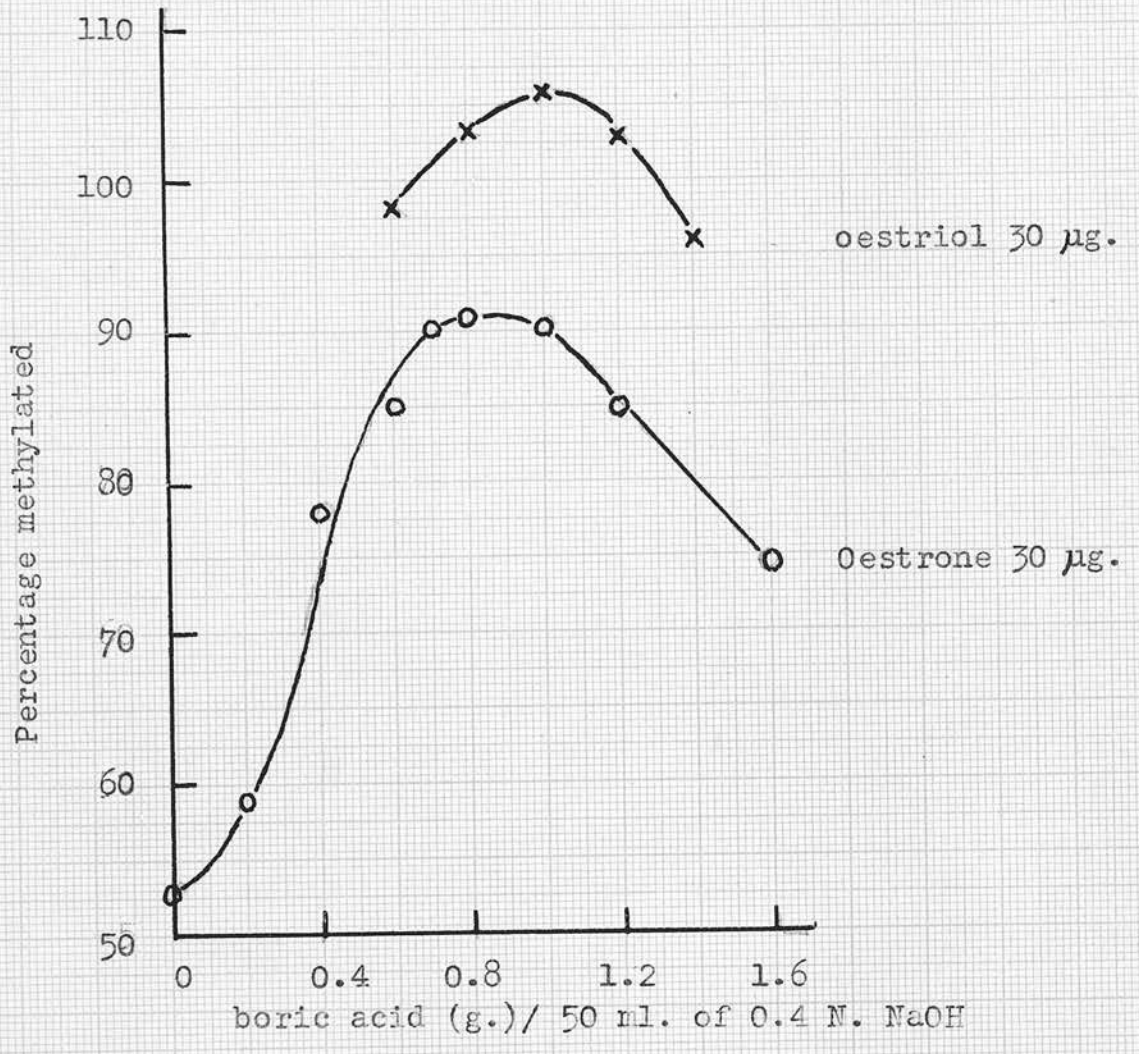


Fig. 19. The methylation at 37°C. of oestriol and oestrone with dimethyl sulphate, showing the relationship between the percentage methylated and the amount of boric acid added to 50 ml. of 0.4 N. NaOH

D_{604}

Time (min.)	Boric acid (g.) in 0.4 N.sodium hydroxide (50 ml.) 1.6	1.2	1.0	0.75	0
10		.131	.138	.132	.084
20	.108	.141	.152	.148	.080
40	.123	.132	.145	.159	.086
80	.126	.140	.151	.153	.086
Overnight		.151	.148	.154	.083
Standard oestrone (20 μg.) D_{604} 0.166					

Using oestrone (20 μg.) the last experiment was repeated by incubating at 37°C for 20 min. only, and varying the amount of boric acid added to 50 ml. of sodium hydroxide.

Boric acid (g.)	0	0.2	0.4	0.6	0.8	No methyl sulphate added
D_{604}	.087	.097	.129	.141	.152	.000

See Fig. 19 also.

At 37°C solution of dimethyl sulphate was rapid; maximum yields of oestrone methyl ether were obtained with 0.7 to 1.0 g. of boric acid in 50 ml. of 0.4 N.sodium hydroxide; and reaction was complete in 20 min.

The methylation of oestriol with dimethyl sulphate and borate buffers at 37°C.

(a) In pure solutions.

Dimethyl sulphate (1 ml.) was dissolved with shaking in solutions of oestriol (30 μg.) and varying amounts of boric acid in 0.4 N.sodium hydroxide (50 ml.) maintained at 37°C. The solutions were incubated for 30 min., 5 N.sodium hydroxide (10 ml.) was added to each and the methylation products were extracted with benzene (50 ml.). The benzene solutions were extracted with water (2 x 5 ml.) to remove alkali, and evaporated to small volumes which were transferred with ethanol to Kober tubes, from which the solvent was removed by heating under nitrogen and reduced pressure.

Boric acid (g.)	0.6	0.8	1.0	1.2	1.4
D_{604}	.192	.202	.206	.203	.187

Standard oestriol 30 µg. D_{604} = 0.195
(See Fig. 19)

(b) In the presence of urine extracts.

Four fractions (100 ml.) of pooled male urine were acid hydrolysed with 15 vols.% hydrochloric acid and extracted with ether. The ether solutions were extracted with alkali and bicarbonate and evaporated. The residues were taken up in ethanol (1 ml.) and benzene (20 ml.) and extracted once with one volume, and three times with half volumes of water. Oestriol (30 µg.) (except the control), boric acid and 5 N.sodium hydroxide (4 ml.) were added to the water extracts, the solutions were warmed to 37°C and dimethyl sulphate (1 ml.) added to each. The mixtures were shaken to dissolve the dimethyl sulphate, kept at 37°C for 30 min., made normal to sodium hydroxide and extracted with benzene (50 ml.). The benzene extracts were washed with water and evaporated to small volumes which were transferred to Kober tubes with ethanol. Oestriol (30 µg.) was added to the "Kober" tube containing the control and solvents were removed by heating under nitrogen and reduced pressure. Oestriol was estimated with the hydroquinone-76% sulphuric acid colour method, colour densities were measured at three wavelengths and corrected for interfering chromogenic material.

	D_{604}	D_{606}	D_{601}	D_{604}	Corrected recovery %
Standard	.199	.004	.052		
Control = urine blank + oestriol	.191	.016	.114	.159	100
Methylation in the presence of 0.8 g. boric acid	.177	.015	.083	.153	96
" " " " " 1.0 g.	.184	.015	.083	.160	100
" " " " " 1.2 g.	.186	.015	.085	.161	101

Urinary material depressed the oestriol colour produced by the hydroquinone-76% sulphuric acid colour method and, therefore, the colour of the control represented 100% recovery depressed by the urine material.

Evidently at 37°C, 1 ml. of dimethyl sulphate and 1.0 g. of boric acid in 50 ml. of 0.4 N.sodium hydroxide caused quantitative methylation of oestriol even when urinary material was present.

The recovery of oestrogen methyl ethers from the free oestrogens by various methylation procedures.

Sodium hydroxide (50 ml.) was added to 100 ml. methylation flasks containing boric acid and oestrogen (30 µg.). Methylation was performed either at 37°C or at room temperature (18°C) by adding dimethyl sulphate and shaking until it had dissolved. When several additions of dimethyl sulphate were made, they were made at intervals of 1 hour and 2 ml. of 5 N.sodium hydroxide was added for every extra 1 ml. of dimethyl sulphate. After the final addition and solution of dimethyl sulphate the mixtures were kept at room temperature overnight and the methylated oestrogens were estimated in the usual manner.

The methylation of oestrone in pure solutions:
(30 µg. Standard D_{604} 0.260, 0.253)

Methylation temperature	Boric acid(g.)/ NaOH	Dimethyl sulphate No. of addns. x ml.	D_{604} methyl ether		Recovery %	
Room	1.25/0.4 N	1	.204	.193	77	73
"	"	2 x 1	.240	.248	90	93
"	"	3 x 1	.246	.242	92	91
37°C	0.8/0.4 N	1	.229		86	
"	"	2 x 1	.248	.249	93	93
"	"	3 x 1	.247		93	
37°C	1.6/0.8 N	1 x 2	.234		88	
"	"	2 x 2	.240		90	
"	"	3 x 2	.242		91	

The methylation of oestriol in pure solutions:
Standard (30 µg.) D_{604} 0.195.

Methylation temperature	Boric acid(g.)/ NaOH	Dimethyl sulphate No. of addns. x ml.	D_{604} methyl ether		Recovery %	
Room	1.25/0.4 N	2 x 1	.204	.208	105	107
"	"	3 x 1	.200	.200	102	102
37°C	0.8/0.4 N	1	.184		94	
"	"	2 x 1	.196	.194	100	100
"	"	3 x 1	.184		94	
37°C	1.6/0.8 N	2 x 2	.200		102	
"	"	3 x 2	.190		97	

The methylation of oestrone in the presence of urine extracts:

Oestrone (30 µg.) in the presence of the "oestrone" phenolic fraction from 100 ml. of acid hydrolysed male urine was methylated at 37°C with dimethyl sulphate in 0.4 N.sodium hydroxide (50 ml.) and boric acid (0.8 g.).

Standard oestrone (30 µg.) D_{604} 0.260.

Dimethyl sulphate added	D_{604} urine + oestrone		D_{604} urine blank	D_{604} difference due to oestrone	Recovery %
2 x 1 ml.	.340	.324	.092	.240	92
3 x 1 ml.	.334		.098	.236	91

Evidently, methylation at 37°C with dimethyl sulphate (2 x 1 ml.) and borate buffer solutions (boric acid, 0.8 to 1.0 g. in 0.4 N.sodium hydroxide, 50 ml.) was a convenient and satisfactory method for the quantitative methylation of oestrogens in pure solutions and in urine extracts.

The recommended procedure follows:

Reagents:

0.4 N.sodium hydroxide (1.6% AnalaR grade).
5 N.sodium hydroxide (20% AnalaR grade).
Dimethyl sulphate; B.D.H. reagent redistilled.
Boric acid powder; B.P. grade.

A solution of the oestrogens in 0.4 N. sodium hydroxide (50 ml.) (the phenolic fraction obtained by benzene-alkali partition) was added to 100 ml. stoppered flasks containing boric acid (approx. 0.9 g.). The flasks were placed in a water bath maintained at 37°C, and when they had warmed for a few minutes, dimethyl sulphate (1 ml.) was added and the flasks were shaken until the dimethyl sulphate and boric acid had dissolved. The flasks were returned to the water bath and incubated 20 - 30 min., and then another 1 ml. of dimethyl sulphate and 2 ml. of 5 N.sodium hydroxide were added. The flasks were shaken until the dimethyl sulphate had dissolved and were either incubated another 45 min. and cooled or allowed to stand at room temperature overnight. Sodium hydroxide (10 ml. of 5 N.) was added to each flask and the contents were extracted with petroleum ether (oestrone and oestradiol methyl ethers) or benzene (oestriol methyl ether).

THE SAPONIFICATION OF METHYLATED MATERIAL TO REMOVE ESTERS OF FATTY ACIDS.

It would be expected that certain fatty acids occurring in acid hydrolysed urine would not be removed from ether by sodium bicarbonate and would travel with the oestrogens into the phenolic fraction and be methylated to their methyl esters. These methyl esters would be saponifiable and thus removed from the phenol methyl ethers.

A preliminary experiment was performed with methylated "oestriol" and "oestrone" phenolic fractions from acid hydrolysed male urine (200 ml.). The benzene and petroleum ether extracts of these were evaporated to about 1 ml. of solvent, ethanol (2 ml.) and an alcoholic potassium hydroxide solution (2 ml.) prepared by dissolving potassium hydroxide (2 g.) in water (2 ml.) and diluting to 25 ml. with ethanol, were added. The solutions were refluxed 1 hour using micro burners and finger condensers, then cooled, diluted with water (50 ml.) and extracted with benzene or petroleum ether (50 ml.). The extracts were washed with water, solvents were distilled and "colours" developed in the usual manner were compared with those produced by equivalent urine fractions without saponification.

	D_{604}	
	"Oestriol" fraction	"Oestrone" fraction
Without saponification	0.145	0.104
With saponification	0.091	0.063

Therefore, saponification had caused considerable purification.

The effect of saponification with alcoholic potassium hydroxide upon the pure oestrogen methyl ethers.

The saponification reagent was prepared by dissolving potassium hydroxide pellets (2 g.) in water (2 ml.) and diluting to 25 ml. with 95% alcohol (Hickinbottom, 1948).

Oestriol, oestrone and oestradiol methyl ethers (30 µg.), ethanol (2 ml.) and saponification reagent (2 ml.) were refluxed under finger condensers for various periods of time. The solutions were cooled, diluted with water (50 ml.) and the oestriol methyl ether was extracted as usual with benzene (50 ml.) and the oestrone and oestradiol methyl ethers with petroleum ether (50 ml.). The extracts were washed with water and distilled to dryness. The residues were transferred with ethanol to Kober tubes and the solvent was removed by heating in a stream of air. Oestrogen methyl ethers were estimated by the hydroquinone-sulphuric acid colour method.

Heating time (min.)	D_{604} methyl ethers. Oestriol	Oestrone	Oestradiol
0	.189	.210	.180
5	.183	.194	.198
10	.182	.182	.190
20	.190	.208	.204
40	.180	.246	.200

Evidently there was no obvious destruction of the methyl ethers during saponification.

The effect of saponification with aqueous sodium hydroxide upon pure methylated oestriol and oestrone and their equivalent urine fractions.

Oestrone (30 µg.) was methylated at 37°C with dimethyl sulphate (2 x 1 ml.) in a solution of boric acid (0.8 g.) in 0.4 N.sodium

hydroxide (50 ml.). Oestriol (30 µg.) was methylated at room temperature with dimethyl sulphate (2 x 1 ml.) in a solution of boric acid (1.25 g.) in 0.4 N.sodium hydroxide (50 ml.).

The methylation mixtures were made normal to sodium hydroxide by adding 5 N.sodium hydroxide (10 ml.) and heated. On cooling, the oestrogen methyl ethers were extracted with benzene or petroleum ether in the usual manner, the solvents were evaporated to pools which were transferred with ethanol to Kober tubes and the solvents were removed under nitrogen with heating and reduced pressure. Oestrogen methyl ethers were estimated by the hydroquinone-sulphuric acid colour method.

Heating time (min.)	0	15	30	15	30
Heating temp.(°C)		80	80	100	100
D_{604} oestrone	.258 .259	.256 .267	.259 .256	.255 .267	.249 .249
D_{604} oestriol	.191 .194	.196 .190	.170 .160	.200 .200	.182 .186

"Oestriol" and "oestrone" fractions obtained by benzene-water and benzene-sodium hydroxide partition from male urine (100 ml.) were methylated in borate buffer (50 ml.), and made normal to sodium hydroxide by adding 5 N.sodium hydroxide (10 ml.). The alkaline solution was heated and on cooling, extracted with benzene or petroleum ether. After removing the solvent, colour was developed with the residue in the usual manner.

Temperature of heating (°C)	Time of heating (min.)	"Oestriol" fraction D_{604}	"Oestrone" fraction D_{604}
Room	0	.220	.100
60	15	.170	.098
"	30	.160	.071
80	15	.113	.084
"	30	.126	.071
100	15	.120	.057
"	30	.117	.061
Saponification by alcoholic potassium hydroxide		.112, .112	.040, .050

Evidently saponification with aqueous N.sodium hydroxide could be safely and very conveniently performed by heating for 15 minutes at 100°C. However, less urinary material was removed by this procedure than by saponification with alcoholic potassium hydroxide.

The evaluation of saponification methods in the complete oestrogen method.

Twelve lots of 200 ml. of pooled male urine were hydrolysed by refluxing with hydrochloric acid (30 ml.) for 1 hour. Oestriol, oestrone and oestradiol (30 µg. of each) were added after hydrolysis to six of these and all were extracted once with one volume and twice with half volumes of ether. The ether solutions were extracted twice with fifth volumes of 5% sodium bicarbonate and the ether was evaporated to a small pool. This was taken up in ethanol (1 ml.) and benzene (20 ml.) and extracted once with one volume and twice with half volumes of water ("oestriol" fraction) and then once with one volume and twice with half volumes of 0.4 N.sodium hydroxide ("oestrone" fraction). Four ml. of 5 N.sodium hydroxide was added to the "oestriol" fraction and both fractions were methylated at 37°C with boric acid

(0.8 g.) and dimethyl sulphate (2 x 1 ml.). 5 N.sodium hydroxide (10 ml.) was added to each methylated mixture. In the case of saponification in aqueous solution, the alkaline methylation mixtures were heated to 100°C for 15 minutes, otherwise the methylated solutions were merely extracted with benzene ("oestriol") or petroleum ether ("oestrone and oestradiol"). The extracts were washed with water to remove alkali. In the case of alcoholic saponification, the extracts were evaporated to a small volume, ethanol (1 ml.) and saponification reagent (2 ml.) were added, and the mixtures were heated under reflux for 30 minutes. Water (50 ml.) was added and the mixture again extracted with organic solvent.

The benzene and petroleum ether extracts were purified by chromatography on alumina columns and thereby divided into oestriol, oestrone and oestradiol fractions. These were evaporated to small volumes, transferred with ethanol to Kober tubes, the solvent was removed by heating under nitrogen and reduced pressure. Colours were developed with the residues using the three hydroquinone-sulphuric acid reagents.

	Saponification procedure		
	None	aqueous	alcoholic
Oestriol Standard (30 µg.) 0.198			
D604 urine blank	.068 .062	.068 .224*	.040 .043
D604 urine + oestriol	.170 .177	.161 .152	.147 .157
Recovery %	55	45	56
Oestrone Standard (30 µg.) 0.255			
D604 urine blank	.047 .038	.031 .277*	.038 .038
D604 urine + oestrone	.230 .213	.174	.198 .194
Recovery %	71	56	62

	Saponification procedure		
	None	Aqueous	Alcoholic
Oestradiol Standard (30 µg.) 0.227			
D604 urine blank	.061 .057	.040 .250*	.043 .041
D604 urine + oestradiol	.235 .230	.211	.200 .201
Recovery %	77	75	70

*Standard (30 µg.) was added to urine blanks before final removal of solvent and therefore this reading was equivalent to 100% recovery. Theoretically these figures should have been equivalent to D604 standard plus D604 urine blank, or for - oestriol 0.266, oestrone 0.286 and oestradiol 0.267.

The discrepancy between the observed and calculated figures indicated that depression of the Kober colour by urine extracts was an important factor in causing the low recoveries. This phenomenon is considered in more detail in the section on colorimetric methods.

In this experiment losses during saponification, especially from the oestrone fraction, were considerable compared with the poor purification achieved. In several similar experiments it was shown that these losses were apparent only after chromatography. Apparently oestrone methyl ether was partly modified by these saponification procedures to a substance which was Kober chromogenic but which was removed by chromatography. Purification of urine extracts by saponification was therefore abandoned.

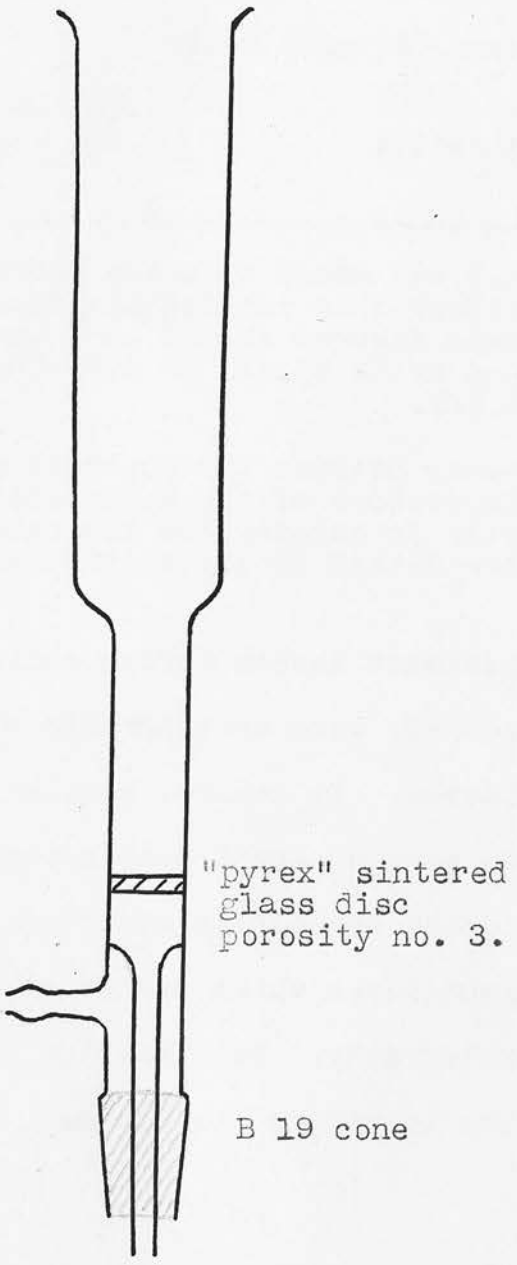


Diagram 3. The chromatogram tube, two-thirds actual size.

