CCXII. THE APPLICATION OF THE KOBER TEST TO THE QUANTITATIVE ESTIMATION OF OESTRONE AND OESTRIOL[1] IN HUMAN PREGNANCY URINE.

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It is well known that both oestrone and oestriol when warmed with concentrated sulphuric acid give a green-fluorescing orange-coloured solution, thus resembling sterols and the bile acids. The colours given by the latter compounds are discharged by dilution with water, but as Kober [1931] showed, the oestrin colour on dilution with water is changed to a clear red, the green fluorescence being retained. His evidence suggested that this test was highly specific for oestrin. Kober also showed that the addition of phenol to the sulphuric acid eliminated the green fluorescence from, and intensified the red colour of, the finally diluted solution. He stated that under the conditions of his procedure, the intensity of the red colour bore a direct but not linear relationship to the amount of oestrin, and he claimed that by the use of this test it was possible to assay the oestrin content of impure extracts.

The obvious desirability of a rapid chemical method for the quantitative determination of the oestrin content of human urines led the authors to investigate the value of the Kober test for such a purpose.

Experimental.

Modifications introduced into the Kober technique.

Kober suggested the following technique. About 5γ of crystalline hormone (presumably oestrone) are heated to 100° for 2 minutes with 0·2 ml. of a mixture of equal parts[2] of concentrated sulphuric acid and phenolsulphonic acid (*o*-+*p*-). The mixture is then cooled, and after the addition of 0·2 ml. of water is heated momentarily to boiling. Finally the solution is made up to 1 ml. with water and immediately cooled. After standing in the cold for 5–10 minutes, the colour is compared with that of a standard solution of acid cresol red (10 mg. in 100 ml.) in a colorimeter. Kober states that 5γ of the hormone give a colour of the same intensity as that of 25γ of cresol red.

Since the greater part of the oestrogenic material in human pregnancy urine is oestriol, most of the earlier experiments described here were carried out on

[1] The names *oestrone* and *oestriol* are here used to designate the two oestrogenic substances present in human pregnancy urine which have been known previously as *ketohydroxyoestrin* and *trihydroxyoestrin*, *theelin* and *theelol*, *etc.* This new system of nomenclature has been approved by the majority of the interested English workers [*Nature*, 1933, **132**, 205]. The name *oestrin* is used where it is desired to refer to the oestrogenic material in urine without specifying its exact chemical nature.

[2] It is assumed here that Kober used equimolecular parts of sulphuric and phenolsulphonic acids.

that compound rather than on oestrone. When attempts were made to repeat Kober's technique with pure oestriol, it was not found possible to obtain a colour of the same order of intensity as that reported by him. Furthermore it was found to be impossible to obtain on final dilution solutions of sufficiently uniform tint to enable a single cresol-red solution of any concentration or p_H to be used as a permanent standard of comparison.

In attempting to overcome these difficulties, certain modifications in the conditions of heating were introduced in order to facilitate a rigid standardisation of technique. Even after the introduction of these modifications, slight variations in the tint of the final solution still occurred. It became necessary therefore to use an instrument for colour measurement in which the colour could be analysed and the intensities of its components separately determined. The Lovibond tintometer proved to be suitable for the purpose.

The modified technique used in the initial stages of this work was as follows: 1 ml. of the reagent was measured[1] into an ordinary test-tube which contained from 0·01 to 0·20 mg. of pure dry oestrone or oestriol. The tube was heated in a boiling water-bath for exactly 10 minutes and then immediately cooled in a freezing mixture. The mixture was transferred with 5 % sulphuric acid[2] to a graduated test-tube of about 1 cm. diameter and the volume made up to exactly 4 ml.

The colour of a solution prepared in this way was invariably a yellow-orange, provided that during the dilution the tube was kept at as low a temperature as possible. On heating the solution in a boiling water-bath, the intensity of the yellow component of the colour rapidly decreased, while that of the red component increased, reaching a maximum at about 2 minutes, and then fell slowly. It was found possible to follow these colour changes quantitatively, by removing the diluted solution from the water-bath at 30 second intervals, cooling in a freezing mixture to "fix" the colour and then matching in the tintometer after transference to the standard cell of that instrument.

