The Determination of Oestrogens in Human Pregnancy Urine. A New Method of Correcting for the Brown Colour Developed in the Kober Reaction by Non-oestrogenic Substances

By MARGARET F. STEVENSON and G. F. MARRIAN
Department of Biochemistry, University of Edinburgh

(*Received* 20 *January* 1947)

The determination of oestrogens in human pregnancy urine by the Kober (1931) method is complicated by the fact that urinary oestrogen concentrates may contain substances which yield a brown colour in the reaction with the phenolsulphonic acid reagent. Since this brown-coloured material shows considerable absorption at the wave-length of the absorption band of the pink colour developed by oestrogens, the latter may be considerably overestimated. The problem of correcting for this interfering brown colour, or of eliminating without loss of oestrogen the substances which give rise to it has been approached by a number of workers in different ways.

Cohen & Marrian (1934) measured the red colour of the final reaction mixture in Lovibond units and then subtracted the red component of the residual brown colour after fading the oestrogen pink colour with H_2O_2. However, as was pointed out by Venning, Evelyn, Harkness & Browne (1937) and by Bachman & Pettit (1941), this method of correction is not satisfactory since treatment with H_2O_2 causes some fading of the non-oestrogen brown colour. Venning *et al.* (1937) showed that the brown-coloured material absorbs strongly at 420 mμ. and that the oestrogen pink-coloured substance, which shows an absorption maximum at 522 mμ., is nearly transparent at the former wave-length. By assuming that the spectrophotometric characteristics of the non-oestrogen brown colour are constant for different urines they were able to calculate from the measured absorption at 420 mμ. the absorption at 522 mμ. due to this brown colour. The validity of the basic assumption underlying this method of correction has, however, been questioned by Bachman & Pettit (1941).

Kober (1938), in the paper in which a modified oestrogen reagent (β-naphthol and H_2SO_4) was described, observed that the oestrogen pink colour developed with this reagent fades in the presence of acetone. This fading in the presence of acetone has been utilized by Jayle, Crépy & Judas (1943) in a new method for correcting for the brown colour. After allowing the Kober reaction mixture to stand in the dark for several hours in the presence of acetone, the residual brown colour was measured spectrophotometrically and the appropriate correction applied to the original measurement of the pink colour.

Bachman & Pettit (1941) attempted to avoid the necessity of a correction by eliminating from the oestrogen concentrates the substances which give rise to the brown colour in the Kober reaction. By separating the oestriol and the oestrone-oestradiol fractions and then purifying these fractions by distribution between various pairs of immiscible solvents they obtained products which gave little brown colour, as judged by absorption at 420 mμ., in the Kober reaction.

A somewhat similar approach to the problem has been made more recently by Stimmel (1946). The total ether-soluble phenolic material obtained from urine was separated into oestriol, oestrone, and oestradiol fractions by adsorption on Al_2O_3 and fractional elution with different methanol-benzene mixtures. The three fractions so obtained gave colours in the Kober reaction which showed little absorption at 420 mμ. Stimmel has suggested that the final coloured reaction mixture should be shaken with ethyl acetate before measurement of the colour. It was claimed that this procedure removed impurities without affecting the pink colour in the aqueous phase.

The object of the present work was to evolve a simplified method for determining the total oestrogen in human pregnancy urine which would be suitable for clinical investigations. In the course of this work it was observed that whereas the pink colour given by oestriol or oestrone in the Kober reaction can be completely faded by heating for 1·5 hr. in the boiling water-bath, the brown colour given by the ether-soluble phenolic fraction of human male urine is affected by this treatment only to a very small extent. On the basis of these observations a simple procedure for correcting for the non-oestrogen brown colour given by human pregnancy urine oestrogen concentrates in the Kober reaction has been worked out. This correction procedure which, in principle, has much in common with the one suggested by Jayle *et al.* (1943), is undoubtedly superior to the H_2O_2 method of Cohen & Marrian (1934) and because of its simplicity would seem to have certain advantages over the methods employed by others for the avoidance of, or correction for, the interfering brown colour.