ADSORPTION CHROMATOGRAPHY OF THE OESTROGEN METHYL ETHERS ON ALUMINA COLUMNS.

EXPERIMENTAL.

Materials.

The alumina was manufactured by Savory & Moore and labelled "Aluminium oxide for chromatographic analysis: standardized according to Brochmann". The behaviour of the oestrogen methyl ethers on standard columns was always noted for each new batch of alumina. Standardization in terms of Brochmann numbers was not attempted because the behaviour of azo dyes on these alumina columns was considered to be irrelevant to the present work. The writer believes that too much reliance on the standardization of alumina by the Brochmann method has done much to discredit alumina chromatography as a routine quantitative procedure.

Sand was boiled with hydrochloric acid, washed with water and extracted thoroughly with alcohol.

Petroleum ether (bp. 40 - 60°C) was redistilled AnalaR reagent.

Benzene was AnalaR reagent dried and purified by azeotrophic distillation.

Ethanol was absolute, refluxed with sodium hydroxide pellets, and twice distilled.

Apparatus.

The chromatogram tube is illustrated in the diagram on the opposite page. The usual proportions recommended for adsorbent column width to column length were not used. The width-length ratio was about 1:1.5 and was adequate for separating the desired components. Decreasing the width and increasing the length would give a greater concentration of

adsorbed substances per unit cross-sectional area and consequently a greater danger of displacement effects when concentrated urine extracts were used. Increasing the length without decreasing the width would make it necessary to use inconveniently large volumes of eluting solvents. The chromatogram tube, through its interchangeable B 19 ground glass joint, was easily connected with either B 19 tubes or flasks for receiving the eluate.

Chromatograms were usually performed in batches of four, suction being applied from a manifold through two-way stop-cocks which allowed suction to be applied to, or released from, individual chromatogram tubes and their receiving flasks or tubes.

Chromatographic procedure.

The narrow portion of the chromatogram tube was filled with dry solvent and the alumina, measured by weight, was added in a thin stream so that entangled air could free itself during passage through the solvent. When the alumina had settled, about ¼ inch over-layer of acid-alcohol washed sand was added to protect the alumina from being disturbed during addition of solvents.

Solvents were usually sucked through to the level of the sand but no further before another solvent was added. The rate of flow of solvents through the alumina columns was not important and was usually about 3 ml. per minute.

The chromatography on active alumina of oestrone, oestradiol and oestriol methyl ethers using completely dry solvents.

The petroleum ether and benzene used in this experiment were carefully dried over sodium wire and then distilled.

Preliminary work showed that oestrone and oestradiol methyl ethers were adsorbed by active alumina from solutions in petroleum ether and

were eluted therefrom with benzene or benzene and ethanol. Oestriol methyl ether was adsorbed by active alumina from solutions in benzene and was eluted with mixtures of benzene and ethanol.

The methyl ethers (100μg.) were measured in ethanolic solution, the ethanol was evaporated and the oestrone and oestradiol methyl ethers were taken up in dry petroleum ether (50 ml.) and the oestriol methyl ether in dry benzene (50 ml.). The petroleum ether solutions were passed through columns of active alumina (3 g.) prepared in petroleum ether and the benzene solution was passed through similar columns prepared in dry benzene. The columns were developed with the corresponding dry solvent (30 ml.) and fractionally eluted with dry solvents following the scheme described in the table. The smaller 8 ml. fractions were eluted directly into "Kober" tubes, and the larger fractions into flasks from which solvent was distilled to small volumes and the residue transferred with ethanol to "Kober" tubes. Solvent was removed from the "Kober" tubes by heating under nitrogen and reduced pressure, and the oestrogen methyl ethers were estimated in the usual manner.

Oestrone (100μg.) in dry pet.ether (50 ml.)			Oestradiol (100μg.) in dry pet.ether (50 ml.)			Oestriol (100μg.) in dry benzene (50 ml.)		
Eluate	ml.	D_{604}	Eluate	ml.	D_{604}	Eluate	ml.	D_{604}
Pet.ether	80	0	Pet.ether	80	0	Benzene	80	.009
Benzene	8	0	Benzene	8	0	1.5% ethanol		
"	8	0	"	8	.125	in benzene	32	.006
"	8	.035	"	8	.216		8	.005
"	8	.158	"	8	.102		8	.004
"	8	.194	"	8	.046	10% ethanol		
"	8	.161	"	8	.022	in benzene	8	.093
"	8	.093	"	8	.009		8	.285
1.5% ethanol			1.5% ethanol				8	.104
in benzene	8	.047	in benzene	8	.043		8	.036
"	8	.066		8	.044		8	.018
"	8	0		8	0		8	.008
"	8	0		8	0		8	.005
							8	.005
Total eluted		.754	Total eluted		.607	Total eluted		.544

When completely dry solvents were used, the methyl ethers of oestrone and oestradiol behaved similarly on the column. When this experiment was repeated the results were not entirely reproducible.

The chromatography on active alumina of oestrone, oestradiol and oestriol methyl ethers dissolved in solvents saturated with water.

In practice the oestrogen methyl ethers would be extracted from aqueous alkaline solution with organic solvents and these extracts would be extracted with water to remove alkali. It would be convenient if these water saturated extracts could be applied, without preliminary vigorous drying, directly to the alumina columns.

The methyl ethers (100 µg.) in ethanol solution, were added to water (50 ml.) and extracted with petroleum ether (oestrone and oestradiol methyl ethers) or benzene (oestriol methyl ether). The organic solvent extracts were washed with water (1 x 20 ml.) and passed through columns of active alumina (3 g.). Development and elution with dry solvents followed by evaporation and colour production were performed in the same manner as in the previous experiment.

Oestrone (100 µg.) in moist pet.ether (50 ml.)			Oestradiol (100 µg.) in moist pet.ether (50 ml.)			Oestriol (100 µg.) in moist benzene (50 ml.)		
Eluate	ml.	D_{604}	Eluate	ml.	D_{604}	Eluate	ml.	D_{604}
Pet.ether	80	0	Pet.ether	80	0	Benzene	80	0
Benzene	8	0	Benzene	8	0	1.5% ethanol	32	0
"	8	.005	"	8	0	in benzene	8	0
"	8	.053	"	8	0	"	8	0
"	8	.162	"	8	0	10% ethanol	8	.310
"	8	.195	"	8	0	in benzene	8	.203
"	8	.134	"	8	0	"	8	0
"	8	.082	"	8	0	"	8	0
"	8	.043	"	8	0	"	8	0
"	8	.032	"	8	0	"	8	0
"	8	.023	1.5% ethanol	8	.200	"	8	0
"	8	.012	in benzene	8	.350	"	8	0
				8	.020			
Total eluted		.741	Total eluted		.570	Total eluted		.513

Evidently the presence of moisture in the petroleum ether or benzene applied to the columns had a profound effect upon the subsequent behaviour of the oestrogen methyl ethers. Less trailing from the columns was observed and oestrone and oestradiol methyl ethers were separable. Results obtained by repeating this experiment were much more consistently reproducible than in the last experiment.

The chromatography of methylated urine extracts.

Male urine (200 ml.) was acid hydrolysed, and extracted with ether. The ether extract was extracted with 5% sodium bicarbonate, the ether was distilled and the residue was taken up in benzene. The benzene solution was extracted with water ("oestriol" fraction) and then with sodium hydroxide ("oestrone" fraction). The oestriol and oestrone fractions were methylated at room temperature with dimethyl sulphate (2 x 1 ml.) and boric acid. The methylated "oestriol" fraction was extracted with benzene (50 ml.) and the "oestrone" fraction with petroleum ether (50 ml.).

The petroleum ether extract was passed through a column of alumina (3 g.) and the chromatogram was developed with dry petroleum ether (30 ml.) and dry benzene (15 ml.). The "oestrone" fraction was eluted with dry benzene (70 ml.) and the "oestradiol" fraction with 10% ethanol in benzene (20 ml.). A yellow band was adsorbed from the petroleum ether extract and remained at the top of the column during development with dry petroleum ether. When benzene was added to the column, this band divided into two, the fainter one migrated slowly with the benzene and was eluted in the "oestrone" fraction, the other remained at the top of the column until the benzene-ethanol was added when it migrated rapidly and was eluted in the oestradiol fraction.

The "oestriol" fraction in benzene was passed through another alumina column, and the chromatogram was developed with dry benzene (30 ml.) and 10% ethanol in benzene (10 ml.). The "oestriol" fraction was eluted with 10% ethanol in benzene (40 ml.). A yellow band was adsorbed from the benzene extract and remained on the top of the column during the development with dry benzene. When the 10% ethanol in benzene was added to the column the band divided into two, one of which migrated rapidly and was eluted in the developing ethanol-benzene, the other remained adsorbed on the top of the column during the elution of the "oestriol" fraction.

The "oestrone", "oestradiol" and "oestriol" fractions from the chromatograms were transferred to Kober tubes and "colours" developed with the appropriate hydroquinone-sulphuric acid reagents. The densities were compared with those of the fractions before chromatography.

	D_{604}
"Oestrone plus oestradiol" fraction before chromatography	.100
"Oestrone" fraction after chromatography	.033
"Oestradiol" fraction after chromatography	.063
Oestriol fraction before chromatography	0.150
Oestriol fraction after chromatography	0.064

Considerable purification of the oestriol fraction but no purification of the oestrone plus oestradiol fractions had been achieved by chromatography.

A search was made for a chromatographic procedure which would separate oestradiol from the urine band which accompanied it.

<u>The purification of the oestradiol fraction.</u>

The "oestrone plus oestradiol" fraction from acid hydrolysed male urine (1,400 ml.) was extracted from benzene solution with 0.4 N.sodium

hydroxide and methylated at room temperature. The methylated material was extracted with petroleum ether and fractions of this were chromatographed on columns of alumina (3 g.) prepared from dry petroleum ether.

(a) Chromatography using graded eluants.

According to Trappe /(1940) (see Techniques of Organic Chemistry, Vol. V) the following series of solvents is arranged in the order of increasing eluting power with polar adsorbents; benzene, chloroform, di-ethyl ether, acetone, ethanol and methanol.

To separate steroids on alumina columns, Reichstein & Von Euw (1941) used the following series of eluents which are arranged in order of increasing eluting powers; benzene, benzene plus di-ethyl ether, di-ethyl ether, di-ethyl ether plus ethanol.

The effect of some of these solvents on the elution of pure oestradiol methyl ether and the above methylated "oestradiol" fraction from urine was investigated.

The same yellow zone was observed as in the last experiment. This zone was not visibly eluted with benzene, but was eluted rapidly with mixtures of benzene and ethanol. The zone moved slowly and diffusely when the eluents were mixtures of benzene and chloroform (2.5%, 5%, 10% and 20% chloroform) and benzene and di-ethyl ether (5% and 10% ether) but moved rapidly as a sharp band with the advancing front of eluents containing as little as 0.5% ethanol or methanol.

Pure oestradiol methyl ether behaved similarly by trailing diffusely when eluted with benzene and mixtures of benzene and di-ethyl ether or chloroform and by being rapidly eluted when ethanol or methanol were present. It soon became apparent that the required separation of

oestradiol methyl ether from the coloured zone could not be achieved by using graded eluents alone.

(b) Chromatography using deactivated alumina.

Two processes were used to deactivate alumina.

1. A layer of alumina was kept for about a week in an atmosphere containing water vapour until no more water was absorbed. The alumina was agitated at frequent intervals to expose fresh surfaces.

2. Alumina was completely moistened with methanol and the methanol was allowed to evaporate in air at room temperature.

Oestradiol methyl ether was quantitatively adsorbed from petroleum ether solution by both of these deactivated aluminas (3 g.) and was completely eluted from them with benzene (100 ml.).

The methylated "oestradiol" fraction obtained from acid hydrolysed male urine (700 ml.) by chromatography on active alumina was added in petroleum ether to similar deactivated alumina columns. The usual yellow zone was adsorbed at the top of the columns and did not migrate with the first 100 ml. of benzene, but was rapidly eluted with 1% ethanol in benzene.

The separation of oestradiol methyl ether from the troublesome urine zone had therefore been achieved.

The effect of water deactivation of alumina used for the chromatography of anthraquinone dye-stuffs was described by Stewart (1949), who showed that the differences in the mean relative movements of bands of these dye-stuffs on alumina columns were beneficially increased by partially deactivating the alumina, and better separation of closely related dye-stuffs was thus achieved. Stewart deactivated alumina by adding liquid water and mixing thoroughly.

The fractional chromatography of oestrone and oestradiol methyl ethers on deactivated alumina.

Alumina was deactivated by keeping it in an atmosphere containing water vapour and agitating occasionally until equilibrium with the water vapour had been reached at room temperature.

Oestrone and oestradiol methyl ethers (50 µg.) in ethanol (0.6 ml.) were added to petroleum ether (50 ml.), the petroleum ether solution was extracted with water to remove ethanol and passed through columns of deactivated alumina (2.5 g.). The chromatograms were developed with dry petroleum ether (50 ml.) and eluted with benzene and benzene plus ethanol in the manner shown in the table. Solvents were evaporated and colours developed in the usual manner.

	Oestrone methyl ether (50 µg.)		Oestradiol methyl ether (50 µg.)	
Eluate	ml.	D_{604}	ml.	D_{604}
Pet.ether	100	0	100	0
Benzene	10	.365	10	0
"	10	.008	10	.133
"	10	0	10	.100
"	10	0	10	.028
"	10	0	10	.003
"	10	0	10	0
"	10	0	10	0
10% ethanol in benzene	10	0	10	0
Total eluted		.373		.264

Evidently oestrone and oestradiol methyl ethers were separated by this chromatographic procedure.

The elution of oestrone methyl ether from deactivated alumina by benzene-petroleum ether mixtures.

The further purification of the oestrone fraction by removing substances which were less strongly adsorbed on alumina columns was investigated.

Petroleum ether (50 ml.) containing oestrone methyl ether (50 µg.) was extracted with water and passed through columns of deactivated alumina (2 g.). The column was developed with dry petroleum ether (25 ml.) and fractionally eluted into Kober tubes with mixtures of petroleum ether and benzene.

Solvents were evaporated by heating under nitrogen and reduced pressure, and oestrone methyl ether was estimated with the hydroquinone-sulphuric acid colour method as usual.

	Composition of eluting solvent			
Eluate (ml.)	Pet.ether 90 + benzene 10	Pet.ether 80 + benzene 20	Pet.ether 60 + benzene 40	Pet.ether 50 + benzene 50
	D_{604}	D_{604}	D_{604}	D_{604}
10	0	0	0	0
10	0	0	.031	.205
10	0	0	.175	.105
10	0	0	.100	.030
10	0	0	.034	.013
10	0	0	.013	0
10	0	0	0	0
10	0	0	0	0

Pre-oestrone substances should therefore be removed by eluting with 10 ml. of a mixture of petroleum ether and benzene (60 vol. + 40 vol.) before eluting the oestrone methyl ether with benzene.

The standardization of solvents for chromatography.

The water content of solvents used in the foregoing chromatographic procedures was not rigidly standardized and it was feared that this might lead to difficulties. Consequently, the drying of extracts in separating funnels, by shaking with anhydrous sodium sulphate before chromatography, was investigated. Even moderate amounts of this mild drying agent caused unpredictable shifts of bands on columns. The effect seemed to be related to the time of contact between extract and drying agent and could not be standardized. Fortunately, the most reproducible conditions were those in which extracts were saturated with water at room temperature, as in preceding experiments. At first, accidental addition of free water to alumina columns was prevented by adding small amounts of anhydrous sodium sulphate to separating funnels, before pouring off the organic solvent extract. This immobilized water films still adhering to the walls of the funnels. However, with care a better method to prevent this was to allow funnels containing extracts to stand for some time after discarding the aqueous layer, and thus to allow water adhering to their sides to drain away completely and be removed.

During these experiments a sample of essentially water-free benzene was accidently prepared by twice treating a new batch of AnalaR reagent benzene with fresh sodium wire and distilling in a carefully dried apparatus. When this particularly dry benzene was used to elute oestrone and oestradiol methyl ethers from deactivated alumina columns, a considerable displacement and overlapping of their bands occurred with diffuse trailing from the columns. Apparently, the dry benzene had dehydrated and therefore reactivated the alumina. This phenomenon

is common knowledge (Borth, 1952), although its description was not found in any of the literature studied on the subject. Fortunately, the best alumina for the chromatography of the oestrogen methyl ethers is one which has been deactivated in an atmosphere saturated with water vapour at room temperature. The deactivated alumina is unlikely to change its activity when kept in containers which are not absolutely air-tight. The water content of the benzene and petroleum ether used for chromatography is easily standardized in the same manner by keeping them for a time in an atmosphere saturated with water vapour, at room temperature.

Using these convenient methods of standardization, it is claimed that the chromatographic procedure is entirely reproducible and quantitative.

The standardization of a new batch of alumina.

Before being used in the chromatographic method, all batches of alumina were standardized in the following manner.

Oestrone and oestradiol methyl ethers.

The alumina was deactivated by keeping for a week in an atmosphere containing water vapour. Petroleum ether solutions of oestrone and oestradiol methyl ethers (50 µg. in 50 ml.) were extracted with water, and added to columns of deactivated alumina (2 g.). The columns were developed with petroleum ether (25 ml.) and a mixture of petroleum ether and benzene (10 ml. of 60 vol. + 40 vol.) and fractionally eluted with benzene. The eluates were evaporated with heating under nitrogen and reduced pressure and colours developed using 66% and 60% sulphuric acid-hydroquinone reagents (See Fig.20).

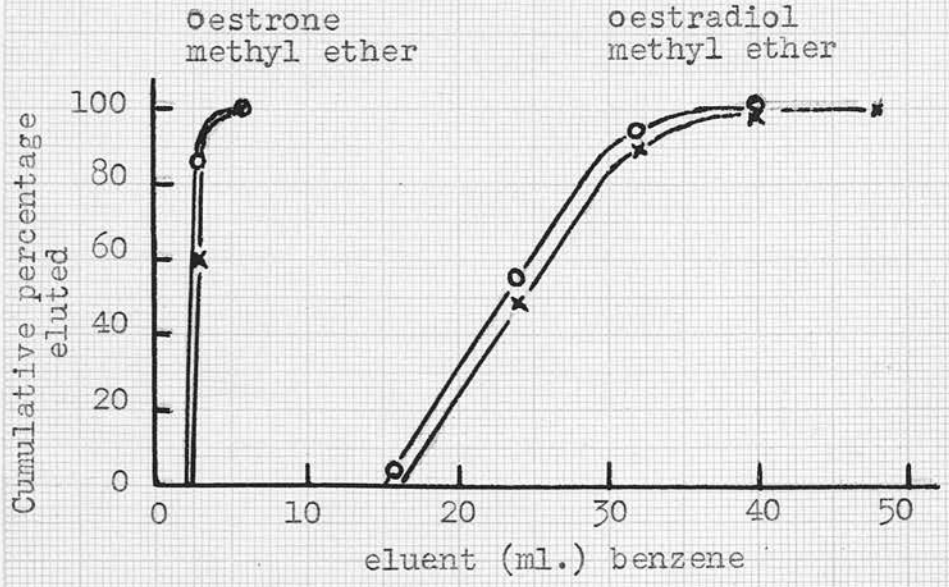


Fig. 20. The standardization of a new batch of alumina for the chromatography of oestrone and oestradiol methyl ethers; 0——0 was performed two months after x——x.