Colour development curves for oestriol obtained by this procedure are shown in Fig. 1. Similar curves were also obtained with oestrone. It will be seen that the exact time required for the red component of the colour to reach a maximum and for the yellow component to reach a minimum varied with the quantity of oestriol used in the experiment. It was therefore impossible to be sure of observing the "red maximum" of an unknown amount of oestriol by merely heating the diluted solution in a water-bath for a definite time. It was clearly necessary to construct a full colour development curve in every case in order to be certain of recording the true maximum red colour.

Preparation of reagents and the effect of composition of the reagent on the intensity of the colour reaction.

The reagents used in these experiments were prepared by measuring out the required amounts of pure concentrated sulphuric acid and of freshly redistilled phenol (warmed to 60°), and mixing thoroughly in a dry, well-stoppered bottle. Owing to the high viscosities of the acid and phenol, prolonged drainage of the pipettes was essential to ensure reasonably accurate measurement.

[1] An ordinary graduated 1 ml. pipette was used. Owing to the high viscosity of the reagent considerably less than 1 ml. was actually delivered. By adopting standard conditions of drainage, the amounts delivered were sufficiently constant to enable reproducible results to be obtained.

[2] Water would be as efficient a diluent as 5 % sulphuric acid for pure oestrone or oestriol, but the latter was found to have certain advantages when colour reactions were carried out on the urine concentrates which were being obtained at this stage of the work.

Since there was some uncertainty in the minds of the authors as to the exact composition of Kober's reagent, an investigation of the effect of varying the

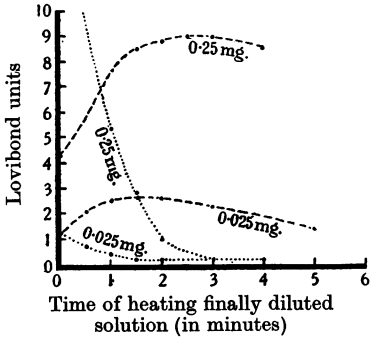


Fig. 1.

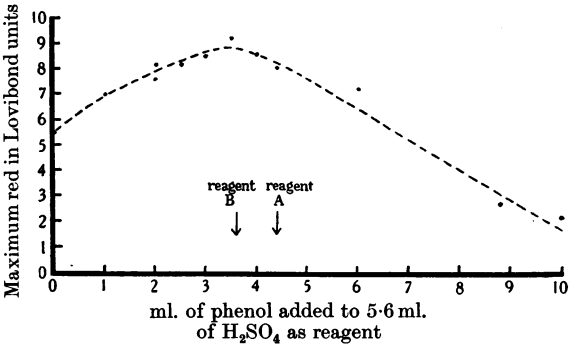


Fig. 2.

Fig. 1. Colour development curves of oestriol treated with 1 ml. of reagent B.
•———• Red. •·····• Yellow.

Fig. 2. The effects of variations in the composition of the reagent on the intensity of the colour reaction. 0·2 mg. oestriol, 1 ml. reagent.

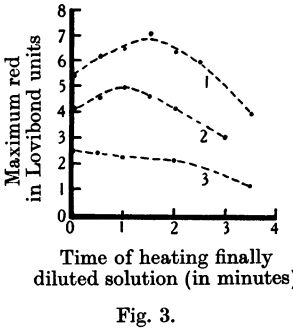


Fig. 3.

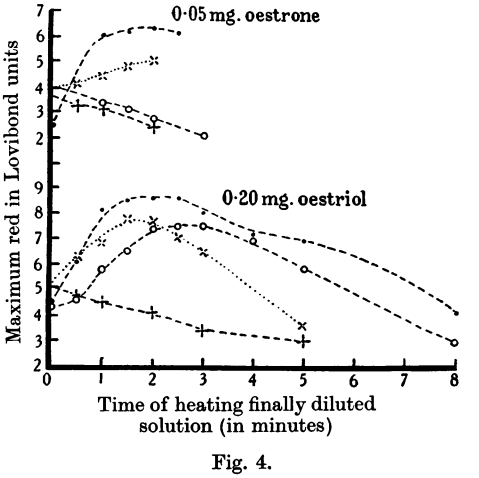


Fig. 4.

Fig. 3. The effect of the addition of water to the reagent upon the colour development curve of oestriol. 0·20 mg. oestriol, 1 ml. reagent A. 1, No added water; 2, 0·05 ml. added water; 3, 0·10 ml. added water.