EXPERIMENTAL

Hydrolysis and extraction of urine

A method of hydrolysis of urine based on that suggested by Smith & Smith (1935) has been employed. These workers added 15% (v/v) of conc. HCl to the urine, heated to boiling

and continued the boiling for 10 min. It was shown by Smith, Smith & Pincus (1938) that the yields of free oestrogen extractable from human pregnancy urine after such a hydrolysis were as high as those obtained after the less convenient method of Cohen & Marrian (1935). Two modifications have been made in the Smith & Smith procedure: (i) In order to ensure accurate timing of the hydrolysis and to minimize oxidative destruction of the oestrogens, the urine specimens have been brought to the boil before the addition of the acid. (ii) It has been found that the yield of free oestrogen, as determined by the Kober method, is not maximal after boiling for 10 min. with 0·15 vol. of conc. HCl. The data available, which will be published in detail later, indicate that maximal hydrolysis of the conjugated oestrogens in human pregnancy urine is attained by boiling with 0·15 vol. of conc. HCl for 30–40 min. In the majority of the experiments reported in this paper, therefore, the hydrolysis has been continued for 30 min.

Urine specimens were collected with toluene as preservative. In order to standardize the procedure as far as possible, 24 hr. specimens were all diluted to a volume of 2·5 l. before removing samples for hydrolysis and extraction. The procedure for hydrolysis and extraction was as follows: 100 ml. of the diluted urine was heated to boiling under reflux and, after the addition of 15 ml. of conc. HCl down the condenser, boiling was continued for the required time (see above). The hydrolyzed urine was rapidly cooled under the tap and then extracted once with 100 ml. and twice with 50 ml. portions of ether. The combined ethereal extracts were washed three times with 25 ml. portions of 5% (w/v) $NaHCO_3$ and the combined washings back-extracted once with 20 ml. of ether. The washed ethereal extract was combined with the back-extract and evaporated to dryness. The residue was warmed with *c.* 3 ml. of ethanol and 100 ml. of benzene added. This benzene solution was then extracted once with 50 ml. and twice with 25 ml. portions of N-NaOH. The combined NaOH extracts were acidified with 15 ml. of conc. HCl and extracted once with 100 ml. and twice with 50 ml. portions of ether. The ethereal extracts were washed twice with 20 ml. portions of 5% (w/v) $NaHCO_3$, and the washings back-extracted once with 20 ml. of ether. The combined ethereal extracts after washing three times with 20 ml. portions of water, were evaporated to dryness. The residue, consisting of the total ether-soluble phenolic fraction of the hydrolyzed urine, was dissolved in ethanol and suitable measured samples of the solution pipetted out for colorimetric assay.

Technique of the Kober reaction

The phenolsulphonic acid reagent was prepared according to the method of Cohen & Marrian (1934). The technique of colour development adopted was essentially similar to that described by Venning *et al.* (1937).

Measured samples of the solution of the urinary phenolic fraction, containing between 10 and 80 μg. of oestrogen, and equivalent to not more than 2%* of the 24 hr. urine specimen, were evaporated to dryness in a stream of air in test tubes *c.* 2 cm. in diameter, bearing graduation marks at 8 and 15 ml. To the dry residues 3 ml. portions of the Kober reagent were added and the tubes were heated in the boiling water-bath for 20 min. After cooling in an ice-salt freezing mixture 3·0 ml. of water were added to each tube and the contents thoroughly mixed. The tubes were then heated for 3 min. in the boiling water-bath, cooled to room temperature by immersion in water and finally made up to the 15 ml. mark with 10% (v/v) H_2SO_4. The intensities of absorption at *c.* 520 mμ. were measured on 7 ml. portions of the final solutions in a Spekker photoelectric absorptiometer using an Ilford 'spectrum green, no. 604' light filter.

The 8 ml. portions of the solutions remaining in the tubes were heated in the boiling water-bath for 1·5 hr. in order to fade the pink colour produced by the oestrogen. After cooling and making good the water lost by evaporation, the absorption at *c.* 520 mμ. was again measured. The amount of oestrogen (as oestriol) originally present in each tube was obtained by referring the difference between the initial and final absorptiometer readings to an oestriol calibration curve.

* When amounts of phenolic fraction equivalent to more than 2% of the 24 hr. urine specimen were used precipitates were sometimes formed during the subsequent fading procedure.