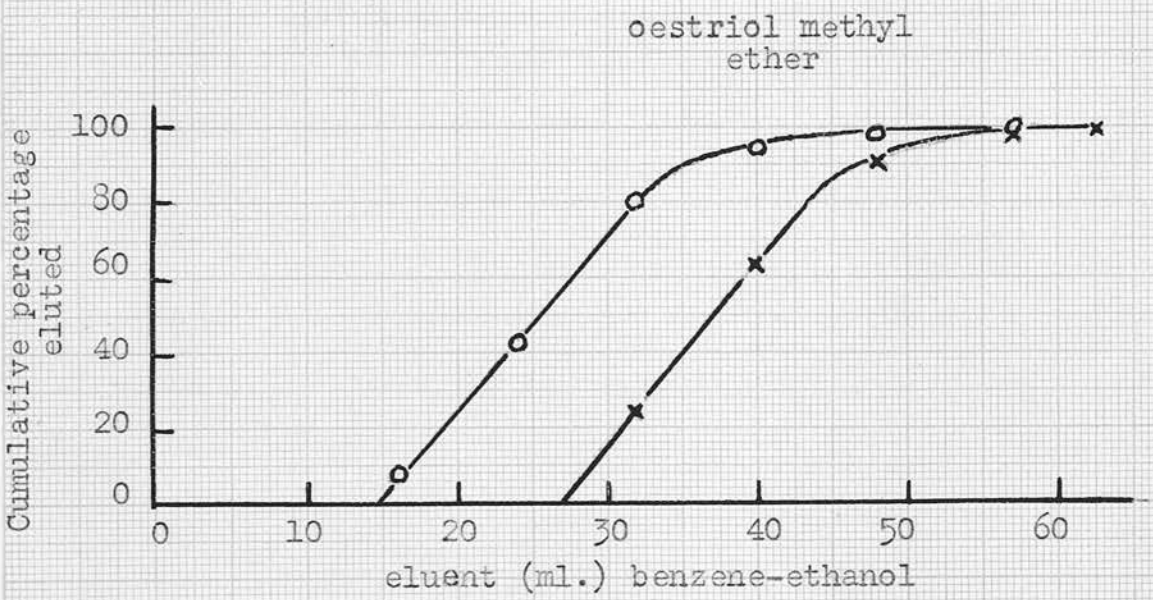


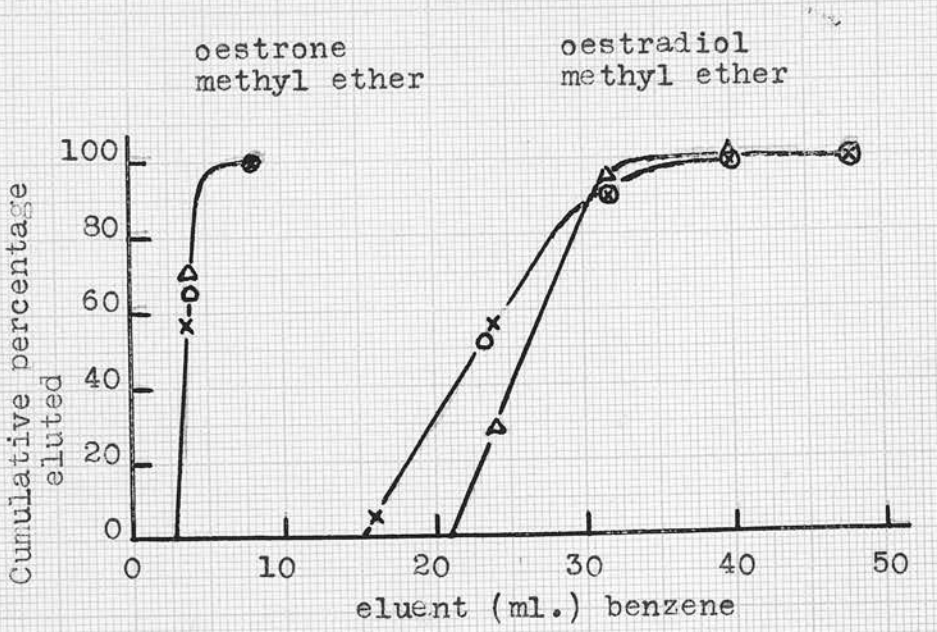
Fig. 21. The standardization of a new batch of alumina for the chromatography of oestriol methyl ether; 0—0 3.5% ethanol in benzene, x—x 3% ethanol in benzene.

Using this batch of alumina and the above procedure, oestrone methyl ether was quantitatively eluted in the first 8 ml. of benzene and oestradiol methyl ether in the next 50 ml. of benzene.

Oestriol methyl ether.

The alumina was Savory & Moore reagent, untreated, Benzene solutions of oestriol methyl ether (50 µg. in 50 ml.) were extracted with water, and passed through a column of alumina (2 g.). The column was developed with a further 25 ml. of benzene and eluted with mixtures of ethanol and benzene (3 and 3.5% v+v ethanol in benzene). Eluates were evaporated and colour developed in the usual manner (See Fig. 21).

Using this batch of alumina, a satisfactory chromatographic procedure was to develop the chromatogram with 25 ml. of benzene and then 20 ml. of 3% ethanol in benzene and elute the oestriol methyl ether with a further 50 ml. of 3% ethanol in benzene.



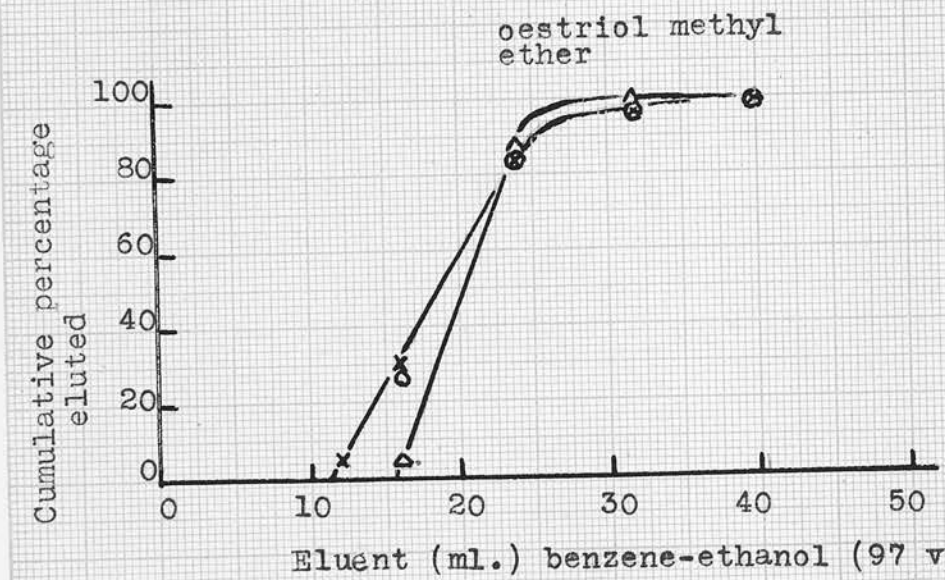


Fig. 22. The effect of speed of elution on the fractional chromatogram curves of the oestrogen methyl ethers. x—x, 9 ml./min.; 0—0, 3 ml./min.;Δ—Δ, 1 ml./min.

The effect of speed of elution upon the fractional elution curves of the oestrogen methyl ethers.

Oestrone and oestradiol methyl ethers in petroleum ether and oestriol methyl ether in benzene, after water extraction, were added to alumina columns which were developed and eluted at different rates of flow of solvents but otherwise under identical conditions.

The fig.22 shows the fractional elution curves obtained:

(a) Without applied suction (approx. 1 ml./min.).

(b) At the normal solvent flow (approx. 3 ml./min.)

(c) At approx. 9 ml./min.

These curves show that very slow rates of solvent flow tend to sharpen slightly the bands in the chromatogram. This effect is small and scarcely justifies the technical inconvenience of standardizing the flow rates through the columns. However, chromatograms can be improved by using slow rates of flow at critical stages, i.e. during the elution of oestrone methyl ether with benzene and during the development of the oestriol chromatogram with mixtures of benzene and ethanol.

The separation of oestrone, oestradiol and oestriol methyl ethers on a single chromatogram.

Up till now oestriol had been separated from oestrone and oestradiol by benzene-water partition before methylation. Theoretically, it should be possible to separate oestriol from oestrone and oestradiol after methylation by petroleum ether-water partition, because oestriol methyl ether is relatively much more soluble in water than in petroleum ether,

(when partitioned between equal volumes, 85% is found in the water layer). The oestrone and oestradiol methyl ethers would be quantitatively extracted by the petroleum ether and the oestriol methyl ether would be left in the aqueous layers (including the water washes of the petroleum ether extract), and would be extractable from them with benzene. Either the petroleum ether and benzene solutions could be chromatographed on separate columns in the usual manner, or on the same column. There are two ways of doing this; the benzene extract containing the oestriol fraction could be used to elute the oestrone and oestradiol methyl ethers, or it could be added to the column after elution of oestrone and oestradiol methyl ethers with benzene. Both of these procedures would recover oestriol otherwise lost by incomplete separation in the benzene-water or petroleum ether-water partitions and thereby finding its way on to the oestrone-oestradiol chromatogram.

The behaviour of oestriol methyl ether on a column of deactivated alumina was first investigated and, using the usual methods of adsorption and elution it was found that 2.5% (v+v) ethanol in benzene was a satisfactory eluting solvent.

The combined chromatogram was performed in the following manner:-

Oestriol, oestrone and oestradiol methyl ethers were added to normal sodium hydroxide (50 ml.) and extracted with petroleum ether (50 ml.). The petroleum ether was extracted twice with water (5 ml.) which was then combined with the sodium hydroxide layer and extracted with benzene (50 ml.). The benzene was extracted twice with water (5 ml.).

The petroleum ether was passed through a column of deactivated alumina (2 g.) followed by petroleum ether (25 ml.) and a mixture of petroleum ether and benzene (60 vol. + 40 vol.). The column was

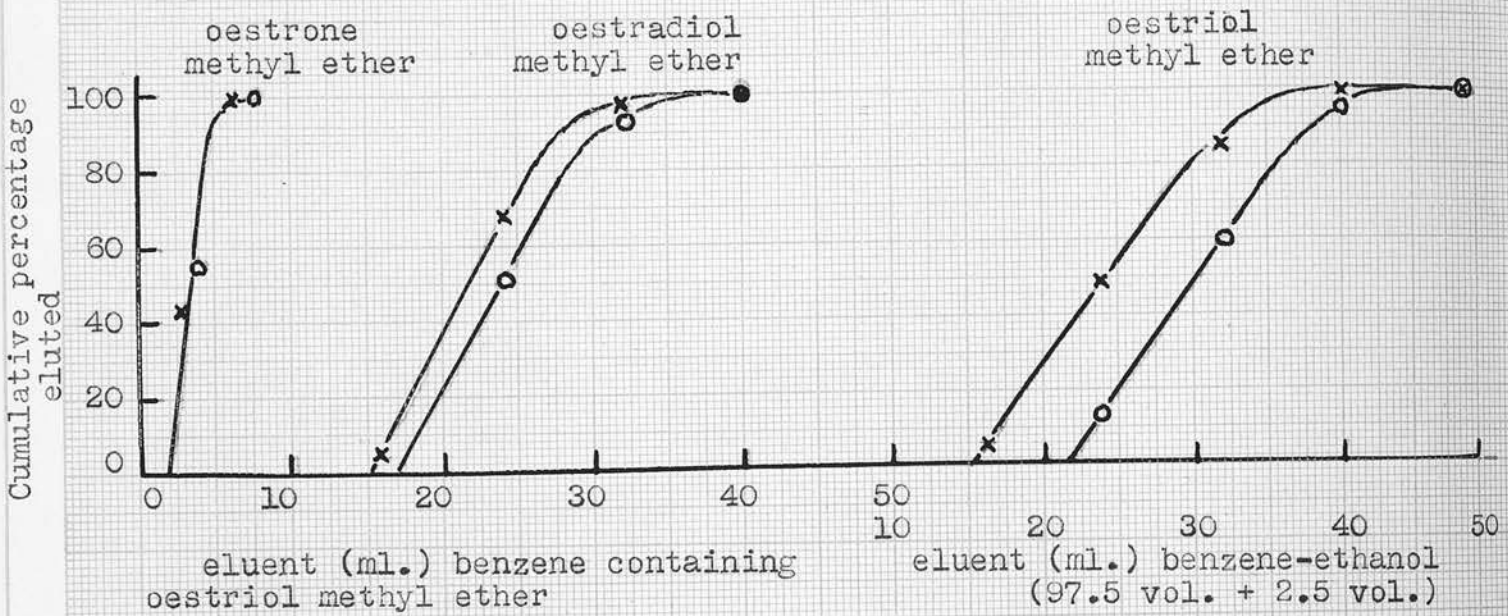


Fig. 23. The fractional elution of oesrtone, oestradiol and oestriol methyl ethers from a single alumina column of deactivated alumina. Experiment x—x was performed one month after experiment O—O.

fractionally eluted with the 50 ml. of benzene solution containing the oestriol methyl ether, and then with benzene ethanol (97.5 vol. + 2.5 vol.). The eluates were evaporated and colours developed in the usual manner.

The fractional chromatogram curves of two such experiments performed at an interval of a month are shown in Fig. 23.

The fractional elution of oestrogen methyl ethers in the presence of methylated urinary material.

Acid hydrolysed male urine (200 ml.), to which 30 µg. of oestriol, oestrone and oestradiol had been added, was extracted with ether. The ether solution was extracted with bicarbonate and alkali and evaporated to near dryness. The residue was dissolved in ethanol (1 ml.) and benzene (20 ml.), and extracted once with 20 ml. and three times with 10 ml. of 0.4 N.sodium hydroxide. The alkali solution was methylated at 37°C with boric acid (0.9 g.) and dimethyl sulphate (2 x 1 ml.), and extracted first with petroleum ether (50 ml.), and then, together with water washes of the petroleum ether solution, with benzene.

The petroleum ether and benzene extracts were fractionally chromatographed in the following manners:-

(a) The petroleum ether extract was added to a column of deactivated alumina (2 g.) which was developed with petroleum ether (25 ml.) and 10 ml. of a mixture of petroleum ether and benzene (60 vol. + 40 vol.) and eluted with benzene.

The benzene extract was passed through a column of active alumina (2 g.) which was developed with benzene (25 ml.) and eluted with a mixture of benzene and ethanol (97 vol. + 3 vol.). Solvents were

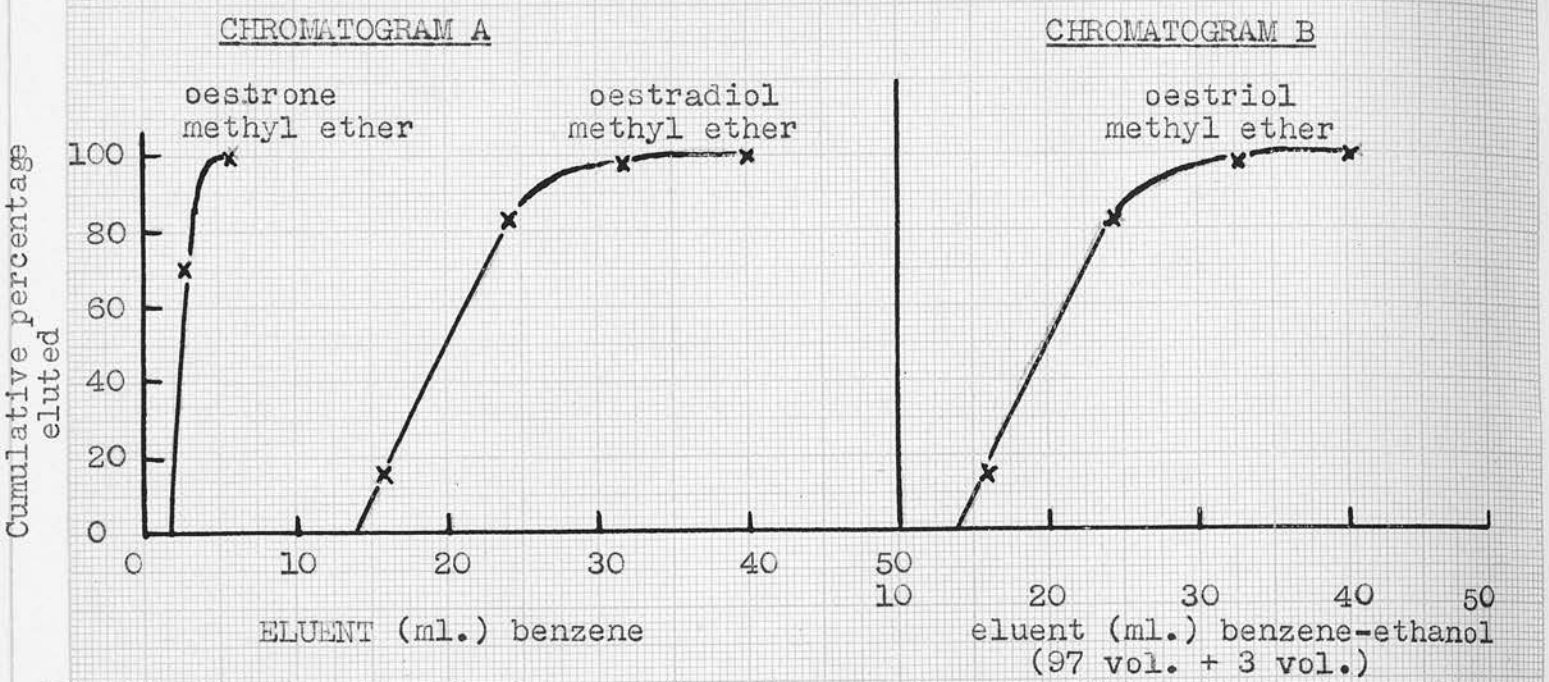


Fig.24. The fractional elution of oestrone and oestradiol methyl ethers from deactivated alumina (A); and oestriol methyl ether from active alumina (B), in the presence of urinary material. Density readings are corrected.

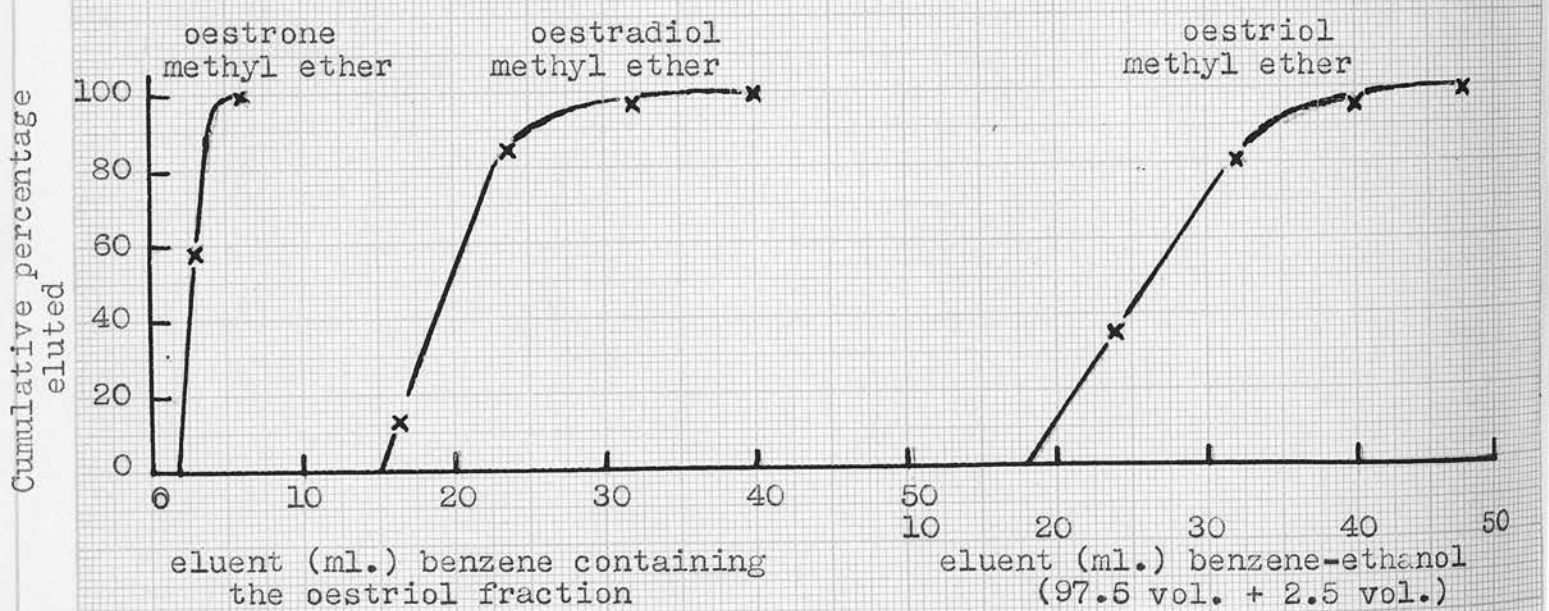


Fig. 25. The fractional elution of oestrone, oestradiol and oestriol methyl ethers in the presence of urinary material from a single column of deactivated alumina. Density readings are corrected.

evaporated and colours developed in the usual manner. Density measurements were made at three wavelengths and the oestrogen present calculated by applying the correction formula. Also see Fig. 24.

Eluate	Cumulative volume (ml.)	D604	D606	D601	corr. D604	Corrn.	Cumulative D604	%	
Benzene	3	.141	.008	.038	.133	.008	.133	70	Oestrone methyl ether
"	6	.070	.007	.037	.058	.012	.191	100	
"	9	.005	0	.013	.002	.003	-	-	
"	12	.005	0	.008	.001	.002	-	-	
"	16	.032	.003	.015	.027	.005	.027	17	Oestradiol methyl ether
"	24	.113	.006	.030	.106	.007	.133	82	
"	32	.027	0	.010	.026	.001	.159	98	
"	40	.004	0	.004	.003	.001	.162	100	
"	50	.006	.003	.015	0	.006	-	-	
Benzene + ethanol (97 + 3)	8	.041	.022	.080	.005	.036	-	-	
"	16	.030	.005	.022	.022	.008	.022	16	Oestriol methyl ether
"	24	.094	0	.034	.090	.004	.112	82	
"	32	.022	0	.015	.019	.003	.131	96	
"	40	.008	0	.009	.006	.002	.137	100	
"	48	.002	0	0	.002	0	-	-	
"	56	.002	0	0	.002	0	-	-	
"	64	0	0	0	0	0	-	-	

Standards (30 µg.)				Recovery from urine
Oestrone	.262	0	.052	74%
Oestradiol	.198	0	.041	82%
Oestriol	.178	0	.042	77%

(b) The petroleum ether extract was added to a column of deactivated alumina (2 g.) which was developed with petroleum ether (25 ml.) and 10 ml. of a mixture of petroleum ether and benzene (60 vol. + 40 vol.) and eluted with the benzene containing the oestriol fraction. The column was developed further with benzene (25 ml.) and eluted with a mixture of benzene and ethanol (97.5 vol. + 2.5 vol.).