Fig. 4. The effects of the addition of cholesterol and pregnandiol on the colour development curves of oestrone and oestriol. 1 ml. of reagent B.

•———• Oestrin alone.
×···× ,, +0·2 mg. pregnandiol.
o———o ,, +0·2 mg. cholesterol.
×———× ,, +0·2 mg. cholesterol + 0·2 mg. pregnandiol.

proportions of phenol and sulphuric acid in the reagent upon the intensity of the colour reaction was carried out. The results of this experiment are shown in Fig. 2. It will be seen that a reagent containing from 3 to 4 parts by volume

of phenol to 5·6 parts by volume of sulphuric acid, gave colours of greater intensity than those given by reagents containing any other proportions of the two components. In some of the experiments described in this paper a reagent was used containing 4·4 parts of phenol to 5·6 parts of acid, which was believed to be identical with Kober's reagent. This will be referred to as "reagent A." In later work a reagent containing 3·6 parts of phenol to 5·6 parts of acid ("reagent B") was used.

At first some difficulty was experienced in preparing different batches of reagent which would produce the same intensities of colour with given amounts of oestrin. It was suspected that these variations might be due to the accidental introduction of traces of water into the reagent. The effect of added water on the course of the colour reaction was therefore quantitatively examined. Small amounts of water were added to the oestrin-reagent mixture before the initial period of heating. As is clear from the results shown in Fig. 3, the addition of water very considerably decreased the intensity of the colour yielded by a given quantity of oestriol. The necessity of keeping the reagent in a tightly stoppered bottle and of ensuring the complete dryness of all pipettes and test-tubes is therefore apparent.

Colour development curves of oestrone and oestriol in the presence of pregnandiol and cholesterol.

Partially purified oestrin concentrates were prepared by extracting acidified pregnancy urine with ether, washing the extract with aqueous sodium carbonate and evaporating. Such concentrates when treated with the reagent gave colour development curves which were quite different from those given by pure oestrone or oestriol. The initial colour was red rather than orange, while on heating, the red component failed to rise to a maximum but immediately fell from its initial value. It was evident that these urine concentrates contained substances which were inhibiting the production of the full oestrin colour and which were accelerating its normal rate of fading.

Urine concentrates prepared in the above manner contain not only the total ether-soluble phenolic fraction of the urine, but also all ether-soluble neutral substances. It was therefore decided to investigate the effects of cholesterol and pregnandiol on the oestrin colour development curve, these being the two principal ether-soluble neutral substances which are so far known to occur in human pregnancy urine. Amounts of each and of both were added to oestriol and to oestrone in approximately the same proportions as are known to be present in urine. Either compound separately considerably decreased the intensity of the red colour yielded by given quantities of oestrone or oestriol. When both substances were added together, the inhibitory effect was greatly enhanced. These results, which are shown in Fig. 4, emphasise the necessity for a separation of the phenolic substances, including the oestrone and oestriol, from the neutral fraction before carrying out the colour test. As will be shown in a later section however cholesterol and pregnandiol were not the only colour-inhibitors which must have been present in these crude urine concentrates.

The separation of oestrone and oestriol from the neutral fraction.

It is a matter of some difficulty to effect the quantitative separation of the total oestrogenic material in urine concentrates from the neutral fraction. Oestriol may be readily extracted from an ethereal solution with dilute aqueous

alkali hydroxides, but this procedure is not so effective in the case of oestrone, owing to the very feebly phenolic character of the latter compound. A method of separating the oestrone quantitatively from the neutral fraction had therefore to be devised. As a preliminary to the elaboration of such a method the distribution of oestrone between different strengths of aqueous alkali hydroxides and various immiscible organic solvents was studied.

Solutions containing weighed amounts of oestrone in different organic solvents were made up, and aliquot portions of these were shaken with equal volumes of aqueous solutions of sodium and potassium hydroxides of different strengths. After complete separation of the phases, aliquot portions of the organic solvent layers were measured off and evaporated to dryness. Colour reactions were then carried out on the residues so obtained in the manner previously described. By reference to an oestrone standardisation curve constructed specially for the purpose, the amounts of oestrone remaining in the organic solvent after equilibration with the alkali were determined. The results obtained with toluene and ethereal solutions of oestrone equilibrated with solutions of $N/10$ and N sodium hydroxide are shown in Table I.