Oestrogen methyl ethers in the fractions were estimated in the same manner as in the previous experiment. Also see Fig. 25.

Eluate	Cumulative volume (ml.)	D_{604}	D_{606}	D_{601}	Corr. D_{604}	Corrn.	Cumulative D_{604}	%	
Benzene containing the oestriol fraction	3	.114	.003	.026	.111	.003	.111	59	Oestrone 75% recovery
"	6	.085	.005	.033	.077	.008	.188	100	
"	9	.005	0	.015	.001	.004	-	-	
"	12	.007	.006	.012	.0	.007	-	-	
"	16	.032	.006	.019	.024	.008	.024	14	Oestradiol 84%
"	24	.129	.008	.039	.120	.009	.144	86	recovery
"	32	.028	.004	.021	.021	.007	.165	98	
"	40	.008	.003	.012	.003	.005	.168	100	
"	50	.008	.004	.012	.002	.006	-	-	
Benzene + ethanol (97.5 + 2.5)	8	.089	.054	.152	.011	.078	-	-	
"	16	.016	.009	.033	.001	.015	-	-	
"	24	.052	.007	.017	.045	.007	.045	38	Oestriol 76%
"	32	.070	.007	.020	.063	.009	.108	79	recovery

Eluate	Cumulative volume (ml.)	D604	D606	D601	Corr. D604	Corrn.	Cumulative D604	%
Benzene + ethanol (97.5 + 2.5)	40	.028	.004	.013	.023	.005	.131	96
"	48	.008	0	.007	.006	.002	.137	100
"	56	.004	0	.007	.002	.002	-	-
"	64	.004	0	.008	.002	.002	-	-

These experiments showed that whichever chromatographic procedure was used, urine material had little effect on the elution of the oestrogen methyl ethers. Both procedures were equally satisfactory for separating and recovering oestrogens from urine extracts.

Of the three possible procedures, separation of the oestriol from the oestrone and oestradiol fractions by benzene-water partition, followed by separate methylation and chromatography, gave the cleanest urine fractions but was the most tedious method. Combined methylation followed by chromatographic separation on one alumina column and elution of the oestrone and oestradiol fractions with the oestriol benzene extract, was the most convenient method but gave the most contaminated urine fractions. Partition after methylation and chromatography on separate chromatograms was a little less convenient and gave somewhat cleaner urine fractions.

THE METHOD.

This section describes a satisfactory method based on data obtained from the preceding sections. The method is composed of the following steps:

a) Hydrolysis.

b) Extraction with ether.

c) Removal of acids and polyhydroxy phenols from the ether extract.

d) Evaporation of the ether and solution of the residue in benzene.

e) Extraction of the benzene with water and then alkali or with alkali alone.

f) Methylation of the aqueous extracts and extraction with petroleum ether and benzene.

g) Chromatography.

h) Removal of solvents, development of colour and measurement in a colorimeter.

i) Calculation of oestrogen concentrations by comparing colour densities with those of standards.

Each of these procedures has been considered in detail in the foregoing sections. The exact details adopted in the method will now be described.

The hydrolysis of the conjugated oestrogens in human urine.

The hydrolysis method adopted in this work was that recommended by Marrian & Bauld (1951) who suggested the following modifications to the procedure described by Smith & Smith (1935), in which urine is hydrolysed by boiling under reflux with 15 vols. per cent of concentrated hydrochloric acid.

1. Urine was hydrolysed by boiling 60 minutes. Stevenson &

Marrian (1947), Marrian (1948), Van Bruggen (1948), Stimmel (1949) and Engel (1950) have shown that 60 minute boiling with 15 vols. per cent of concentrated hydrochloric acid is necessary to obtain maximal hydrolysis of the conjugated oestrogens in human urine.

2. As suggested by Stevenson & Marrian (1947), the hydrochloric acid was added to the urine after it had been brought to the boil. By doing so, accurate timing of the hydrolysis was facilitated and destruction due to heating with acid in the presence of oxygen was decreased.

3. Van Bruggen (1948) and Rosenmund (1948) suggested that a mild reducing agent should be added to urine to protect the oestrogens against oxidative destruction. Rosenmund (1948) showed that urine contains a natural protective agent which is not always present in a concentration sufficient to prevent oxidation. In this work, hydroquinone, pyrocatechol, o & p-amino phenol, 1-amino 2-naphthol 4-sulphonic acid and ascorbic acid were investigated for this purpose, but apart from neutralizing hypothetical substances such as ferric chloride added to urine, they did not replace the natural protective agents when they were not present. The search for a completely satisfactory protective agent is still continuing.

4. Twenty-four hour specimens of urine were diluted to 2400 ml. with distilled water and fractions of diluted urine were used for hydrolysis. In this way, concentrations of urinary constituents were roughly standardized.

In this work, the term "acid hydrolysed urine" implies urine which has been boiled under reflux with concentrated hydrochloric acid (15 vols. per cent added through the condenser to the boiling

urine), followed by rapid cooling under tap water.

Extraction with ether.

When the oestrogens were partitioned between equal volumes of ether and dilute hydrochloric acid (see section on partition experiments), 100% of the oestrone and oestradiol, but only 85% of the oestriol were found in the ether layer. Quantitative extraction of the three oestrogens should therefore be obtained by extracting hydrolysed urine once with an equal volume and twice with half volumes of ether. Theoretically, this procedure should extract 99% of the oestriol into the ether phase. That this is true in practice is shown by the following experiments with pure solutions.

Oestriol (30 µg.) in ethanol (0.6 ml.) was added

a) directly to ether (350 ml.)

b) to a solution of hydrochloric acid (30 ml. of concentrated acid) and water (200 ml.). This was extracted by shaking 100 times, once with 200 ml. of ether, and twice with 100 ml. of ether. The combined ether extract was washed with 8% sodium bicarbonate solution (10 ml.) to neutralize the acid and twice with water (2 x 10 ml.).

The ether solutions were evaporated until about 1 ml. of ether remained and this was transferred with the aid of ethanol (3 x 1½ ml.) to "Kober" tubes. The solvent was removed by heating under nitrogen and reduced pressure. Residues were dissolved in "pure" ethanol (0.2 ml.) and colours were developed with the 5% hydroquinone-79% sulphuric acid reagent. Densities were measured with the three light filters, corrected by the formula, and compared with that of a standard from ethanol developed in the same manner.

Oestriol	D_{604}	D_{606}	D_{601}	corr. D_{604}	Recovery %
Standard 30 µg.	.196	0	.032	.196	
Directly from ether	.196	.003	.046	.192	98
" " "	.196	.003	.046	.192	98
" " "	.198	0	.050	.193	98
" " "	.204	.001	.052	.198	101
" " "	.202	.001	.052	.196	100
" " "	.212	.004	.058	.200	102
Extracted from dilute HCl	.212	.007	.048	.205	104
	.205	.005	.045	.200	102

Figures given in the section on partition experiments showed that similar recoveries could certainly be expected for oestrone and oestradiol.

The removal of acids and polyhydroxy phenols.

The method adopted for removing acids and polyhydroxy phenols was that described in the section on the subject. It was shown there that when this procedure was used, about 91% of added oestriol was recovered in the ether phase, and quantitative recovery of oestrone and oestradiol could be expected. The next experiment shows that this figure for oestriol is bettered by using the improved colorimetric procedure.

Oestriol (30 µg.) was added to a solution of hydrochloric acid (30 ml. of concentrated acid) in water (200 ml.) and extracted once with 200 ml. and twice with 100 ml. of ether. The combined ether extract was shaken with concentrated carbonate buffer pH 10.5 (80 ml.), the aqueous layer was discarded, and the ether was shaken with 2 N.sodium hydroxide (20 ml.); 8% sodium bicarbonate (80 ml.) was then added and the whole was shaken again. The aqueous layer was discarded. The ether solution was washed with 8% sodium bicarbonate solution (20 ml.), then either once or twice with water (10 ml.) and evaporated

to near dryness. The residues were transferred with ethanol to "Kober" tubes, solvents were evaporated by heating under nitrogen and reduced pressure. Oestriol was estimated as in the preceding experiment by the ethanol-5% hydroquinone-79% sulphuric acid colour method.

Oestriol	D_{604}	D_{606}	D_{601}	corr. D_{604}	Recovery %
Standard 30 µg.	.200	0	.048	.198	
By experiment	.240	.034	.126	.193	98
	.238	.035	.126	.190	96
1 x 10 ml. water washes	.236	.034	.129	.188	95
	.236	.034	.126	.189	96
2 x 10 ml. water washes	.203	.010	.046	.194	98
	.205	.012	.048	.194	98

These results agreed with those expected from theory.

In this experiment, the D_{604} readings were erroneously high owing to a slight cloudiness in the final colour, and were corrected by measuring the colour densities at the three wavelengths and applying the correction formula. Oestrone and oestradiol also gave erroneously high D_{604} readings when partitioned between ether and bicarbonate solutions (see section on partition experiments) but the spectrophotometric correction method had not been developed then.

The evaporation of the ether and solution of the residue in benzene.

After the discovery that anaerobic conditions were necessary to prevent oxidative destruction of oestrogen methyl ethers when their solutions were heated, the precaution was taken to evaporate anaerobically and with the minimum of heating all oestrogen solutions irrespective of whether they contained the free or the methylated oestrogen. Consequently, when evaporating ether extracts, they were not allowed to

distil to dryness but were removed from the source of heat before all the ether had distilled (about 1 ml. left). Also, towards the end of the distillation, care was taken to swirl the contents of the distilling flask so that those parts no longer covered with ether did not overheat.

Early recovery experiments showed that when ether solutions were evaporated to dryness, oestriol in the residue did not completely dissolve in the benzene added later, and inconsistently low oestriol recoveries were thus obtained. This loss of oestriol was prevented by dissolving the residue in ethanol (1 ml.) before adding the benzene. Added precautions taken were to evaporate the ether incompletely (see above) and to transfer the ether plus ethanol solution from distilling flask to separating funnel with about a third volume of the benzene, and to rinse the flask with the rest of the benzene before adding it to the separating funnel. The aqueous solvents used for extracting the phenolic fraction were also used to rinse the distilling flask.

The presence of these volumes of ether and ethanol in the benzene did not affect the partition of the oestrogens between benzene and aqueous solvents (see next section).

Extraction of the oestrogens from benzene with aqueous solvents and methylation with dimethyl sulphate.

At previous stages, the limiting factor was the water solubility of oestriol; at this stage the limiting factor was the relative insolubility of oestrone in alkalis. The design of the method for separating the phenolic from the neutral fraction also depended upon limitations imposed by the methylation procedure. Therefore, these

two steps are considered together. Methylation was conveniently performed in borate buffer solutions prepared by dissolving boric acid in 0.4 N.sodium hydroxide and fortunately this concentration of sodium hydroxide was satisfactory for extracting oestrone from benzene. Experiments performed at this stage were not completely satisfactory because recoveries of oestrone from benzene with sodium hydroxide were not quantitative or entirely reproducible. Although carbonate-free sodium hydroxide has been investigated without improving the results, it is suspected that carbonate in the sodium hydroxide is really the cause of the inconsistent recoveries.

The following experiments are typical of the many performed at this stage. The first shows the effect of ethanol and ether on the extraction of the oestrogens from benzene with water and then with 0.4 N.sodium hydroxide.

Benzene solutions (25 ml.) of oestriol, oestrone and oestradiol (30 µg.) with and without added ethanol and ether (2 ml.) were extracted once with 25 ml. and twice with 12.5 ml. of water or dilute borate buffer pH 9.2. The water or buffer extracts were extracted once with 50 ml. and twice with 25 ml. of ether, the ether extracts were evaporated to near dryness and the residues transferred with ethanol to "Kober" tubes. The benzene solutions were further extracted once with 25 ml. and twice with 12.5 ml. of 0.4 N.sodium hydroxide. The soda extracts were acidified, and extracted once with ether (50 ml.). These ether extracts were washed with water until neutral and then evaporated to near dryness. The residues were transferred with ethanol to "Kober" tubes. Solvents were removed anaerobically and oestrogens were estimated using the original hydroquinone-sulphuric acid colour methods.

	Oestriol (D_{604} standard 30 µg. 0.180)		Oestrone (D_{604} standard 30 µg. 0.252)			Oestradiol (D_{604} 30 µg. 0.230)		
	Water extract		Water	NaOH extract		Water	NaOH extract	
	D_{604}	recovery	D_{604}	D_{604}	Recovery	D_{604}	D_{604}	Recovery
Benzene 25 ml.	.162	90%	.006	.244	97%	.010	.215	94%
Benzene + ether 2 ml.	.152	85%	.008	.236	94%	.010	.210	92%
Benzene + ether 2 ml. + ethanol 2 ml.	.156	87%	.008	.238	95%	.010	.225	98%
	Borate buffer extract							
Benzene 25 ml.	.164 .162	93% 92%						

Evidently, when oestriol is extracted in this manner from benzene, water recovers 90% and dilute borate buffer recovers 92% of added oestriol. Within experimental error, these results are identical. The recovery of both oestrone and oestradiol by soda extraction of the water extracted benzene, was approximately 95%. The presence of small amounts of ether and ethanol in the benzene did not interfere with the recoveries.

A similar experiment was performed using toluene instead of benzene, in which, however, the oestriol was estimated as its methyl ether.

Toluene solutions (25 ml.) of oestriol, oestrone and oestradiol (30 µg. each) containing ethanol (1.0 ml.) and ether (1.0 ml.) were first extracted once with 25 ml. and twice with 12.5 ml. of water or borate buffer pH 9.0, and then once with 25 ml. and twice with 12.5 ml. of 0.4 N.sodium hydroxide. Extracts containing oestriol were made 0.4 N. to sodium hydroxide and methylated at room temperature with

dimethyl sulphate (twice with 1 ml.) in the presence of boric acid (1.24 g.) (the "first methylation procedure"). The methylated oestriol was extracted with benzene, the benzene extract was washed with water to remove alkali and evaporated to a small volume and this was transferred with ethanol to Kober tubes. Extracts containing oestrone and oestradiol were acidified and extracted with an equal volume of ether. The ether was extracted with water to remove acid and was distilled to a small volume and this was transferred to Kober tubes. Solvents were removed under nitrogen and reduced pressure. Oestrogens were estimated by the original hydroquinone-sulphuric acid colour methods.

Aqueous solvent	Oestriol (Standard 30 µg. 0.194, 0.197)		Oestrone (Standard 30 µg. 0.261, 0.269)			Oestradiol (Standard 30 µg. 0.190, 0.190)		
	D_{604} aqueous	Recovery aqueous	D_{604} aqueous	D_{604} NaOH	Recovery NaOH	D_{604} aqueous	D_{604} NaOH	Recovery NaOH
Water	.170 .168	87% 86%	0	.240 .239	90% 90%	0	.176 .188	93% 99%
borate buffer pH 9.	.160 .170	82% 87%	0	.238 .230	90% 87%	0	.168 .188	89% 99%

These experiments showed that benzene and toluene were equally good organic solvents for the separation of the neutral from the phenolic fraction by partitioning between organic and aqueous solvents. Benzene was finally chosen for this step because it was used later in the method and there was no reason for unnecessarily introducing another solvent. The extraction of the oestrogens from benzene and conversion into their methyl ethers is satisfactorily performed in the following manner. The four extractions were introduced to recover oestriol and oestrone which seemed to be lost when only three extractions were used.

A solution of the oestrogens in benzene (20 ml.), ethanol (1 ml.) and di-ethyl ether (approx. 1.5 ml.) was extracted either - (a) once with 20 ml. and three times with 10 ml. of water to extract the oestriol, and then once with 20 ml. and three times with 10 ml. of 0.4 N.sodium hydroxide to extract the oestrone and oestradiol (partition before methylation); or (b) once with 20 ml. and three times with 10 ml. of 0.4 N.sodium hydroxide to extract the total phenol fraction for combined methylation and chromatography on one chromatogram.

The water extracts (50 ml.) were made approximately 0.4 N. by adding 5 N.sodium hydroxide (4 ml.). Boric acid (0.9 g.) was added to each alkaline extract, the mixtures were warmed to 37°C, dimethyl sulphate (1 ml.) was added, and the mixtures were shaken until the boric acid and dimethyl sulphate had dissolved. The solutions were incubated at 37°C for 20 - 30 minutes, further dimethyl sulphate (1 ml.) and 5 N.sodium hydroxide (2 ml.) were added, the mixtures were shaken until the dimethyl sulphate had dissolved, and then allowed to stand at room temperature overnight. Ten ml. of 5 N.sodium hydroxide was added to each methylation mixture and oestrone and oestradiol methyl ethers were extracted with petroleum ether (50 ml.), and oestriol methyl ether with benzene (50 ml.). Organic solvents were washed twice with water (5 to 10 ml.) to remove alkali. When oestriol, oestrone and oestradiol were methylated together, the water washes (2 x 10 ml.) of the petroleum ether extract were added to the aqueous phase and the oestriol methyl ether was extracted from this with benzene.

The recovery of oestriol and oestrone by these procedures was determined by the experiment next described, in which:-

1. Oestriol (30 µg.) or oestrone (30 µg.) was added to 0.4 N.sodium hydroxide (50 ml.) and methylated.

2. Oestriol (30 µg.), ethanol (1 ml.) and di-ethyl ether (1.5 ml.) were added to benzene (20 ml.) and extracted once with 20 ml. and three times with 10 ml. of water. The oestriol in the water extract was methylated.

3. Oestriol (30 µg.) or oestrone (30 µg.), ethanol (1 ml.) and di-ethyl ether (1.5 ml.) were added to benzene (20 ml.) and extracted once with 20 ml. and three times with 10 ml. of 0.4 N.sodium hydroxide. The oestrogen in the alkali extract was methylated. The methylation conditions and extraction of the methyl ethers were exactly as described above. Solvents were evaporated under anaerobic conditions and oestriol was estimated by the ethanol-5% hydroquinone-79% sulphuric acid colour method and the oestrone by the 2% hydroquinone-66% sulphuric acid colour method. Colour densities were measured with the three filters and corrected.

Oestriol	D_{604}	D_{606}	D_{601}	corr. D_{604}	Recovery %
Standard	.198	0	.036		
	.193	0	.031		
Direct methylation	.204	.011	.062	.190	97
	.210	.014	.074	.191	98
	.208	.009	.052	.198	102
Benzene-water partition	.196	.010	.063	.181	93
	.197	.010	.060	.184	94
	.201	.010	.062	.187	96
Benzene-NaOH partition	.215	.009	.056	.204	105
	.224	.011	.069	.209	107
	.217	.008	.059	.206	106

Oestrone	D_{604}	D_{606}	D_{601}	corr. D_{604}	Recovery %
Standard (30 µg.)	.288	0	.055		
	.288	.001	.059		
Direct methylation	.283	.008	.057	.277	96
	.280	.008	.056	.274	96
Benzene-NaOH	.231	.008	.047	.225	78
partition	.238	.008	.054	.230	80

The chromatography of the methyl ethers, evaporation of solvents and colorimetry.

The technique of chromatography of the oestrogen methyl ethers on alumina columns, the anaerobic distillation of solvents, development of colour, colorimetry, and spectrophotometric correction for interfering chromogenic material are discussed elsewhere.

Two methods were devised, one separates the oestriol fraction from the oestrone plus oestradiol fraction by benzene-water partition before methylation and chromatographs the two fractions on separate alumina columns; the other methylates oestriol, oestrone and oestradiol together and separates them on a single column of alumina. Partition before methylation gave cleaner urine fractions, lower (but reproducible) recoveries of oestriol, and was more time consuming than the other method. Recoveries of oestrogens from dilute hydrochloric acid and from acid hydrolysed male urine were determined by both methods and are recorded in the appendix. It is shown there that both methods are satisfactory for the separate estimation of the three oestrogens in urines containing them at individual concentrations between 120 and 3,600 µg. per 24 hr. specimen. In practice, the oestrogen content of two urines in duplicate can easily be determined by one worker in 2⅓ days by the first method, and in 2 days by the second.

PART III

A DESCRIPTION OF THE COMPLETE METHOD GIVING THE EXPERIMENTAL PROCEDURE IN DETAIL.

A DETAILED DESCRIPTION OF THE TWO METHODS.

Definition of terms used in the description of the methods:

"Oestradiol" implies the isomer oestradiol-17β.

Oestriol, oestrone or oestradiol "fraction" implies that fraction in which the free oestrogen or its methyl ether would normally occur.

"Oestrogen" implies either oestriol, oestrone or oestradiol.

"Extraction" of solvents implies shaking immiscible solvents together a hundred times.

"Washing" of solvents implies shaking immiscible solvents together ten to twenty times.

Apparatus:

Flasks were 500 and 250 ml. round bottom flasks with standard B24 sockets and 100 ml. round bottom flasks with standard B19 sockets.

Methylation flasks were 100 ml. conical flasks fitted with standard B19 sockets and stoppers.

Two sizes of separating funnels were used, these had capacities of 500 and 150 ml.

The chromatogram tubes are described in the section dealing with chromatography. They were usually used in a battery of four, suction being applied from a manifold through two-way stop-cocks which allowed suction to be applied to, or released from, individual chromatogram tubes and their receivers.

"Kober" tubes were 6 x $\frac{3}{4}$" pyrex tubes with standard B19 sockets and graduated at 15 ml.

The apparatus for the anaerobic distillation of solvents from Kober tubes is described in the section on colour methods, Page 52.

The colorimeter was a Spekker Absorptiometer equipped with Ilford no.601, no.604 and no.606 light filters, and glass cells with a light path of 1 cm.

Distilling flasks and Kober tubes were cleaned after use by steeping in chromic acid-sulphuric acid cleaning fluid, washing in tap water, and rinsing with an acid sulphite solution (sodium sulphite, approx. 0.2%, acidified with sulphuric acid) to destroy traces of chromic acid which would be harmful in the method. They were then rinsed thoroughly with tap water and then distilled water, and dried. Other glassware was simply rinsed after use with tap water and distilled water unless visibly dirty, when it was cleaned with chromic acid as described above.

Reagents:

Di-ethyl ether was A.R. grade, washed with saturated ferrous sulphate solution and water and distilled. The purified ether was kept in dark coloured "Winchester" bottles and used within a fortnight of purification.

Benzene was A.R. grade, redistilled. Already used benzene was recovered by washing three times with water to remove ethanol and distilled. Benzene for chromatography was further distilled through an efficient fractionating column to remove traces of water and ethanol by azeotropic distillation. This benzene was moistened with water before use.

Petroleum ether (A.R. grade boiling between 40 and 60°C) was redistilled. Petroleum ether which had already been used was purified by simple distillation. Recovered solvent was not used for chromatography.

Ethanol was usually 95% spirit refluxed with sodium hydroxide pellets to remove aldehydes and twice distilled. Any batches of this which were not chromogenic when heated with hydroquinone-sulphuric acid reagents were kept especially for use in the oestriol Kober colour method. Absolute ethanol for chromatography was also purified by refluxing with sodium hydroxide and distilling twice.

Hydrochloric and sulphuric acids were A.R. grade.

Dimethyl sulphate was B.D.H. reagent redistilled.

Boric acid powder was B.P. grade.

Sodium hydroxide was A.R. grade in the form of pellets.

5 N.sodium hydroxide was prepared by dissolving sodium hydroxide pellets (200 g.) in water so that the final volume of solution was 1 litre.

0.4 N.sodium hydroxide was prepared similarly by dissolving sodium hydroxide (16 g.) in 1 litre of aqueous solution.

8% sodium bicarbonate solution was prepared by dissolving A.R. grade sodium bicarbonate (80 g.) in water so that the final volume of solution was 1 litre.

Concentrated carbonate solution, pH 10.5, was prepared by adding 5 N.sodium hydroxide (150 ml.) to 8% sodium bicarbonate solution (1 litre).

Alumina for chromatography was Savory & Moore reagent labelled "Aluminium oxide for chromatographic analysis; standardized according to Brochmann". This was deactivated by keeping in an atmosphere saturated with water vapour at room temperature until the desired activity had been reached. The behaviour of the oestrogen methyl ethers on standard columns of this deactivated alumina was determined

before it was used in the method.

Sand for overlaying the alumina columns was boiled with hydrochloric acid, washed with water, and extracted with ethanol and dried.

"Kober" reagents:

Pure sulphuric acid was diluted with water to 79%, 66% and 60% v/v sulphuric acid. For instance, the 79% v/v acid was prepared by diluting, and cooling, 79 ml. of pure sulphuric acid to 100 ml. with water.

The oestriol reagent was prepared by dissolving hydroquinone (5 g.) in 79% v/v sulphuric acid (100 ml.). Solution was hastened by warming. The hydroquinone was B.D.H. reagent.

The oestrone and oestradiol reagents were prepared similarly by dissolving hydroquinone (2 g.) in 66% and 60% v/v sulphuric acid respectively.

Borth (1952) has shown that when the hydroquinone is added to cold diluted sulphuric acid and dissolved by warming, lighter coloured reagents are obtained than when the sulphuric is already warm when the hydroquinone is added. This has been confirmed.

Reagents were kept at least 24 hours before use. They had a light brown colour and were stable almost indefinitely.

Sulphuric acid 30% (v+v) was prepared by adding pure sulphuric acid (300 ml.) to water (700 ml.).

Experimental procedure:

Hydrolysis: A 24 hour specimen of urine containing toluene (3 ml.) as a preservative, was diluted to 2400 ml. with distilled water, and a 200 ml. fraction of the diluted urine was added to a 500 ml. flask.

Determinations were usually performed in duplicate. The urine was heated to boiling under a reflux condenser and concentrated hydrochloric acid was added to it through the condenser. The urine and hydrochloric acid were boiled together for exactly 60 minutes and cooled rapidly under tap water.

Extraction: The cooled urine was added to a 500 ml. separating funnel and extracted once with 200 ml. and twice with 100 ml. of ether which had first been used to rinse the hydrolysis flask.

Removal of acids and polyhydroxy phenols: The ether layers were added to another separating funnel and extracted with concentrated carbonate solution, pH 10.5 (80 ml.). The carbonate layer was discarded; 2 N.sodium hydroxide (20 ml.) was added and shaken thoroughly with the ether layer to oxidize the polyhydroxy phenols, 8% sodium bicarbonate solution (80 ml.) was added, the whole was shaken again one hundred times and the carbonate-alkali layer was discarded. The ether solution was washed with 8% sodium bicarbonate solution (20 ml.) to neutralize alkali and then with water (10 ml.). The water was drained off as completely as possible.

Evaporation of the ether extract and solution of the residue in benzene: The ether solution was added to a 500 ml. flask and the ether was distilled in a water bath. Towards the end of the distillation care was taken to swirl the contents of the flask so that those parts no longer covered with ether did not overheat. The ether was distilled until about 1 ml. remained. Ethanol (1 ml.) was added before it had cooled. A 20 ml. volume of benzene was measured and about a third of it was added to the cold distilling flask. The contents were mixed and poured into a 150 ml. separating funnel. The flask was rinsed with the remainder of the benzene and this was also added to the

separating funnel.

Separation of the phenol fraction: The procedure at this stage depended upon the method being followed.

Method 1. - Partition before methylation.

The benzene solution was extracted once with 20 ml. and three times with 10 ml. of water which had been used to rinse the distilling flask. The water extracts contained the oestriol fraction and were added to a methylation flask containing boric acid (0.9 g.). The benzene was further extracted once with 20 ml. and three times with 10 ml. of 0.4 N.sodium hydroxide. The sodium hydroxide extracts contained the oestrone and oestradiol fractions and were added to a methylation flask containing boric acid (0.9 g.). During these extractions, separating funnels were shaken gently to minimize emulsion formation and the two layers were separated as completely as possible.

The methylation flasks were placed in a water bath maintained at 37°C. When they had warmed somewhat, 5 N.sodium hydroxide (4 ml.) was added to the flask containing the oestriol water extracts and dimethyl sulphate (1 ml.) was added to both flasks. The dimethyl sulphate was handled cautiously and added with a 1 ml. pipette equipped with a rubber bulb to manipulate volumes. The flasks were shaken until the boric acid and dimethyl sulphate had dissolved and were incubated at 37°C for 20 - 30 minutes. Sodium hydroxide (2 ml. of 5N.) and another 1 ml. of dimethyl sulphate were added to each flask. The flasks were shaken until the dimethyl sulphate had dissolved, and were either incubated another 45 minutes and cooled, or allowed to stand at room temperature overnight. Sodium hydroxide (10 ml. of 5N.) was then added to each flask. The contents of the flasks were added to 150 ml.

separating funnels.

The oestriol fraction was extracted with benzene (50 ml.) and the oestrone and oestradiol fractions were extracted with petroleum ether (50 ml.). The organic solvents were used first to rinse their respective methylation flasks. The alkaline aqueous layers were discarded and the organic solvent layers were washed twice with 5 ml. of water. The water was allowed to drain as completely as possible from the sides of the funnels and was removed.

Chromatography: Chromatogram columns were prepared by filling the narrow portion of the chromatogram tube with benzene, in the case of oestriol, and with petroleum ether, in the case of the oestrone and oestradiol columns, and adding deactivated alumina (2 g.) in a thin stream so that entangled air could free itself during passage through the solvent. The alumina was allowed to settle for a minute or two and was then overlain with approximately a quarter inch of sand to protect the alumina from being disturbed during addition of solvents. Solvents were usually sucked through the columns to the level of the sand but no further. Columns were never allowed to "go dry" during chromatography. Solvent flows were usually about 3 ml. per minute and were regulated by the suction applied.

The oestriol fraction in benzene was chromatographed by pouring it carefully into a prepared chromatogram tube and sucking the solution through the alumina column. Benzene (10 ml.) which had been used to rinse the separating funnel, was likewise added to the column and sucked through. The exact details of the development and elution of the column depended upon the activity of the alumina used. The following is a typical example. The column was developed by passing through 15 ml.

of a mixture of benzene and ethanol (97.5 vol. + 2.5 vol.) using a slow rate of solvent flow (approx. 1.5 ml. per min.). The oestriol fraction was eluted at normal speeds into a 100 ml. flask with a further 50 ml. of the same mixture of 2.5% ethanol in benzene.

The methylated oestrone and oestradiol fraction, in petroleum ether, was carefully poured into a prepared chromatogram tube and sucked through the alumina column. The separating funnel was rinsed with petroleum ether (10 ml.) and this was similarly added to the column. The chromatogram was developed with 10 ml. of a mixture of benzene and petroleum ether (40 vol. + 60 vol.). The exact details of elution of the column depended on the activity of the alumina. A typical example is described. The oestrone was eluted with 9 ml. of benzene directly into Kober tubes without applied suction, and the oestradiol was eluted at normal speeds into 100 ml. flasks with a further 40 ml. of benzene.

Method 2. - Combined methylation and partition on a single chromatogram.

The benzene solution containing the neutral and phenolic urine fractions was extracted once with 20 ml. and three times with 10 ml. of 0.4 N.sodium hydroxide. The funnel was shaken gently to minimize emulsion formation and layers were allowed to separate as completely as possible. The sodium hydroxide extracts were added to a methylation flask containing boric acid (0.9 g.) and methylated in the same manner as the oestrone and oestradiol fraction in the first method.

Sodium hydroxide (10 ml. of 5 N.) was added to the methylation flask; the strongly alkaline methylation mixture was added to 150 ml. separating funnels and extracted with petroleum ether (50 ml.) which had first been used to rinse the methylation flask. The alkaline aqueous layer was added to another separating funnel and the petroleum

ether layer was washed twice with 10 ml. of water. The water washes were added to the other separating funnel, and the combined aqueous solution was extracted with benzene (50 ml.) which had first been used to rinse the methylation flask. The aqueous layer was discarded, and the benzene layer was washed twice with 5 ml. of water.

The funnels containing the petroleum ether and the benzene extracts were allowed to stand for some time until water adhering to their walls had drained away as completely as possible and was thus removed.

Chromatography: A column of deactivated alumina (2.0 g.) was prepared in petroleum ether as described in the first method, and the petroleum ether extract was passed through the alumina column. The funnel was rinsed with petroleum ether (10 ml.) and this was also added to the column. The column was developed with 10 ml. of a mixture of petroleum ether and benzene (60 vol. + 40 vol.). The benzene extract containing the oestriol fraction was added to the chromatogram tube and used to elute the oestrone and oestradiol fractions. The exact details of this elution depended on the activity of the alumina. A typical example is described. The oestrone was eluted with the first 9 ml. of benzene directly into Kober tubes without applied suction, and the oestradiol with the remaining 41 ml. of benzene solution. The oestradiol was eluted at normal speed into 100 ml. flasks. The separating funnels were rinsed with benzene (10 ml.) and this was added to the column. The column was eluted with 60 ml. of a mixture of benzene and ethanol (97.5 vol. + 2.5 vol.), the first 15 ml. of eluate was sucked through very slowly (about 1.5 ml. per min.) and was discarded. The next 45 ml. of eluate contained the oestriol fraction and was

collected in a 100 ml. flask.

Removal of solvents: Solvents were distilled from the flasks containing the oestriol and oestradiol fractions by using reduced pressure and porous pot to prevent "bumping". Care was taken to remove the flasks from the water bath before the solvent had completely evaporated. The flasks and their contents were allowed to cool, and the residues were transferred to Kober tubes with the aid of three lots of approximately 1.5 ml. of ethanol.

Solvents were completely evaporated from all the tubes by heating under nitrogen and reduced pressure in the special apparatus.

Colorimetry: Specially pure ethanol (0.2 ml.) was added to the oestriol Kober tubes so that their contents were dissolved in it. Four ml. of the 5% hydroquinone-79% sulphuric acid reagent was added to the oestriol tubes and mixed with the ethanol solution. Four ml. of the 2% hydroquinone-66% sulphuric acid reagent was added to the oestrone tubes, and 4 ml. of the 2% hydroquinone-60% sulphuric acid reagent was added to the oestradiol tubes. The tubes were immersed for 20 min. in a boiling water bath and were shaken individually during the first 5 min. of heating. The tubes were cooled in ice water. Water (1.0 ml.) was added to the oestriol tubes and 0.5 ml. of water to the oestrone tubes; the contents were mixed and the oestriol and oestrone tubes were immersed for 5 min. in boiling water. The tubes were cooled in ice water and the contents of all the tubes were diluted to 15 ml. with 30% v+v sulphuric acid. The tubes were allowed to stand at room temperature at least 10 min. after dilution; colour densities were measured in a colorimeter against similarly treated hydroquinone-sulphuric reagent blanks, and within 60 min. of dilution. Densities were

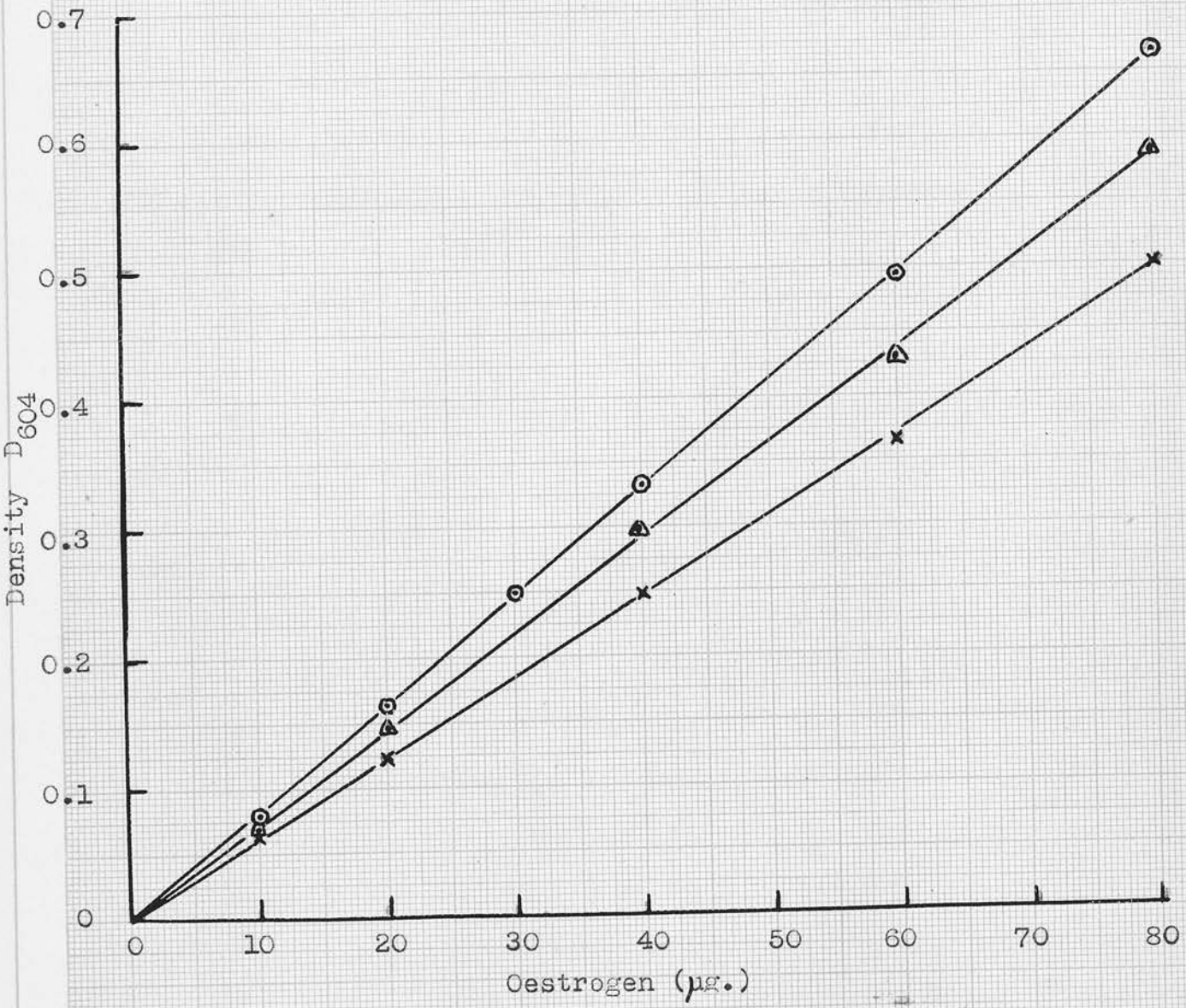


Fig. 26. The relationship between concentration of oestrogen and colour density using hydroquinone-sulphuric acid reagents and anaerobic distillation of solvents.

x—x oestriol, ethanol-5% hydroquinone-79% sulphuric acid reagent.

O—O oestrone, 2% hydroquinone-66% sulphuric acid reagent.

△—△ oestradiol, 2% hydroquinone-60% sulphuric acid reagent.

measured with the Ilford no.604 green, no.606 yellow and no.601 violet light filters. The density (corr. D_{604}) due to the oestrogen component at the wavelength of light transmitted by the no.604 green filter was calculated by the following formula:

$$\text{corr. } D_{604} = \frac{4D_{604} - 3D_{606} - D_{601}}{3.8}$$

where D_{604}, D_{606}, and D_{601} were the observed densities obtained with the no.604, 606 and 601 filters.

The oestrogen content of the extract was calculated from the corr. D_{604} by comparison with a standard curve prepared with pure oestrogens (see Fig.26), and the content of the 24 hour specimen of urine was calculated by multiplying this figure by 12.

When colour densities were greater than 0.500, either smaller volumes of urine were treated, or fractions of extracts were taken after chromatography before adding them to the Kober tubes.

APPENDIX

(Including recovery experiments.)

APPENDIX.

A description of the crystalline oestrogens used in this work:

Melting points were determined in sealed evacuated capillary tubes immersed in a sulphuric acid bath fitted with a thermometer and stirrer. They were uncorrected.

The crystalline oestriol was a gift to Professor G.F. Marrian from Dr. O. Kamm, Parke Davis, Detroit. The sample melted at 282-283°C. Its identity was confirmed by preparing its triacetate in the following manner:

Oestriol (13.2 mg.) was dissolved in redistilled pyridine (1 ml.) and acetic anhydride (0.7 ml.). The mixture, in a stoppered tube, was heated 8 hours in a boiling water bath; water was added, and the resulting curdy precipitate was washed with water and crystallized from dilute ethanol. The crystalline material weighed 10.2 mg. and melted at 127°C. When mixed with an authentic sample of oestriol triacetate (m.p. 128°C) supplied by Professor Marrian, the melting point was unchanged.

The crystalline oestrone melted at 255°C.

The crystalline oestradiol-17β was a gift to Professor Marrian from Charles and E. Frosst & Co., Montreal. Its melting point was not checked.

The crystalline oestrogen methyl ethers:

The crystalline methyl ethers were prepared from the pure oestrogens as follows.

Oestrone methyl ether.

Oestrone methyl ether was prepared by the method described by Marrian & Haslewood (1932) as follows: Oestrone (19 mg.) was dissolved with warming in normal sodium hydroxide (6 ml.). Insoluble material

was removed by centrifugation. Dimethyl sulphate (0.25 ml.) was added to the alkaline solution and the mixture was shaken for 90 min. A white precipitate began to separate almost immediately. More dimethyl sulphate (0.5 ml.) was added, shaking was continued another 60 minutes, and the mixture was heated on a boiling water bath for 15 minutes. The precipitate was separated by centrifugation, resuspended in 0.5 N.sodium hydroxide and heated in a water bath another 15 min. The solid was filtered off, washed with water, and crystallized from 95% ethanol. The crystalline oestrone methyl ether weighed 11 mg., m.p. 171-172°C. Marrian & Haslewood recorded a m.p. of 164-167°C.

Oestradiol methyl ether.

Pure oestradiol (50 mg.) in dry methanol (12 ml.) was methylated with diazo methane prepared according to the instructions given by Hickinbottom (1948) as follows.

Nitroso methyl urethane (2 ml.) was added to dry di-ethyl ether (60 ml.) and the solution was cooled in ice water. Cold 25% potassium hydroxide in methanol was added and the mixture was distilled into cold methanol (20 ml.) until the condensing ether was colourless.

The resulting yellow distillate, containing diazo methane, was added to the solution of oestradiol in methanol, and the mixture was allowed to stand overnight at room temperature. In the morning, di-ethyl ether (250 ml.) was added and the solution was extracted four times with 2 N.sodium hydroxide, and with water to remove alkali. The ether solution was evaporated to dryness and the residue was crystallized from 50% methanol-water. Oestradiol methyl ether separated in the form of hexagonal plates - weight 30 mg., m.p. 98.5°C. Butenandt & Goergens (1937) recorded a m.p. of 97-98°C.

Oestriol methyl ether.

Oestriol methyl ether was prepared by two methods.