Table I. *Distribution of oestrone between ether or toluene and aqueous sodium hydroxide solutions.*

Organic solvent	Concentration of NaOH	mg. of oestrone in 10 ml. of organic solvent: Before equilibration	mg. of oestrone in 10 ml. of organic solvent: After equilibration	Oestrone in NaOH / Oestrone in organic solvent
Ether	$N/10$	0·49	0·39	Approx. 1/4
„	N	0·49	0·17	„ 2/1
Toluene	$N/10$	0·47	0·28	„ 2/3
„	N	0·47	0·10	„ 4/1

These results indicated that whereas $N/10$ sodium hydroxide should only slowly extract oestrone from ethereal solution, N sodium hydroxide should effect complete extraction from toluene with ease. This suggested that not only would it be possible to separate all the oestrogenic material from the neutral fraction of urine concentrates, but that a quantitative separation of the oestrone from the oestriol fractions might at the same time be effected. The suggested scheme for such a separation is shown in Table II.

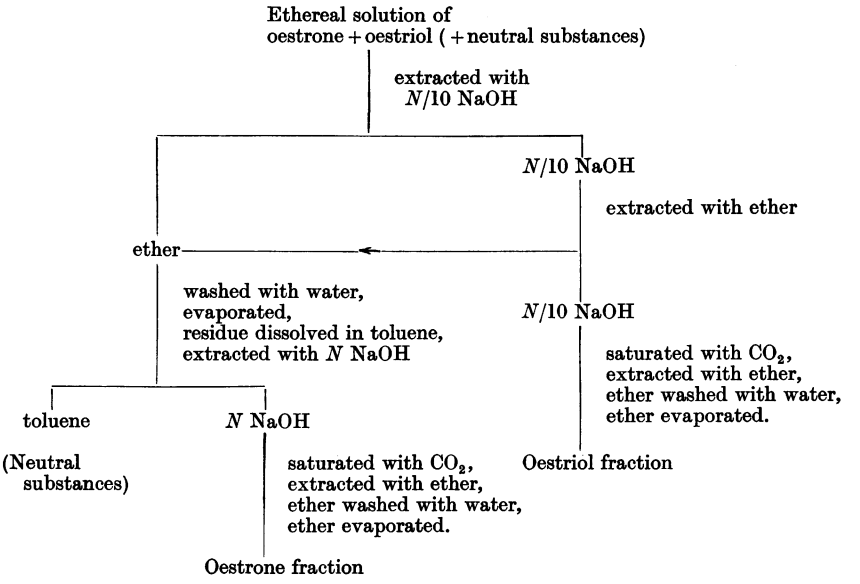
Preliminary experiments on ethereal solutions containing known amounts of oestrone and oestriol indicated that a nearly quantitative separation could be effected by this process. Since these experiments had later to be repeated using the exact technique finally adopted for the urine concentrates (see p. 1611), the preliminary results are not reported.

The estimation of oestrone and oestriol in pregnancy urine.

(*a*) *The preparation of oestrone and oestriol fractions from pregnancy urine.* Acid-hydrolysed urine was extracted with ether, the extract was freed from acidic substances by washing with aqueous sodium carbonate and subjected to the further concentration and separation of the phenolic fractions as outlined in Table II.

(*b*) *Further modifications in the colour reaction.* As a result of the discovery that pregnandiol and cholesterol inhibited the development of the maximum red colour produced by oestrone and oestriol, the hope was entertained that the two separated phenolic fractions would give colour development curves

Table II. *Method of separating oestrone and oestriol mixtures.*



similar to those given by the pure substances. This however did not prove to be the case. The colour development curves of the separated phenolic residues still failed to show an increase of the red value up to a maximum, indicating that inhibiting substances persisted.

At first it was feared that this might necessitate the abandonment of the method entirely, since further purification of the phenolic fractions without loss of oestrone and oestriol seemed to be difficult. Fortunately it was found that the substitution of 2 ml. of reagent for 1 ml. in the colour reaction, led to the production of nearly normal colour development curves from these phenolic urine concentrates. The higher concentration of reagent in the finally diluted solution appeared to overcome to a large extent the effect of the colour-inhibitors

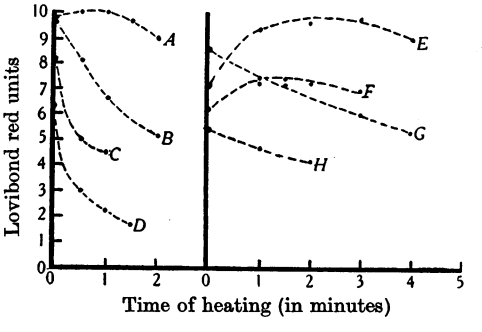


Fig. 5. Colour development curves of oestrone and oestriol fractions of human pregnancy urine. The effects of 1 ml. and 2 ml. of reagent, and of 5 % H_2SO_4 and water as diluents.