1) Methylation with diazo methane:-

Pure oestriol (37 mg.) was methylated with the diazo methane prepared from nitroso methyl urethane (2 ml.) (for preparation see the preparation of oestradiol methyl ether). The solution of oestriol and diazo methane in methanol and ether was allowed to stand overnight at room temperature. The solution was colourless in the morning; it was diluted with ether (250 ml.) and extracted four times with 2 N.sodium hydroxide. The ether layer was washed with water until neutral and the ether was evaporated. The residue was crystallized from methanol. Oestriol methyl ether separated in the form of colourless sheaves; weight 14 mg., m.p. 149°C. Recrystallization from absolute ethanol failed to raise the melting point. The mother liquors yielded another 14 mg. of crystals melting at 149°C.

2) Methylation with dimethyl sulphate:-

Oestriol (20 mg.) was dissolved in 0.5 N.sodium hydroxide (6 ml.) and methylated with dimethyl sulphate (0.5 ml.) according to the instructions given by Marrian & Haslewood (1932). The crude methylated material was crystallized from ethanol and gave 10 mg. of crystals melting at 148-149°C. This was indistinguishable from the material obtained by methylation with diazo methane.

A melting point of 149°C is the lowest recorded for apparently pure oestriol methyl ether. Marrian & Haslewood (1932) recorded a melting point of 162.4-164°C. Butenandt, Stormer & Westphal (1932) methylated oestriol with diazo methane and purified the product by distillation and crystallization. It melted at 159-160°C.

Thayer, Levin & Doisy (1931) prepared oestriol methyl ether using dimethyl sulphate, and purified it by distillation and crystallization from ethanol, and drying in vacuo at 110°C. Their product melted at 154.8°C.

Our oestriol methyl ether was compared with the original oestriol methyl ether prepared by Professor Marrian and with a sample prepared by him from oestriol glucuronide methyl ether. In our hands, the samples, either alone or mixed with our oestriol methyl ether, melted at 149°C. Drying in vacuo at 100°C over phosphorus pentoxide did not change this. The melting points of samples seemed to depend upon the amount used for the melting point determination and also upon previous heat treatment. For instance, when an eighth inch packed column of oestriol methyl ether was used in the melting point capillary, the sample prepared by Professor Marrian softened at 148°C and melted between 152-157°C. Our preparation softened at 148°C and melted between 150-160°C. Mixtures of the two samples behaved similarly. When a few crystals were heated in a sealed evacuated capillary tube, they melted at 148°C. On cooling, the melt solidified immediately and on reheating melted at 149-150°C. The melt was heated to 160°C and on cooling it did not solidify immediately but did so overnight. On reheating, the melting point was 160-167°C. This phenomenon is still being investigated.

The solubilities of the oestrogen methyl ethers in some organic solvents at 20°C.

Oestriol, oestrone and oestradiol methyl ethers, in near saturated ethanol solution (0.2 ml.), were added to small glass phials with capacities of about 0.5 ml. and made from clear glass tubing. The ethanol was removed in vacuo over anhydrous calcium chloride. Pure solvent (about 0.2 ml.) was added, the phials were sealed and allowed to stand for 14 days at room temperature (20°C). The phials were centrifuged and opened carefully. Micro volumes were transferred to Kober tubes with Pasteur pipettes graduated to a mark with mercury. Solvent was removed with heating in a stream of air and oestrogen methyl ethers in the residue were estimated with the hydroquinone-sulphuric acid reagents.

In some phials, the methyl ether was completely dissolved in the solvent so that saturation had not been obtained. In these cases, figures for the saturated solutions will be greater than those given. Solubilities in ethanol were derived gravimetrically by evaporating saturated ethanolic solutions and weighing the residues. The petroleum ether was a grade which boiled between 40 and 60°C.

Oestriol methyl ether (50 µg. standard D_{604} 0.290)

	Ethanol	Ethyl ether		Benzene		Petroleum ether		Chloroform	
Vol. of satd. soln. (ml.)	0.6	.014	.028	.011	.022	.018	.036	.009	.018
Whether saturated	yes	yes		yes		yes		yes	
D_{604}		.043	.067	.016	.029	.0025	.006	.058	.110
Methyl ether content (µg.)	4.8mg.	7.4	11.5	2.8	5.0	.43	1.03	10	19
Solubility (mg.%)		53	41	25	23	2.4	2.9	110	105
Mean solubility (mg.%)	800	47		24		2.7		107	

Oestrone methyl ether (50 μg. standard D_{604} 0.380)

	Ethanol	Ethyl ether		Benzene		Petroleum ether		Chloroform	
Vol. of satd. soln. (ml.)	0.7	.018	.009	.011	.006	.014	.028	.009	.0045
Whether saturated	yes	yes		no		yes		no	
D_{604}		.440	.268	.615		.082	.143	.540	
Methyl ether content (μg.)	3.9mg.	58	35	81		11	19	70	
Solubility (mg.%)		320	390	1,350		78	68	1,550	
Mean solubility (mg.%)	560	360		>1,350		73		>1,550	

Oestradiol methyl ether (50 μg. standard D_{604} 0.257)

	Ethanol	Ethyl ether		Benzene		Petroleum ether		Chloroform	
Vol. of satd. soln. (ml.)		.014	.007	.011	.006	.018	.036	.009	.0045
Whether saturated	very	yes		no		yes		no	
D604	soluble	.238	.172	.297	.157	.117	.229	.292	.148
Methyl ether content (μg.)	-	46	33	58	30	23	45	57	29
Solubility (mg.%)		330	470	530	500	130	125	630	640
		400		>520		127		>640	

RECOVERY EXPERIMENTS.

Recovery experiments were performed by adding known amounts of the three oestrogens to 200 ml. of dilute hydrochloric acid or acid hydrolysed male urine and estimating them in these solutions by one of the methods. Recoveries were calculated by comparing the densities of colours developed from standards with those developed from extracts obtained by the methods. Quantities for the standards were measured by volume directly into Kober tubes at the same time as they were added to the dilute hydrochloric acid or hydrolysed urine. As in previous work, free oestrogen standards were thus used, even though the substance being measured in the final extract was the methyl ether.

The constants for the spectrophotometric correction formula were derived from densities of colours developed with a series of standards and urine blanks and measured with the three filters. Many of the colour densities used for deriving the correction formula are given in the recovery tables, not collected into special tables, but dispersed among results grouped in terms of the urine specimen examined.

The results, which are unselected and include the earliest obtained by the methods, were obtained by four different workers, two of whom had had no previous experience with the methods. It was usual for a new worker to obtain satisfactory results in their second attempt.

Either method seems to be satisfactory for estimating oestriol, oestrone and oestradiol in urine at individual concentrations between 120 and 3,600 µg. per 24 hr. specimen.

EXPERIMENTAL RESULTS.

Partition before methylation with chromatography on two alumina columns.

(Method 1.)

a) From dilute hydrochloric acid (30 ml. of concentrated hydrochloric acid in 200 ml. of water).

Experiment number.	Oestrogen added µg.	Fraction for colorimetry.	D_{604} Standard	D_{604} Experiment		Recovery %	
Oestriol							
1	10	1.0	.071	.049	.043	69	61
2	20	1.0	.138	.088	.091	64	66
3	40	1.0	.280	.211	.209	75	75
4	150	0.2	.200	.134	.142	67	71
5	300	0.1	.200	.140	.153	70	76
Oestrone							
1	10	1.0	.091	.077	.074	85	81
2	20	1.0	.184	.159	.160	86	86
"	"	"	"	.158	.144	86	79
3	40	1.0	.367	.292	.330	80	90
4	150	0.2	.275	.224	.233	82	85
5	300	0.1	.275	.230		84	
Oestradiol							
1	10	1.0	.077	.068	.069	88	89
2	20	1.0	.151	.144	.132	95	87
"	"	"	"	.130	.132	87	89
3	40	1.0	.301	.262	.272	88	90
4	50	1.0	.362	.320	.316	89	87
5	100	0.5	.362	.325		90	

b) From acid hydrolysed male urine (200 ml.)

Oestriol:

Urine or standard	Oestriol µg.	D_{604}		D_{606}		D_{601}		D_{604} corr.		Recovery %	
Standard	30	.189		0		.038					
R.C.B.	10	.068	.062	.015	.011	.066	.058	.043	.042	70	68
0.1	300	.137	.142	.002	0	.034	.036	.134	.140	73	76
Standard	30	.201		0		.042					
J.B.(i)	0	.026		.014		.049		.003			
	30	.172	.178	.017	.020	.080	.088	.145	.148	73	74
Standard	30	.188	.198	0	0	.042	.035				
J.B.(ii)	0	.040	.034	.022	.018	.079	.073	.004	.002		
	10	.080	.082	.018	.020	.073	.077	.051	.050	82	81
0.1	300	.160	.150	.004	.004	.048	.038	.150	.145	81	78
Standard	30	.189		.007		.057					
W.B.	0	.033	.032	.019	.020	.071	.065	.001	.001		
	30	.162	.169	.021	.020	.091	.100	.130	.135	70	73
Standard	30	.182		.005		.044					
J.O.	0	.037	.027	.021	.017			.007	.004		
	10	.083	.087	.020	.019			.046	.052	74	84
	30	.188		.029		.087		.152		82	
0.1	300	.143	.152	.007	.008	.043	.054	.134	.139	72	75
Standard	30	.198	.184	.007	.004	.057	.039				
S.N.	0	.042	.037	.023	.023	.085	.078	.004	0		
	10	.107		.041		.139		.044		71	
	30	.173	.188	.025	.030	.099	.120	.136	.143	73	77
0.1	300	.164	.149	.013	.002	.049	.033	.150	.147	81	79
Standard	30	.220	.187	.018	.004	.088	.044				
F.T.	0	.085	.058	.048	.034	.137	.112	.010	.005		
	10	.091	.079	.027	.020	.096	.078	.049	.047	79	76
	30	.180	.195	.037	.038	.121	.135	.128	.140	69	75
0.1	300	.160	.142	.010	.002	.050	.029	.147	.141	79	76
Standard	30	.193		0		.055					
H.B.	0	.030	.030	.013	.013	.063	.061	.005	.005		
	10	.076	.076	.013	.013	.066	.066	.052	.052	80	80
	30	.153	.153	.013	.013	.072	.072	.132	.132	68	68
0.1	300	.146	.148	0	0	.028	.028	.146	.148	75	76
Standard	30	.195	.198	0	0	.038	.046				
D.H.	0	.048	.046	.028	.028	.088	.088	.005	.003		
	10	.080	.082	.025	.025	.090	.093	.041	.042	63	65
	30	.190	.187	.028	.024	.103	.098	.151	.152	77	78
0.1	300	.158	.160	0	0	.048	.050	.154	.155	79	79

Oestrone:

Urine or standard	Oestrone µg.	D_{604}		D_{606}		D_{601}		D_{604} corr.		Recovery %	
Standard	10	.086	.262	0	0	.015	.058				
H.B.	0	.012		.006		.044		.003			
	10	.092	.088	.008	.008	.044	.042	.079	.075	92	87
	30	.200	.230	.008	.008	.062	.064	.189	.220	72	84
0.3	100	.204	.200	.004	.004	.048	.046	.200	.198	76	76
Standard	10	.088	.265	0	0	.026	.054				
N.T.	0	.047		.016		.081		.015			
	10	.092	.092	.014	.014	.074	.074	.067	.066	76	75
	30	.240	.234	.017	.016	.083	.081	.217	.212	81	80
0.3	100	.252		.008		.061		.243		92	
Standard	10	.087	.270	0	0	.023	.048				
D.H.	0	.018	.018	.014	.014	.029	.030	0	0		
	10	.090	.092	.012	.012	.052	.052	.072	.073	83	84
	30	.230	.232	.018	.019	.079	.080	.206	.208	78	79
0.3	100	.226	.228	.004	.004	.049	.050	.222	.224	84	85
Standard	10	.088	.264	0	0	.018	.052				
J.O.	0	.014	.016	.007	.007	.033	.034	0	.002		
	10	.072	.072	.007	.007	.043	.043	.059	.059	67	67
	30	.234	.232	.010	.009	.074	.072	.220	.218	83	82
0.3	100	.222	.222	.003	.003	.047	.047	.220	.219	83	82
Standard	10	.083	.250	.004	.005	.017	.048				
C.B.	0	.007	.010	.004	.004	.016	.022	0	.001		
	10	.069	.076	.004	.004	.024	.031	.063	.069	76	83
	30	.203	.192	.008	.008	.048	.041	.196	.196	74	74
0.3	100	.202	.226	.006	.006	.041	.041	.197	.222	74	84
Standard	10	.074	.262	.002	.003	.016	.052				
R.W.	0	.002	.004	.006	.004	.007	.007	-.004	-.001		
	10	.055	.052	.007	.008	.026	.026	.040	.036	54	49
	30	.198	.184	.007	.007	.044	.044	.191	.177	72	67
Standard	10	.086	.264	.002	0	.012	.045				
C.B.	0	.015	.007	.008	.005	.027	.010	.002	.001		
	10	.082	.086	.012	.010	.037	.037	.059	.064	69	74
	30	.223	.233	.012	.009	.058	.054	.210	.213	79	80
0.3	100	.210	.210	.008	.010	.044	.044	.203	.201	77	76
Standard	30		.266		.002		.046				
J.B.	0	.019	.030	.012	.017	.029	.043	.003	.006		
	10	.081	.076	.009	.009	.031	.023	.074	.067	88	80
	30	.227		.015		.061		.211		80	
0.3	100	.203	.218	.004	.005	.037	.040	.201	.215	76	81

Oestrone (cont.):

Urine or standard	Oestrone µg.	D_{604}		D_{606}		D_{601}		D_{604} corr.		Recovery %	
Standard	10 30		.272		.002		.053				
C.L.	0	.018	.029	.009	.013	.023	.049	.006	.007		
	10	.082	.080	.009	.008	.040	.037	.069	.068	82	81
	30	.219	.219	.009	.013	.048	.068	.211	.202	80	76
0.3	100	.195		.006		.043		.189		72	
Standard	10 30	.086	.260	0	0	.015	.044				
H.B.	0	.014	.020	.008	.009	.042	.042	-.003	.003		
	10	.089	.094	.013	.014	.063	.063	.067	.071	81	84
	30	.230	.234	.011	.011	.076	.076	.214	.217	81	82
0.3	100	.220	.226	.004	.004	.048	.048	.216	.222	82	84
Standard	30	.261		0		.054					
D.M.	0	.019	.011	.005	.0	.063	.054	-.001	-.002		
	30	.230	.243	.022	.019	.092	.079	.200	.220	80	87

Oestradiol:

Urine or standard	Oestradiol µg.		D_{604}		D_{606}		D_{601}		D_{604} corr.		Recovery %	
Standard	8	30	.056	.210	0	0	.008	.037				
	50		.326		.001		.078					
H.B.	0		.014	.012	.008	.008	.042	.044	-.003	-.005		
	8		.064	.060	.008	.008	.044	.040	.049	.046	89	84
	30		.184	.188	.010	.012	.074	.072	.166	.169	79	80
	50		.300	.294	.011	.011	.092	.090	.273	.278	79	81
Standard	8	30	.053	.210	0	0	.044	.040				
	50		.352		0		.068					
N.T.	0		.052	.033	.034	.020	.108	.066	-.001	.002		
	8		.089	.062	.019	.017	.075	.070	.059	.034	107	62
	30		.262	.255	.034	.032	.109	.108	.220	.215	105	102
	50		.380		.020		.124		.352		100	
Standard	8	30	.056	.212	0	0	.009	.045				
	50		.366		0		.072					
D.H.	0		.018	.022	.011	.013	.043	.044	-.001	.001		
	8		.076	.074	.013	.012	.056	.056	.055	.054	100	100
	30		.220	.222	.012	.013	.088	.090	.200	.200	95	95
	50		.350	.354	.015	.015	.106	.110	.328	.332	95	96
Standard	8	30	.054	.207	0	0	.027	.033				
	50		.370		.001		.081					
J.O.	0		.014	.012	.007	.006	.044	.044	-.002	-.004		
	8		.070	.068	.017	.017	.094	.094	.035	.033	64	60
	30		.204	.202	.007	.007	.066	.066	.192	.190	92	91
	50		.368	.360	.020	.019	.142	.140	.334	.328	97	95
Standard	30	50	.192	.338	.005	.006	.041	.067				
C.B.	0		.008	.008	.004	.002	.020	.015	0	.003		
	8		.056	.044	.004	.004	.024	.024	.049	.037	89	67
	30		.192	.178	.010	.009	.052	.044	.180	.169	86	81
	50		.304	.313	.013	.011	.070	.067	.292	.303	85	88
Standard	30	50	.208	.340	.005	.007	.044	.062				
R.W.	0		.014	.012	.011	.007	.036	.032	-.004	-.001		
	8		.050	.047	.012	.010	.034	.034	.034	.034	62	62
	30		.182	.188	.013	.013	.054	.054	.167	.173	80	83
	50		.262	.252	.020	.017	.069	.064	.242	.235	70	68
Standard	30	50	.200	.350	.005	.017	.034	.079				
C.B.	0		.015	.009	.007	.007	.032	.021	.002	-.002		
	8		.057	.058	.006	.006	.030	.030	.047	.048	85	88
	30		.191	.206	.012	.014	.053	.061	.178	.190	85	98
	50		.270	.296	.012	.012	.064	.064	.258	.286	75	83

Oestradiol (cont.):

Urine or standard	Oestradiol µg.		D_{604}		D_{606}		D_{601}		D_{604} corr.		Recovery %	
Standard	30	50	.231	.354	.004	.005	.038	.057				
J.B.	0		.028	.032	.013	.013	.048	.048	.007	.011		
	8		.072	.062	.013	.010	.042	.031	.054	.049	98	89
	30		.207		.012		.057		.194		92	
	50		.307	.320	.014	.014	.070	.073	.294	.307	85	89
Standard	30	50	.222	.340	.002	.005	.037	.070				
C.L.	0		.021	.026	.014	.015	.044	.051	.001	.002		
	8		.072	.072	.014	.013	.058	.058	.050	.050	91	91
	30		.210	.209	.013	.016	.068	.078	.194	.184	92	88
	50		.281	.307	.013	.017	.087	.097	.263	.285	77	83
Standard	8	30	.058	.216	0	0	.008	.046				
	50		.355		.002		.059					
H.B.	0		.015	.014	.007	.007	.036	.036	.001	0		
	8		.072	.068	.014	.014	.090	.090	.041	.037	75	67
	30		.210	.208	.009	.008	.076	.076	.194	.192	92	92
	50		.346	.350	.015	.015	.108	.110	.324	.328	94	95
Standard	30		.216		.003		.053					
D.M.	0		.035	.031	.021	.015	.077	.078	0	0		
	30		.210	.219	.022	.028	.089	.094	.181	.184	87	88

Partition after methylation by chromatography on one alumina column.

(Method 2.)

a) From dilute hydrochloric acid (30 ml. of concentrated hydrochloric acid in 200 ml. of water).

	D_{604}	D_{606}	D_{601}	Recovery %
Oestriol 30 µg.				
Standard	.203	0	.042	
Method	.170	0	.030	85
"	.170	0	.031	85
"	.173	0	.032	86
"	.170	0	.031	85
Oestrone 30 µg.				
Standard	.270	0	.048	
Method	.220	0	.037	80
"	.224	0	.037	82
"	.218	0	.035	80
"	.226	0	.038	82
Oestradiol 30 µg.				
Standard	.213	0	.041	
Method	.193	.005	.039	90
"	.195	.005	.040	92
"	.197	.006	.040	92
"	.180	.005	.034	85

b) From acid hydrolysed male urine (200 ml.)

Oestriol:

Urine or standard	Oestriol µg.	D_{604}		D_{606}		D_{601}		D_{604} corr.		Recovery %	
Standard	30	.178		.003		.037					
R.C.B.(i)	0	.048	.022	.019	.009	.100	.051	.009	.003		
	30	.173	.169	.019	.019	.080	.080	.145	.142	76	75
Standard	30	.187		.002		.036					
J.O.(i)	30	.232	.229	.047	.039	.128	.117	.174	.179	93	96
	30	.247	.212	.050	.037	.142	.110	.183	.165	98	88
Standard	30	.178		0		.025					
H.B.(i)	0	.028	.028	.012	.014	.039	.039	.010	.008		
	30	.186	.182	.014	.014	.070	.067	.166	.163	92	91
H.B.(ii)	30	.160	.153	.018	.008	.058	.029	.139	.147	77	82
	30	.150	.172	.014	.016	.054	.054	.133	.155	74	86
Standard	30	.183		0		.043					
J.B.(i)	0	.033	.033	.014	.014	.073	.076	.004	.004		
	30	.207	.207	.018	.018	.096	.100	.179	.177	99	98
Standard	30	.178		0		.032					
R.C.B.(ii)	0	.024	.020	.009	.006	.042	.038	.007	.006		
	30	.194	.194	.012	.010	.075	.077	.175	.176	97	98
Standard	30	.188		0		.041					
J.O.(ii)	0	.033	.033	.014	.014	.053	.050	.010	.010		
	30	.200	.204	.017	.019	.084	.089	.175	.176	93	93
Standard	30	.183		.005		.040					
J.O.(iii)	0	.031	.024	.016	.012	.062	.050	.004	.003		
	30	.183	.196	.014	.016	.079	.087	.161	.171	88	93
Standard	30	.197		0		.044					
J.B.(ii)	30	.189	.189	.018	.026	.097	.097	.159	.153	81	78
	30	.185	.177	.015	.011	.072	.072	.164	.159	83	81
Standard	30	.202		0		.062					
H.B.(iii)	30	.208	.215	.022	.024	.100	.104	.175	.180	87	89
	30	.220	.228	.016	.016	.080	.082	.197	.205	98	101

Oestrone:

Urine or standard	Oestrone µg.	D_{604}	D_{606}	D_{601}	D_{604} corr.	Recovery %
Standard	30	.277	.004	.056		
R.C.B.(i)	0	.021 .033	.010 .018	.043 .064	.003 .004	
	30	.247 .237	.015 .010	.088 .077	.225 .221	83 81
Standard	30	.263	0	.049		
J.O.(i)	30	.220 .229	.026 .022	.107 .100	.183 .197	70 75
	30	.228 .230	.025 .029	.111 .098	.191 .196	73 74
Standard	30	.260	0	.048		
H.B.(i)	0	.033 .033	.015 .015	.057 .057	.008 .008	
	30	.271 .268	.018 .016	.100 .098	.245 .244	93 93
Standard	30	.256	0	.052		
H.B.(ii)	30	.280 .263	.027 .015	.122 .092	.241 .241	94 94
	30	.263 .268	.022 .023	.103 .104	.233 .236	91 92
Standard	30	.268	0	.044		
J.B.(i)	0	.023 .025	.008 .010	.048 .048	.005 .005	
	30	.270 .270	.022 .022	.118 .118	.236 .236	89 89
Standard	30	.265	.004	.044		
R.C.B.(ii)	0	.022 .018	.012 .010	.044 .042	.002 0	
	30	.225 .242	.013 .012	.073 .075	.208 .226	79 86
Standard	30	.260	.003	.046		
J.O.(ii)	0	.033 .035	.015 .018	.058 .063	.008 .006	
	30	.262 .257	.033 .032	.138 .131	.214 .211	82 81
Standard	30	.278	.007	.060		
J.O.(iii)	0	.030 .028	.017 .019	.058 .058	.003-.001	
	30	.235 .229	.021 .018	.093 .093	.206 .202	73 74
Standard	30	.287	0	.052		
J.B.(ii)	30	.290 .287	.019 .020	.120 .128	.258 .252	90 88
	30	.285 .289	.016 .015	.118 .110	.256 .264	89 92
Standard	30	.290	0	.052		
H.B.(iii)	30	.282 .306	.035 .035	.170 .170	.224 .249	77 86
	30	.290 .300	.026 .026	.130 .132	.250 .260	86 90

Oestradiol:

Urine or standard	Oestradiol µg.	D_{604}	D_{606}	D_{601}	D_{604} corr.	Recovery %
Standard	30	.197	.010	.046		
R.C.B.(i)	0	.069 .041	.043 .029	.135 .091	.003 -.004	
	30	.235 .235	.037 .033	.129 .129	.184 .187	92 94
Standard	30	.202	0	.027		
J.O.(i)	30	.271 .254	.039 .038	.137 .137	.218 .201	108 100
	30	.252 .252	.039 .039	.130 .136	.201 .200	100 99
Standard	30	.200	0	.028		
H.B.(i)	0	.048 .048	.023 .023	.076 .072	.012 .013	
	30	.243 .240	.025 .025	.106 .108	.208 .204	104 102
Standard	30	.217	0	.039		
H.B.(ii)	30	.274 .213	.044 .020	.162 .092	.211 .199	97 92
	30 60	.238 .400	.028 .029	.112 .132	.185 .365	85 84
Standard	30	.201	0	.033		
J.B.(i)	0	.056 .054	.031 .030	.112 .110	.005 .004	
	30	.260 .262	.038 .038	.145 .148	.206 .207	103 103
Standard	30	.200	.004	.038		
R.C.B.(ii)	0	.042 .046	.024 .030	.082 .086	.004 .002	
	30	.242 .248	.032 .034	.125 .128	.197 .201	99 100
Standard	30	.190	.004	.033		
J.O.(ii)	0	.093 .097	.060 .066	.148 .154	.011 .009	
	30	.249	.035	.148	.195	98
Standard	30	.190	.007	.039		
J.O.(iii)	0	.058 .081	.036 .055	.119 .142	.001 .004	
	30	.239 .219	.059 .040	.167 .131	.161 .164	85 86
Standard	30	.218	.003	.035		
J.B.(ii)	30	.241 .250	.032 .033	.110 .121	.200 .205	92 94
	30	.249 .247	.033 .029	.111 .111	.207 .208	95 95
Standard	30	.200	0	.044		
H.B.(iii)	30	.276 .268	.048 .045	.203 .172	.199 .201	100 100
	30	.250 .242	.036 .028	.146 .140	.196 .196	98 98

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SOME OBSERVATIONS ON THE KOBER COLOUR AND FLUORESCENCE REACTIONS OF THE NATURAL OESTROGENS

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SUMMARY

The two-stage Kober reaction with oestriol, oestrone and oestradiol-17β has been investigated. The factors involved in the maximum production of colour are summarized.

Reducing agents were important in both the first and second stages of the Kober reaction. Oestriol in small amounts did not form the red Kober colour when reducing agents were not present in the first stage.

In the absence of water, sulphuric acid did not give optimal results in the first stage. Oestradiol-17β failed to give the Kober colour reaction in the presence of high concentrations of sulphuric acid. Optimal sulphuric acid-water ratios differed for the three oestrogens and were 76 % sulphuric acid for oestriol, 66 % acid for oestrone and 60 % acid for oestradiol-17β. The development of fluorescence was similarly affected by sulphuric acid concentration.

The phenol-sulphuric acid reagent used by many workers appears to owe its efficacy to the action of the phenol, partly as a reducing agent and partly as a diluent of the sulphuric acid.

Oestriol reacted in the first stage at a slower rate than oestrone and oestradiol.

Further water and heating was usually required after the first stage (i.e. for the second stage of the Kober reaction) for complete formation of the red colour. Maximum intensity and stability of the red colour in the second stage was obtained in the presence of 50–60 % sulphuric acid and water. Under these optimum conditions the second heating time was not critical.

In the second stage, concentrations of sulphuric acid lower than 50 % caused instability of the colour and fading during heating.

In the second stage of the Kober reaction, reducing agents with oxidation-reduction potentials of the order of the hydroquinone-quinhydrone couple were also required for the production of maximum density and stability of the red colour.

A colour method based on these findings is presented.

Marrian [1930] reported that when oestriol was warmed with concentrated sulphuric acid, an orange colour with a green fluorescence was produced. Kober [1931] noted that when this orange fluorescent solution was diluted with water and warmed, the colour changed to a clear red, also with a greenish fluorescence, and that this change appeared to be almost specific for the natural oestrogens. Phenol added to the reaction mixture quenched the final fluorescence making the red colour both more intense and suitable for visual colorimetry. This colour reaction is known generally as the Kober reaction, although many modifications have since been proposed.

Kober's original method was thus carried out in two stages, the first being the formation of the orange (or under certain conditions, yellow) intensely fluorescing colour and the second the conversion of this to the red colour. The intense greenish fluorescence obtained in the first stage of the Kober colour reaction has been used by a number of workers [Jailer, 1947; Bates & Cohen, 1947], for the fluorimetric estimation of the natural oestrogens. These fluorimetric methods, though not highly specific, are very sensitive and for this reason are attractive for the estimation of the small amounts of oestrogens which occur in non-pregnancy urine.

In general, neither these colorimetric nor fluorimetric methods have been completely satisfactory. It was therefore felt that a thorough reinvestigation of the Kober reaction might lead to better knowledge of the factors concerned in the production of fluorescence and colour and that this knowledge might suggest improved methods for determining the natural oestrogens both in pure form and in extracts from biological material. This paper is a report of a study, using pure oestrogens, of the many variable factors concerned in the Kober reaction and describes a number of phenomena which previously do not seem to have been fully appreciated.

EXPERIMENTAL

Materials and apparatus

Pure crystalline oestriol, oestrone and oestradiol-17β* were used throughout these experiments. They were dissolved in redistilled 95% ethanol (5 mg./100 ml.) and the appropriate volumes added to $6 \times \frac{3}{4}$ in. Pyrex test-tubes, from which the ethanol was then removed under nitrogen by heating at reduced pressure. (More consistent results were obtained following this anaerobic procedure than when the ethanol was removed by heating in a stream of air.) No special precautions were taken to prevent entrance of water vapour or air during colour or fluorescence development. Throughout this paper, the term 'heating' implies 'heating in a boiling water bath'. All reagents were of A.R. quality.

Colour densities were measured against a reagent blank in a Spekker absorptiometer using 1 cm. cuvettes. Ilford spectrum green no. 603 or no. 604 light filters were used, according to the wave-length of maximum absorption which varied with the reagent used for developing the colour.

Density-wave-length curves were measured in a Unicam diffraction grating spectrophotometer.

Fluorescence intensities were measured in a Spekker fluorimeter against a standard developed from oestrone. Reagent blanks were usually negligible. The primary light filter was a Hilger type OB1 blue transmitting maximally at about 430 mμ. The secondary filter was a yellow Corning no. 3486 filter opaque to blue light.

The influence of phenol and ethanol in the Kober reaction

Until recently, it has been a general practice to employ phenols in colour methods based on the Kober reaction. However, Cohen & Bates [1947] published a method for the development of the Kober colour in which no phenol was added. Their procedure was carried out in two stages. In the first stage, the oestrogen dissolved in 0·4 ml. of absolute ethanol was heated with concentrated sulphuric acid without removal of the ethanol. In the second stage, dilute sulphuric acid was added and the solution was heated again. Their final colours had densities similar to those obtained by using Kober's phenol-sulphuric acid reagent. From this they suggested that the increase in colour density in the presence of phenol, reported by Kober, was only visually apparent owing to the quenching of the green fluorescence, and that it was not a real increase when measured photometrically.

Preliminary experiments in this laboratory showed that when the procedure of

* This nomenclature follows that used by Fieser & Fieser [1949] and replaces the older term 'α' oestradiol.

Cohen & Bates was applied to small amounts of pure oestriol in the absence of ethanol, no red colour was obtained. It was also found that water added with the sulphuric acid for the first stage caused increased colour densities, and that the sulphuric acid diluent recommended by Cohen & Bates was not optimal. These latter findings will be considered in more detail below. Experiments in which the quantities of ethanol and water added to the reaction mixture were varied are summarized in Fig. 1, which shows the relation between red colour density and ethanol concentration, other factors being optimal. The first stage of the reaction was carried out by adding to

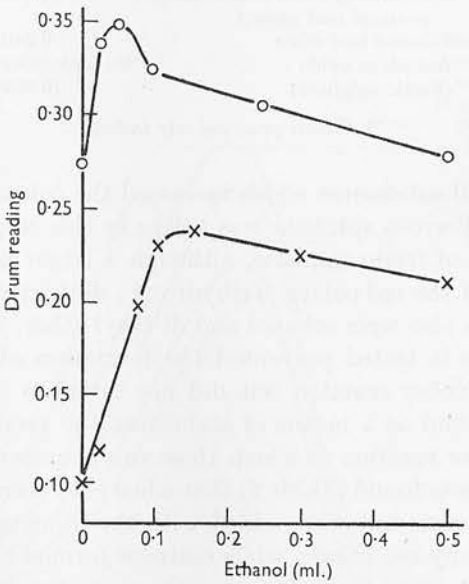

Fig. 1. The influence on Kober colour production of varying amounts of ethanol added to oestriol and oestrone and heated with the sulphuric acid in the first stage of the colour reaction. ×—× oestriol, 50 μg; ○—○ oestrone, 50 μg.

50 μg. of oestrogen the ethanol, then a measured amount of water, and finally 3 ml. of concentrated sulphuric acid. The mixture was heated for 20 min., further water was added for the second stage—and the mixture heated another 4 min. After cooling, the solution was diluted to 15 ml. with 30 % (v + v) sulphuric acid and the density was read in the absorptiometer using Ilford green no. 603 light filters. In the absence of ethanol, oestriol did not form any red colour; small amounts of ethanol allowed the formation of red colour, but large amounts increased the rate of fading of the final colour. Oestrone gave the red colour even in the absence of ethanol, but the colour was more intense in the presence of small amounts of ethanol.

A search was made for other substances which would similarly enhance the Kober colour. Substances investigated included other organic hydroxyl compounds stable to sulphuric acid. Since many of these have reducing properties, some reducing agents and their oxidized forms were also investigated, of which Table 1 lists a few. The investigation was made by adding a small amount of the test substance to 50 μg. of oestrone and then adding sulphuric acid. Colour was developed using approximately optimum conditions.

Table 1. *The intensity of red colour produced with oestrone* (50 μg.) *when certain 'reducing' agents were added to the sulphuric acid in the first stage of the Kober reaction. The reaction conditions were the same in all cases, though not optimal*

'Reducing' agent	Drum reading (Ilford no. 603 filter)
None	0·280
Catechol and hydroquinone	0·405
Ferrous sulphate	0·389
β-Naphthol	0·381
α-Naphthol, cresol (*o*-, *m*- and *p*-) guaiacol and phenol	0·350
Ethanol and ether	0·340
Arsenious oxide	No pink colour formed
(Ferric sulphate)	(0·092)*

* Colour progressively fading.

It was found that all substances which increased the colour density had potential reducing properties. Ferrous sulphate was active in this respect even when it contained small amounts of ferric sulphate, although a larger proportion of the latter caused rapid fading of the red colour. Trihydroxy-, dihydroxy- and mono-hydroxy-phenols were active as also were ethanol and di-ethyl ether. Arsenious oxide and all stronger reducing agents tested prevented the formation of the red colour in the second stage of the Kober reaction but did not interfere in the first stage. This finding has proved useful as a means of stabilizing the production of fluorescence. The stage of the Kober reaction at which these compounds exerted their effect was next investigated. It was found (Table 2) that when they were omitted from the first stage of colour production but were added with the diluting water for the second, oestriol did not form any red colour, while oestrone formed almost the same density of colour as when the agent was added for the first stage. It appears that when oestriol is heated with sulphuric acid in the absence of reducing agents a yellow colour forms which does not undergo the second stage of the Kober reaction.

Table 2. *The influence of a reducing agent added at various stages of the Kober reaction upon the final intensity of colour*

Stage of addition of reducing agent (hydroquinone 60 mg.)	Drum reading	
	Oestrone (50 μg.)	Oestriol (50 μg.)
In sulphuric acid for the first stage	0·378	0·289
In water for the second stage	0·364	0·120
In 30 % sulphuric acid for final dilution	0·275	0·116
None added	0·276	0·116

The influence of the sulphuric acid-water ratio in the second stage of the Kober reaction

The second stage of the Kober reaction is usually performed by diluting the products of the first stage with water and heating, during which process the characteristic red colour appears. Kober, in his original method, added 0·2 ml. of water for each 0·2 ml. of phenol-sulphuric acid used in the first stage. These proportions have been varied somewhat by subsequent workers but Cohen & Bates [1947] were

the first to investigate this factor thoroughly. They heated oestrone with 2 ml. of concentrated sulphuric acid for the first stage and then added 8 ml. of a series of dilutions of sulphuric acid and water before heating for the second stage. Diluents which contained more than 50 % (v + v) sulphuric acid did not permit the formation of any red colour from the yellow colour formed in the first stage, whereas those containing 25 % (v + v) or less of sulphuric acid allowed a complete transition from yellow to red. This change was followed with a spectrophotometer which showed the transition by a shift in the wavelength of maximum light absorption from 460 to

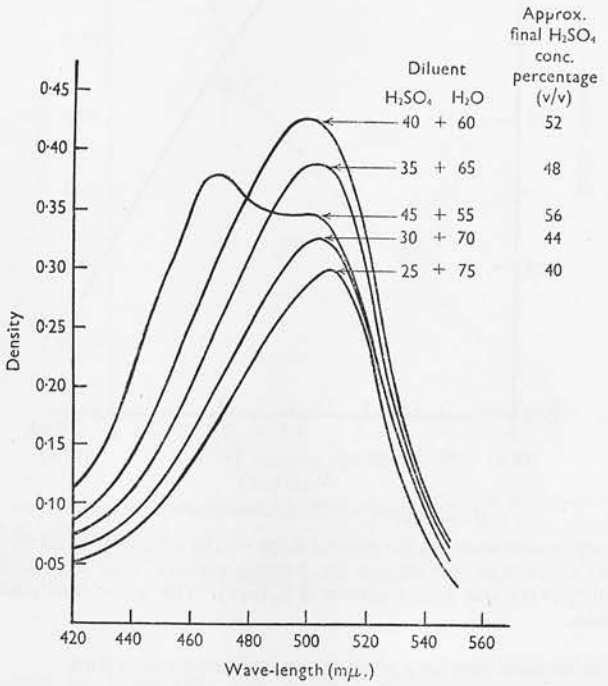

Fig. 2. The influence of sulphuric acid concentration at the time of second heating upon colour formation with oestrone (50 μg.), showing the spectral characteristics of the colours produced.

505 mμ. On the basis of these results, Cohen & Bates chose 25 % (v + v) sulphuric acid for their diluent at the second stage, but did not study diluents with sulphuric acid concentrations between 25 and 50 %. This sulphuric acid range has now been investigated using their procedure (Fig. 2). The density of red colour increased as the sulphuric acid concentration of the diluent was increased to 40 % (v + v), but decreased thereafter owing to incomplete conversion of the yellow colour. The important point appeared to be the final sulphuric acid-water ratios. For most systems the optimum for this was found to be between 50 and 60 % (v + v) sulphuric acid-water, which is higher than that recommended by most workers.

In the subsequent work during the present investigation the amount of water required for maximum colour production in the second stage was always determined as the first step in the examination of any new set of conditions. An example is shown in Fig. 3 which summarizes the results of an investigation of a phenolic reagent. The

reagent was prepared by dissolving hydroquinone (2 % w/v) in 76 % (v/v) sulphuric acid and water. The first heating time was 20 min. during which a considerable conversion to red colour had already occurred.

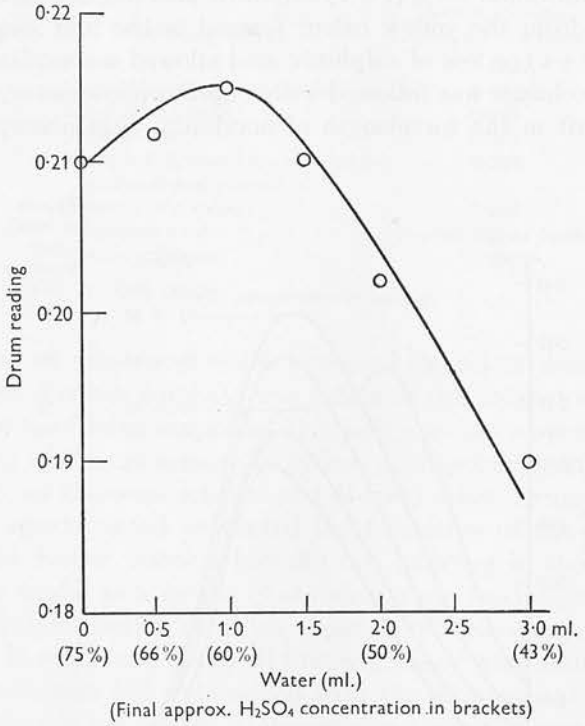

Fig. 3. The effect of varying the water in the second stage on the intensity of colour produced by oestriol (30 μg.). The first stage was carried out by heating oestriol with a reagent (4 ml.) containing sulphuric acid 76 % (v/v) and hydroquinone 2 % (w/v). The water was added and heating was continued for 4 min.

The influence of the second heating time upon colour production

In many published modifications of the Kober colour reaction the duration of heating at the second stage has been stated to be critical. This has largely been due to the use of sulphuric acid concentrations in the second stage which were not optimal. When the correct amount of water is added, the red colour, once formed, is remarkably stable, and the heating time is no longer critical. Fig. 4 shows the effect of the second heating time upon colour production with oestriol using a reagent containing hydroquinone (2 % w/v) and sulphuric acid (76 % v/v). The upper curve shows the time reaction with an optimal amount of water (1·0 ml.) and the lower curve with an excess of water (3·0 ml.). Evidently excess water prevents the red colour from reaching its maximum intensity, and accelerates fading. To obtain maximum colour under these conditions the second heating would require careful timing.

Dilution of the red colour for colorimetry

Dilution of the Kober red colour after development is frequently desirable for convenience in colorimetry. For instance, Kober [1931] diluted his red colour with water, but later [1938] used 65 % sulphuric acid. Cohen & Marrian [1934] used 5 %

sulphuric acid and Venning, Evelyn, Harkness & Browne [1937] used 10% acid. From the work described in the preceding sections on the optimum sulphuric acid concentration for the second stage, it would appear that a diluent containing between 50 and 60% (v+v) sulphuric acid ought to be the best. In practice, diluting with 60% sulphuric acid did give complete stability to the final red colour.