A, oestriol fraction, 2 ml. reagent, 5 % H_2SO_4.
B, " " " " water.
C, " " 1 ml. " 5 % H_2SO_4.
D, " " " " water.
E, oestriol fraction, 2 ml. reagent, 5 % H_2SO_4.
F, oestrone " " " " "
G, oestriol " 1 ml. " " "
H, oestrone " " " " "

still present. When water instead of 5 % sulphuric acid was used to effect the final dilution, colour development curves less like the normal resulted. These effects are clearly illustrated in Fig. 5.

Kober observed that the red colour in his test was rapidly and completely destroyed by the addition of hydrogen peroxide. This has been confirmed and the fact has been utilised to eliminate errors due to the presence of "non-oestrin" colour in the finally diluted colour mixture. On adding to this colour mixture a few drops of hydrogen peroxide and heating to 100° for a few minutes, the red colour rapidly disappeared and a pale brown solution usually resulted. (From experiments on pure oestrone and oestriol it was found that heating with peroxide for 2 minutes was sufficient to discharge the colour completely.) The colour of this solution could then be analysed by means of the tintometer and the value of the red component subtracted from the value of the original maximum red. This correction has been applied to all the colour reactions carried out on urine concentrates.

(*c*) *Standardisation curves for oestrone and oestriol.* Before the red values obtained on urine concentrates could be calculated in terms of oestrone and oestriol, curves showing the relationship between weights of the pure compounds and maximum intensities of the red colours had to be constructed. The oestrone curve was constructed from a sample of the International Oestrone Standard.

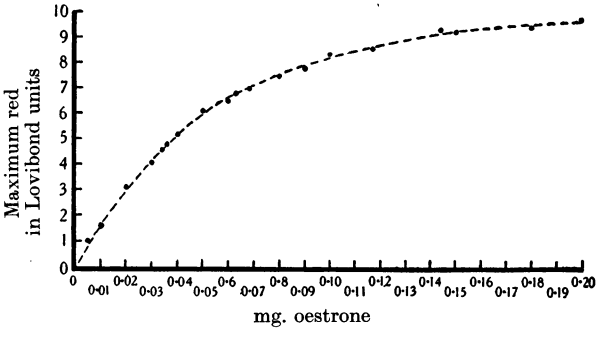


Fig. 6. Standardisation curve for oestrone.

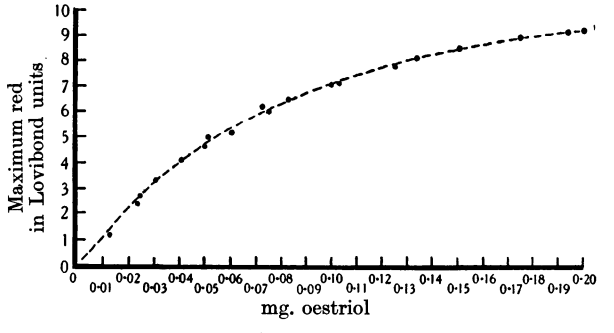


Fig. 7. Standardisation curve for oestriol.

For the oestriol curve, a highly purified specimen, prepared by Dr André Girard and very kindly presented to one of the authors, was used. Colours were developed by 2 ml. of reagent B and 5 % sulphuric acid as diluent. The standardisation curves are shown in Figs. 6 and 7. It will be seen that, as Kober pointed

out, the relationship between intensity of maximum red colour and quantity of hormone is not linear. Owing to the shape of the curves, the most accurate results are obtained from colours between 3 and 7 Lovibond red units.

(*d*) *Hydrolysis of the ether-insoluble "oestrin esters" in urine.* Zondek [1934] has recently stated that for human pregnancy urine (but not for pregnant mare's urine) no preliminary treatment with acid is required for the complete removal of the oestrin by extraction with immiscible solvents. The majority of other workers in the field believe, on the contrary, that the yields of oestrin obtainable from human urine can be very considerably increased by a preliminary treatment with acid [*cf.* Marrian, 1933; Borchardt *et al.*, 1934]. Hence it is generally assumed that much of the oestrin in urine is present as a water-soluble, ether-insoluble ester. Owing presumably to the great labour involved in carrying out large numbers of biological assays of oestrin, no systematic attempt has hitherto been made to determine the optimum conditions for the hydrolysis of these "oestrin esters" by acid. Furthermore, little attention has been paid in the past to the possibility of effecting the hydrolysis by other means than acids, although Marrian [1933] reported experiments in which alkaline hydrolysis at 100° was found to be ineffective, while allowing the urine to putrefy was found to be more effective than acid hydrolysis for one hour at 100°. Both these facts have been confirmed by the present authors.

Before this colorimetric method could be applied to the quantitative estimation of oestrin in urine, it was obviously necessary to determine the optimum conditions for the preliminary hydrolysis. Experiments have therefore been carried out in which urines have been hydrolysed under varying conditions for varying times before extraction and fractionation. In this way it has been possible to determine the rate of release from an ether-insoluble form, on hydrolysis under different conditions, of the chromogenic material which appeared in both the oestrone and oestriol fractions. As will be shown later, the assumption that all the chromogenic material released on hydrolysis was oestrogenic proved to be justified.

The results obtained by these means may conveniently be summarised at this point. It was found that on heating pregnancy urine at 100° after acidification with hydrochloric acid to p_H 1–2, a rapid and almost linear increase of ether-soluble chromogen in both oestrone and oestriol fractions occurred for about 2 hours. From 2 to 16 hours the amount still increased but at a much slower rate. On heating for more than 16 hours, definite evidence of destruction of the hormone of both phenolic fractions was obtained. On incubating urine at 37°, a gradual increase of ether-soluble chromogens occurred. A maximum higher than that obtained by heating at 100° at p_H 1–2, was arrived at after about 2 weeks' incubation. On further incubation, destruction of the chromogens took place. By heating the urine acidified with hydrochloric acid to about p_H 1–2 in an autoclave at 15 lbs. for 2–4 hours, a degree of hydrolysis as great as the optimum for incubation at 37° was obtained. On more prolonged autoclaving, slow destruction occurred. Some of the values obtained for pregnancy urine (batch PU_7) are given in Table III to illustrate most of these points. Confirmatory results for the conclusions stated above were also obtained with other batches of urine.

It is as yet impossible to state definitely whether or not complete hydrolysis of the "oestrin esters" in urine has been effected by any of these methods. So far, however, no method of hydrolysis giving a yield higher than that given by autoclaving at p_H 1–2 for 2–4 hours has been found. As a temporary working hypothesis, therefore, the assumption will be made that hydrolysis under these conditions is complete.

Table III. *Effect of hydrolysis of pregnancy urine on the oestrin content of ether extracts.*

		mg. oestrin per 100 ml. urine							
			Acid-hydrolysed at 100°			Incubated at 37°		Acid-autoclaved	
Urine batch		Not hydrolysed	2 hrs.	16 hrs.	48 hrs.	2 weeks	4 weeks	1 hr.	4 hrs.
PU_7	Oestriol	0·420	0·540	0·800	0·620	1·120	0·560	0·620	1·000
	Oestrone	0·024	0·046	0·070	0·065	0·080	0·047	0·070	0·082

(*e*) *The recovery of oestrone and oestriol added to mixtures.* The conditions necessary for the production of normal colour development curves from the phenolic fractions of pregnancy urine having been established, it was thought desirable to test the efficacy of the whole method by experiments on solutions containing known amounts of oestrone and oestriol. For this purpose solutions in 400 ml. of water or of male urine, containing known weights of oestriol and/or oestrone, and in one case also cholesterol and pregnandiol, were made up. These were acidified with hydrochloric acid, in some cases heated to 100° for 4 hours, and then ether-extracted. The ethereal extracts were washed with aqueous sodium carbonate solution and then subjected to the oestrone-oestriol separation process as shown in Table II. Colour tests on aliquot portions of the oestrone and oestriol fractions were then carried out using 2 ml. of the reagent. The results are shown in Table IV.