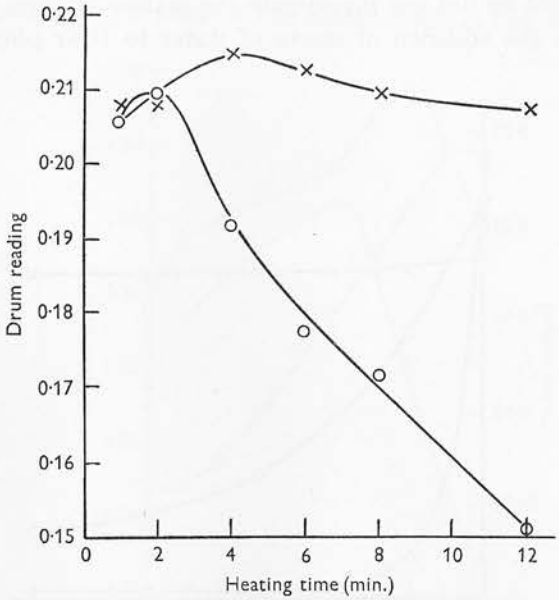

Fig. 4. The effect of varying the heating time and the amount of water added in the second stage upon the intensity of Kober colour produced with oestriol (30 μg.). The first stage was carried out by heating for 20 min. the oestriol with a reagent (4 ml.) containing sulphuric acid 76 % (v/v) and hydroquinone 2% (w/v). ×—× optimum water added (1·0 ml.); ○—○ excess water added (3·0 ml.).

Such permanency is, however, not always desirable. For instance, Stevenson & Marrian [1947] described a method of correcting for interfering chromogenic material when the colour method is applied to urine extracts. They heated the final red solution obtained by the procedure of Venning *et al.* [1937] and thereby caused complete fading of red colour due to oestrogens, whilst the colour due to other urinary material remained practically unchanged. The influence of the final sulphuric acid concentrations upon the rate at which the oestrogen colour fades during heating has now been examined. Fig. 5 shows the influence of the sulphuric acid concentration of the diluent upon the rate of fading at 100° C. of colour produced with oestriol by a hydroquinone (2% w/v) sulphuric acid (76% v/v) reagent. Diluted with 60% (v+v) sulphuric acid the colour was practically stable to heating, whereas diluting with 30 % (v+v) sulphuric acid caused considerable fading in 2 hr., and diluting with 10 % (v+v) sulphuric acid caused complete fading in 40 min.

It is easy therefore to select a diluent which contains a sulphuric acid concentration which will confer sufficient stability upon the final diluted colour and yet allow this fading correction to be used.

The influence of the sulphuric acid-water ratio in the first stage of the Kober colour reaction, determined colorimetrically after the second stage

It has been known for some time that water affects the first stage of the Kober reaction. For instance, Cohen [1936] showed that the addition of water to the sulphuric acid in the first stage had an enhancing effect on the colour density similar to that of phenol, but he did not investigate the matter further. Cohen & Marrian [1934] showed that the addition of traces of water to their phenol-sulphuric acid

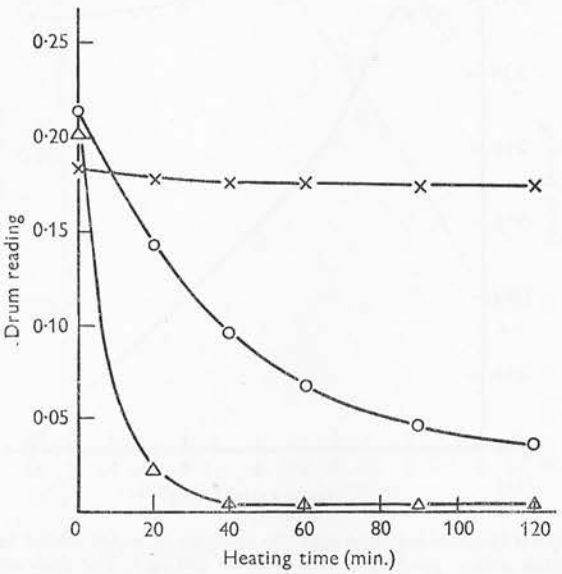

Fig. 5. The influence of the final diluent upon the fading of colour at 100° C. The Kober colour was produced with oestriol (30 μg.) under optimum conditions using a reagent containing sulphuric acid 76 % (v/v) and hydroquinone 2 % (w/v). Before fading, the colour was diluted to 15 ml. with dilutions of sulphuric acid. ×—× diluent 60 % (v+v) sulphuric acid; ○—○ diluent 30 % (v+v) sulphuric acid; △—△ diluent 10 % (v+v) sulphuric acid.

reagent in the first stage of the Kober reaction decreased the colour produced from oestriol. Salter, Humm & Oesterling [1948] described a satisfactory phenol-sulphuric acid reagent in which phenol was partly substituted by water. However, up till now a more complete investigation of the water effect in the first stage of the Kober reaction has not been reported.

This has now been examined for a number of colour reagents and, as an example, results with a reagent containing 2 % (w/v) hydroquinone in sulphuric acid are described.

Reagents were prepared by dissolving 2 g. of hydroquinone in 100 ml. of various dilutions of sulphuric acid and water. Colour reactions were performed with 30 μg. of oestriol, oestrone or oestradiol-17 β. The amount of reagent added was 4 ml. and the heating time for the first stage was 20 min. For the second stage the products of the first stage were diluted with water so that the final acid concentration was approximately 60 % (v/v), and the mixture was heated for 4 min. The reaction mixture was cooled and diluted to 15 ml. with 30 % (v+v) sulphuric acid for

colorimetry. The density of colour produced with each of the three oestrogens plotted against the sulphuric acid concentration of the reagent is shown in Fig. 6. Although the responses of the three oestrogens were similar, the optimum sulphuric acid concentration in the first stage was different for each oestrogen. For oestriol this was approximately 76 % (v/v) acid, for oestrone 65 % acid and for oestradiol-17β 60 % acid. Oestriol gave rapidly decreasing intensities of red colour when the sulphuric acid concentration in the first stage of the Kober reaction was decreased below 73 %.

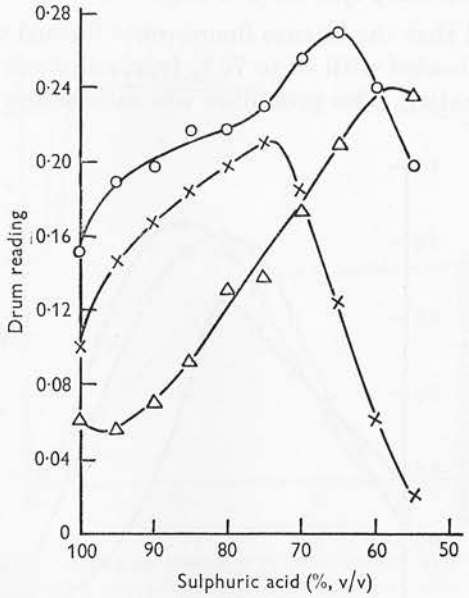

Fig. 6. The influence of the sulphuric acid-water ratio in the first stage of the Kober reaction upon the intensity of colour. The reagent was hydroquinone-sulphuric acid-water and other conditions were optimal. ×—× oestriol, 30 μg.; ○—○ oestrone, 30 μg.; △—△ oestradiol-17β, 30 μg.

Oestrone produced intense colours over a wide range of sulphuric acid concentrations. Oestradiol-17β did not form a visible red Kober colour in the second stage when the concentration of sulphuric acid was greater than 76 % in the first stage. These differences in response to the sulphuric acid concentrations in the first stage are probably the main cause of the different properties of the various Kober reagents which have been described in the past. For instance, the addition of water in the first stage of the reaction to the phenol-sulphuric acid reagent described by Cohen & Marrian [1934], caused a decrease in colour with oestriol but increased the colours produced by oestrone and oestradiol-17β. This phenol-sulphuric acid reagent, which contains almost as much phenol as sulphuric acid, behaves similarly to a reagent containing hydroquinone in 74 % sulphuric acid and water. It appears that the phenol acts both as a reducing agent and also as a diluting agent with the same effect as water in the first stage of the Kober reaction. Szego & Samuels [1940] described a reagent consisting of guaiacol dissolved in concentrated sulphuric acid, which gave no red colour with oestradiol-17β (although it permitted development of the initial yellow colour), presumably because its sulphuric acid concentration was too high.

Oestradiol-17β forms both the yellow and the red Kober colours with lower

concentrations of sulphuric acid than do oestriol and oestrone. It is thus possible that the response of oestradiol-17β to such agents as succinic anhydride and phthalic anhydride, containing antimony trichloride as described by Clark & Thompson [1948] might really be variations of the Kober reaction in which these weaker agents replace the sulphuric acid.

The influence of the sulphuric acid-water ratio in the first stage of the Kober reaction, determined fluorimetrically after the first stage

Jailer [1947] showed that the intense fluorescence formed when oestriol, oestrone or oestradiol-17β was heated with 60 to 70 % (v/v) sulphuric acid, could be used to measure their concentration. This procedure was satisfactory as a method of deter-

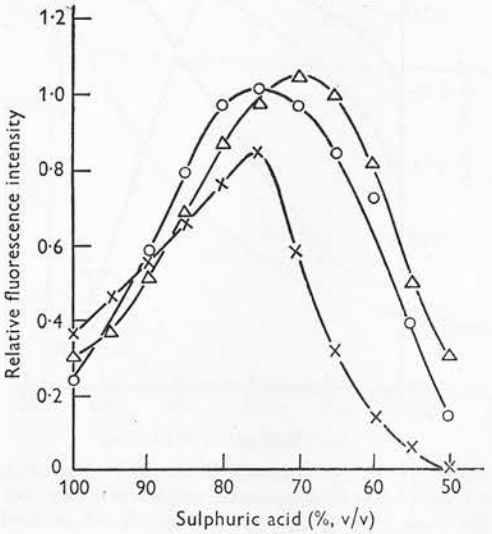

Fig. 7. The influence of sulphuric acid concentration upon the intensity of fluorescence in the first stage of the Kober reaction. ×—× oestriol, 4 μg.; ○—○ oestrone, 4 μg.; △—△ oestradiol-17β, 4 μg.

mination for oestrone and oestradiol-17β but was not sufficiently sensitive for oestriol. Bates & Cohen [1947] also described a fluorimetric method giving a more intense fluorescence with oestriol in which, however, the reagent was 90 % (v+v) sulphuric acid. In a later paper, Bates & Cohen [1950] further investigated the effect of sulphuric acid concentration on fluorescence production and, using oestrone, showed that between 70 and 85 % sulphuric acid concentration gave optimum results, but they did not report experiments to show that this also applied to oestriol and oestradiol-17β. They recommended dissolving the oestrogen in ethanol, water or toluene before adding the sulphuric acid because better duplicate results were thus obtained.

The effect of sulphuric acid concentration upon production of fluorescence by all three oestrogens has now been examined with the results shown in Fig. 7. Reducing agents in the form of arsenious acid and traces of catechol were added to the systems since these agents had been found to increase the fluorescence produced by oestriol and to make all fluorescence intensities more reproducible, and were superior in this respect to the solvents used by Bates & Cohen. These findings will be discussed in

more detail in a later paper. The relation of fluorescence intensity to sulphuric acid concentration followed a similar pattern to that of the colour densities. The optimum sulphuric acid concentrations (v/v) were 76 % for oestrone and oestriol and about 70 % for oestradiol-17β.

With sulphuric acid concentrations above 76 %, oestradiol-17β fluoresced with a bluish-white fluorescence instead of the more usual green. Umberger & Curtis [1949] showed that at these high sulphuric acid concentrations oestradiol-17β forms

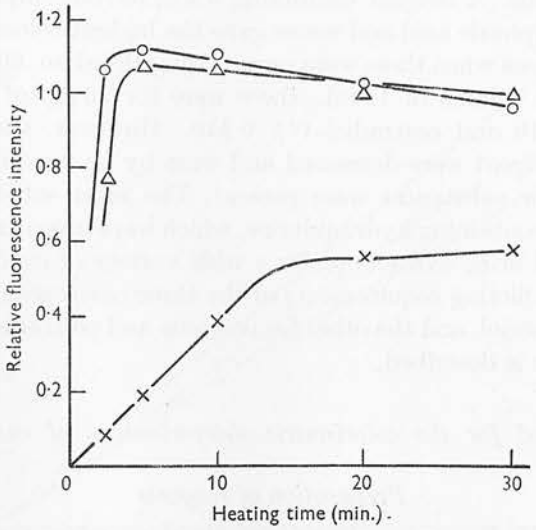

Fig. 8. The influence of heating time upon intensity of fluorescence. The reagent was sulphuric acid 68 % (v/v), arsenious acid 0·2 % (w/v), catechol 0·002 % (w/v). ×—× oestriol, 4 μg.; ○—○ oestrone, 4 μg.; △—△ oestradiol-17β, 4μg.

a yellow colour which absorbs light maximally at a wave-length of 430 mμ., whereas the colour usually formed in the first stage of the Kober reaction absorbs maximally at 460 mμ. It has been noted in the present investigation that oestradiol-17β does not readily form the red colour in the second stage of the Kober reaction when high concentrations of sulphuric acid are used in the first. It would therefore appear that the yellow pigment of Umberger & Curtis is the one which fluoresces with a bluish-white fluorescence and that it does not undergo the change to red in the second stage of the Kober reaction.

The influence of the heating time in the first stage of the Kober reaction

The formation of the yellow fluorescent colour in the first stage of the Kober reaction is a time reaction which depends upon a number of factors. These include temperature of heating and sulphuric acid concentration. It is well known that oestriol reacts at a slower rate than oestrone and oestradiol.

In this work the influence of the first heating time has been examined for each colour or fluorescence system studied. Fig. 8 records such a study and shows the rate of development of fluorescence in the first stage with oestriol, oestrone and oestradiol-17β and a reagent containing 68 % (v/v) sulphuric acid. The order of the

rates of reaction is typical. At this sulphuric acid concentration the fluorescences formed by oestrone and oestradiol-17β had reached their maximum in 5 min., while that formed by oestriol was still increasing after 20 min. heating.

Reagents for the optimum production of Kober colour

Sulphuric acid reagents containing hydroquinone, catechol, phenol, *p*-cresol, β-naphthol and ferrous sulphate have been investigated in the search for improved Kober colour methods. A reagent containing 0·2 % ferrous sulphate and a trace of ferric sulphate in sulphuric acid and water gave the highest colour densities recorded for the three oestrogens when these were pure. Using Ilford no. 603 light filters, 1 cm. cuvettes and a final volume of 15 ml., these were for 50 μg. of oestrogen: oestriol 0·400, oestrone 0·510 and oestradiol-17β 0·440. However, the colour intensities produced by this reagent were decreased and were by no means reproducible when mere traces of other substances were present. The most satisfactory reagents in practice were those containing hydroquinone, which have given excellent results with pure oestrogens and urine extracts under a wide variety of conditions.

To satisfy the conflicting requirements of the three oestrogens, two reagents were prepared, one for oestriol, and the other for oestrone and oestradiol-17β. A procedure using these reagents is described.

An improved method for the colorimetric determination of oestriol, oestrone and oestradiol-17β

Preparation of reagents

Oestriol reagent. Hydroquinone (2 g.) was dissolved with warming in 100 ml. of 76 % (v/v) sulphuric acid, prepared by diluting, and cooling, 76 ml. of pure sulphuric acid to 100 ml. with water.

Oestrone and oestradiol-17β reagent. Hydroquinone (2 g.) was dissolved similarly in 100 ml. of 65 % (v/v) sulphuric acid.

These reagents kept well at room temperature. A certain maturing took place in the first week during which the solutions darkened to a light brown colour. After this the reagents were stable and colour densities formed by them were reproducible.

Sulphuric acid 30 % ($v+v$). 30 ml. of conc. sulphuric acid added to 70 ml. of water.

Colour production

Four ml. of the appropriate reagent were added to the oestrogens in tubes and heated for 20 min. in a boiling water bath. The tubes were shaken once during the first 5 min. (notes 1 and 2). After heating, the tubes were cooled in ice water for about 5 min. (note 3). One ml. of water was added to the oestriol tubes and 0·5 ml. of water to the oestrone and oestradiol tubes (note 4). After mixing, the tubes were heated for 5 min. (note 2) and then cooled in ice water for about 5 min. (note 3). The contents were diluted to 15 ml. with 30 % (v + v) sulphuric acid. The tubes were allowed to stand at room temperature for at least 5 min. and were read in the colorimeter within 45 min. of dilution. Colours absorbed light maximally at wave lengths between 515 and 520 mμ.

Notes on the hydroquinone colour method

Note 1. Small amounts of water did not interfere so that, during heating, no precautions were required to prevent entrance of water vapour.

Note 2. The heating times were not critical. For oestriol the first heating time could be varied between 15 and 45 min. and for oestrone and oestradiol-17β, between 10 and 30 min., without appreciable change in colour density.

Note 3. At these stages reaction products were almost indefinitely stable.

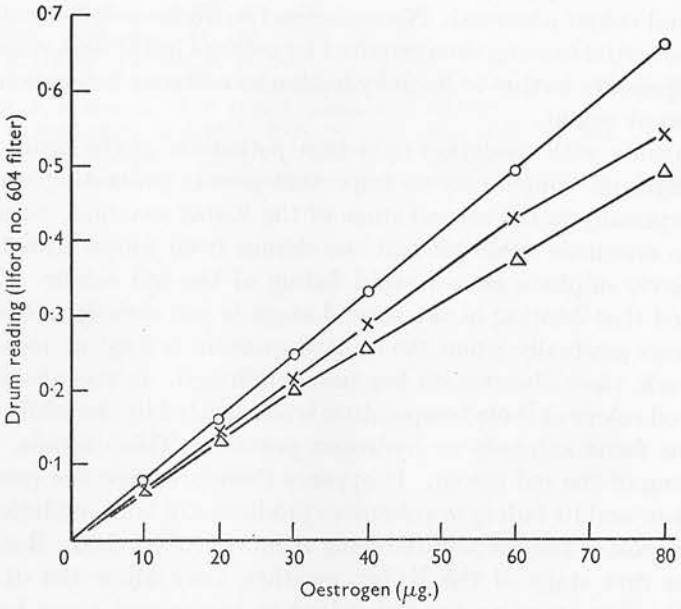

Fig. 9. Relationship between concentration of oestrogen and colour density using the hydroquinone-sulphuric acid reagents. ×—× oestriol; ○—○ oestrone; △—△ oestradiol-17β.

Note 4. The water required for the maximum colour production was not critical. For oestriol the water added could be varied between 0·5 and 1·5 ml. and for oestrone and oestradiol-17β the addition of water and second heating could be omitted without significant change in colour densities.

This method gave good correspondence between the amount of oestrogen present and the density of colour produced (Fig. 9).

DISCUSSION

While this work does not furnish sufficient data to warrant the presentation of a theory concerning the actual mechanism of the Kober reaction, there are a few features which should be recorded.

Any theory which attempts to explain the mechanism of the Kober reaction must account for the extreme intensity of the colour produced. This is so great that as little as 10 μg. of oestrogen in a final volume of 15 ml. can be estimated accurately in the above procedure. The fluorescence produced in the first stage is also extremely intense, being approximately twice that given by an equal weight of sodium fluorescein, so that the system must rank among the most strongly fluorescent known.

Optimum results in the first stage of the Kober reaction occur with mixtures of sulphuric acid and water. The sulphuric acid concentration which produces maximum colour is approximately 76 % for oestriol, 66 % for oestrone and 60 % for oestradiol-17β. Since these mixtures of sulphuric acid and water give better results than concentrated sulphuric acid alone, it is unlikely that dehydration or sulphonation are important processes in the Kober reaction. It is possible that the sulphuric acid may act as an isomerizing agent causing double bond or ring rearrangement within the molecule and thereby gives rise to resonant structures which produce the intense fluorescence and colour observed. Nevertheless the higher sulphuric acid concentration and longer initial heating time required by oestriol in the first stage of the Kober reaction may possibly be due to its dehydration to oestrone before conversion to the yellow fluorescent colour.

Reducing agents with oxidation-reduction potentials of the order of the hydroquinone-quinhydrone couple play an important part in promoting maximum colour production, especially in the second stage of the Kober reaction. Stronger reducing agents such as arsenious oxide prevent the change from yellow to red in the second stage while ferric sulphate causes rapid fading of the red colour. Cohen & Bates [1947] recorded that heating in the second stage is not strictly necessary since the red colour forms gradually when the diluted mixture is kept at room temperature and in this work, their observation has been confirmed. It was also found that the formation of red colour at room temperature is accelerated by the addition of oxidizing agents such as ferric sulphate or hydrogen peroxide. These agents, however, also cause the fading of the red colour. It appears therefore, that the production of the Kober red colour and its fading to colourless products are both oxidation phenomena. If this is so, the role of the effective reducing agents becomes clear. Besides protecting oestriol in the first stage of the Kober reaction, they allow the oxidation of the products of the first stage to the red colour in the second stage but prevent the further oxidation of this to colourless products. In other words, the reducing agents and their oxidized forms may 'poise' the reaction conditions so that the red colour can form by oxidation without simultaneous destruction, by still further oxidation.

The role of water in the second stage is obscure. For any given method of colour development the three oestrogens have identical water requirements in the second stage. On the basis of the oxidation theory a possible explanation might be the effect of decreased acid concentration on the oxidation-reduction system. It would be reasonable to expect that the oxidation-reduction potential of the change from yellow to red would depend upon acid concentration. A decrease in acid concentration should increase the reduction potential and thereby increase the tendency of the yellow colour to oxidize to the red colour. This theory is supported by the finding that the more strongly the oxidation-reduction system is poised, the less critical are the water requirements in the second stage of the reaction.

The author wishes to thank Prof. G. F. Marrian for his interest in the work and Mr H. A. F. Blair for his skilled technical assistance. The oestriol and oestradiol-17β were gifts to Prof. G. F. Marrian from Dr O. Kamm, Parke Davis, Detroit and Charles and E. Frosst and Co., Montreal.

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