Table IV. *The recovery of oestrone and oestriol from mixtures of known composition.*

	mg. added to 400 ml. water					mg. recovered		% recovery	
	Oestriol	Oestrone	Cholesterol	Pregnandiol	Hydrolysis	Oestriol fraction	Oestrone fraction	Oestriol fraction	Oestrone fraction
I	2·000	—	—	—	None	1·600	0·053	80	—
II	—	0·200	—	—	,,	0·012	0·190	—	95
III	1·000	0·100	—	—	,,	0·870	0·106	87	106
IV	1·000	0·100	—	—	4 hrs. HCl at 100°	0·870	0·100	87	100
V	0·500	0·100	—	—	,,	0·465	0·100	93	100
VI	1·500	0·400	—	—	,,	1·250	Fraction lost	83	—
VII	1·000	0·200	—	—	,,	0·780	0·192	78	96
VIII	1·000	0·100	1·0	1·0	,,	0·890	0·106	89	106
IX	1·000	0·100 (in 400 ml. of male urine)			,,	0·965	0·093	97	93

It will be seen that there is an average loss of about 13 % of oestriol in these experiments, which is hardly surprising when the somewhat elaborate nature of the whole process is taken into consideration. At first sight it might appear that the recovery of the oestrone was nearly quantitative. It will be seen, however, that from the solution containing oestriol but no oestrone, a small amount of "oestrone" was "recovered." This is undoubtedly due to the fact that traces of oestriol had escaped extraction from the ethereal solution by the $N/10$ alkali and were therefore later extracted by the N alkali and hence appeared in the oestrone fraction. It should be pointed out that since the mixtures contained approximately ten times more oestriol than oestrone—the proportion usually present in pregnancy urine—a negligible loss of oestriol would cause a not inconsiderable error in the oestrone figure. The oestrone-oestriol separation process is therefore not strictly quantitative. Nevertheless the figures obtained seem to justify the conclusion that for most practical purposes the separation can be

considered to be nearly quantitative. It is believed that in determinations on urines the error in the oestrone value should not be large, provided that abnormally high amounts of oestriol and low amounts of oestrone are not present.

(*f*) *Comparison of the colorimetric and biological methods of assay.* Finally it had to be shown that the chromogenic material obtained in the ether-soluble phenolic fractions of pregnancy urine was all oestrogenic. Comparisons of the biological and colorimetric values obtained for the oestrone and oestriol contents of fractions obtained from different samples of urine subjected to various types of preliminary hydrolysis were made.

The biological tests were carried out on ovariectomised mice by the method of Marrian and Parkes [1929]. Standardisation curves for the mouse colony for oestrone and oestriol were first constructed[1], using the same standard preparations as those used for the colorimetric standardisation curves.

Table V shows the values obtained. The close agreement between the figures obtained by the two methods of assay clearly shows that for human pregnancy urine the modified Kober test is specific for oestrone and oestriol.

Table V. *Comparison of the values for the oestrin content of urinary phenolic fractions obtained by the biological and colorimetric methods of assay.*

		Oestriol mg./100 ml. urine		Oestrone mg./100 ml. urine	
Urine batch	Conditions of hydrolysis of the urine	Biological	Colorimetric	Biological	Colorimetric
P.U. 3	4 hrs.; acid at 100°	0·243 0·231	0·205 0·240	—	—
P.U. 4	,,	0·171 0·178	0·182 0·178	0·024 0·031	0·033 0·042
P.U. 5	8 hrs.; acid at 100°	0·265 0·218 0·224 0·231 —	0·316 0·306 0·275 0·280 0·300	0·070 —	0·071 0·072
P.U. 6	16 hrs.; acid at 100°	0·624 0·607 —	0·691 0·700 0·630	0·107 —	0·100 0·100
P.U. 6	4 weeks; incubation	—	—	0·126 0·115	0·127 0·100
P.U. 7	16 hrs.; acid at 100°	0·593 0·558	0·680 0·705	—	—
P.U. 7	4 hrs.; acid-autoclaving	—	—	0·092	0·100 0·077 0·077
P.U. 8	,,	1·07	1·07 1·00 0·970 1·00	0·125 0·121 —	0·127 0·123 0·107
I.P.U., i, B	,,	0·887	1·20 1·13	—	—
I.P.U., i, C	,,	1·22 0·989	0·800 0·875	—	—

[1] It was previously found by one of us (G. F. M.) that oestrone was slightly more than twice as active as oestriol when tested on ovariectomised mice. In the present work the potencies of oestrone and oestriol have been found to be 13,000 m.u. and 8130 m.u. per mg. respectively. It appears therefore that the strain of mice used in Toronto is relatively more sensitive to oestriol than were the English mice previously used.

Discussion.

The authors are of the opinion that the evidence presented in this paper has shown that the oestrone and oestriol in human pregnancy urine may be separated and estimated with a reasonable degree of accuracy by means of a modified Kober colour reaction. The separation of the oestrone and oestriol, although not strictly quantitative, is nevertheless sufficiently complete to permit the detection of any abnormal amounts of either compound in pregnancy urine. The quantitative colour test *per se* is certainly as accurate as, if not more accurate than, the biological method of assay and can be carried out in a few minutes, whereas several days at least are required for the performance of a test by the latter method.

For the convenience of any other worker who may wish to use this method of assay, the exact procedure finally adopted is as follows:

(*a*) *Preparation of the phenolic fractions from urine.* A 300 ml.[1] sample of pregnancy urine is adjusted to p_H 1–2 with hydrochloric acid and heated in an autoclave for 2–4 hours at a pressure of 15 lbs. After cooling, the hydrolysed urine is extracted once with 150 ml. and then three times with 100 ml. lots of freshly redistilled ether[2]. The combined ethereal extracts are then washed four times with 30 ml. lots of 10 % aqueous sodium carbonate solution, these washings being discarded. The "strong phenolic" fraction containing the oestriol is removed from the ethereal solution by extracting four times with 30 ml. volumes of $N/10$ sodium hydroxide. This alkaline extract is then washed three times with 30 ml. lots of ether, in order to remove any oestrone that may have been extracted.

The two ethereal solutions are combined, washed four times with 30 ml. lots of water and evaporated to dryness. The residue is dissolved in 100 ml. of toluene, and the "weak phenolic" fraction containing the oestrone is removed from the solution by four extractions with 30 ml. volumes of N sodium hydroxide.

The $N/10$ and N sodium hydroxide solutions containing respectively the oestriol and oestrone phenolic fractions are saturated with carbon dioxide until they are no longer alkaline to phenolphthalein and then extracted four times with 30 ml. lots of ether. The two ethereal solutions are finally washed four times with 25 ml. lots of water and evaporated to dryness. The residues containing the oestriol and oestrone fractions are then dissolved in alcohol, and aliquot samples of each solution are removed for the colorimetric assay.

(*b*) *The colorimetric assay on the phenolic fractions.* The sample for assay is measured into a test-tube and evaporated to dryness at 100° under a stream of nitrogen. To the residue are added 2·0 ml. of the phenolsulphonic acid reagent, the tube is heated for 10 minutes at 100° and then immediately cooled in a freezing mixture. The reaction mixture is transferred to a graduated test-tube (1 cm. diameter) with 5 % sulphuric acid and the volume made up to exactly 4 ml. The initial colour of the solution and the colours after heating at 100° for successive 30 second periods and cooling are analysed in the Lovibond tintometer. For the actual colour measurement, the solution is transferred to the standard 1 cm. tintometer cell. For subsequent heating and cooling, the solution

[1] All pregnancy urines so far tested contained sufficient oestrone and oestriol to enable the tests to be carried out with no more than 300 ml. This may not be the case with urines obtained in the early stages of pregnancy.

[2] The purity of the ether is of the utmost importance. In experiments in which pure oestriol was treated with impure ether, marked inhibition of colour development occurred. This was presumably due to the peroxide content of the ether.

is returned to the graduated test-tube. Colour measurements at 30-second intervals are continued until the maximum red value has been attained and passed. 0·5 ml. of 3 % hydrogen peroxide is then added and the solution heated at 100° for 2 minutes. The remaining colour of the solution, if any, is analysed in the tintometer and the value of the red component subtracted from the red maximum originally obtained, after making the necessary correction for the change in volume caused by the addition of the peroxide. The weight of oestrone or oestriol corresponding to the corrected red maximum is finally read from the appropriate standardisation curve. To ensure the greatest possible accuracy in the colorimetric determination, it is necessary to conduct several estimations on different amounts of the phenolic fractions.

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