'Blank' determinations on human male urine

As a necessary preliminary to recovery experiments in which oestrone and oestriol were added to male urine a series of 'blank' determinations was carried out on the latter.

24 hr. urine specimens (preserved with toluene) were collected from a number of healthy adult men. Samples from each were hydrolyzed and extracted according to the procedure described above, and Kober reactions carried out on amounts of the phenolic fractions equivalent to 1% and/or 2% of the 24 hr. specimens. The results are shown in Table 1.

It will be seen that the brown colours developed underwent only small changes in absorption at *c.* 520 mμ. as a result of heating at 100° for 1·5 hr. These small changes, however, correspond to 'apparent oestrogen' contents (calculated as oestriol) varying from 0·0 to 0·7 mg./24 hr. and averaging *c.* 0·3 mg.

The significance of this small 'apparent oestrogen' content of male urine is difficult to assess. The variations in the values obtained for the same urine specimen and between the values obtained for different urines must be to some extent due to unavoidable small errors in the absorptiometer readings. Thus an error of only 0·004 in the difference between the initial and final readings would correspond to an error of 0·1 mg./24 hr. in the 'apparent oestrogen' value when the determination is carried out on the equivalent of 1% of the 24 hr. specimen. Nevertheless, since the small observed changes in absorption at *c.* 520 mμ. resulting from heating the solutions were all decreases, they cannot be due only to the multiplication of small absorptiometer reading errors. This small decrease in absorption at *c.* 520 mμ. may be due largely to changes in the reaction mixture other than that resulting from the

Table 1. *'Blank' determinations on normal human male urine*

Urine	Hydrolysis time (min.)	Fraction of 24 hr. urine specimen used (%)	Absorptiometer readings (spectrum green filter) Initial (*A*)	After heating for 1·5 hr. at 100° (*B*)	Oestriol equivalent of *A* − *B* (μg.)	'Apparent oestrogen' content of urine (mg./24 hr.)
A	10	1	0·089	0·087	0·5	0·05
	10	2	0·167	0·163	1·0	0·05
	10	2	0·220	0·212	2·0	0·10
B	10	1	0·116	0·099	4·5	0·45
	10	2	0·152	0·138	4·0	0·20
	10	2	0·150	0·139	3·0	0·15
B.C. (i)	10	1	0·097	0·080	5·0	0·50
	10	1	0·112	0·089	6·5	0·65
G.A. (i)	10	1	0·090	0·078	3·5	0·35
	10	1	0·068	0·059	2·5	0·25
	10	1	0·077	0·064	3·5	0·35
	10	1	0·066	0·058	2·0	0·20
R.J. (i)	10	1	0·123	0·102	6·0	0·60
	10	1	0·109	0·083	7·3	0·73
R.J. (ii)	10	1	0·098	0·098	0·0	0·0
	10	1	0·082	0·081	0·0	0·0
B.C. (ii)	30	2	0·147	0·141	1·5	0·08
	30	2	0·154	0·151	0·75	0·04
G.A. (ii)	30	1	0·046	0·038	2·0	0·20
	30	1	0·062	0·044	5·0	0·50
	30	2	0·101	0·081	5·5	0·28
	30	2	0·111	0·099	3·0	0·15
	30	2	0·108	0·102	1·5	0·08
	30	2	0·120	0·082	10·8	0·54
R.J. (iii)	30	2	0·158	0·157	0·0	0·00
M.G.	30	1	0·091	0·070	5·8	0·58
	30	1	0·101	0·084	4·5	0·45
G.J.	30	1	0·067	0·057	2·3	0·23
	30	1	0·064	0·052	2·8	0·28
	30	2	0·137	0·124	3·0	0·15
	30	2	0·137	0·120	4·0	0·20
K.W.	30	1	0·050	0·044	1·5	0·15
	30	1	0·049	0·043	1·5	0·15
	30	2	0·106	0·090	4·0	0·20
	30	2	0·098	0·098	0·0	0·00

fading of an oestrogen pink colour. However, the alternative possibility that the 'apparent oestrogen' values are in fact due to oestrogen cannot be entirely disregarded. Callow, Callow, Emmens & Stroud (1939) have recorded oestrogen contents for human male urine, determined by bioassay, of *c.* 30–40 i.u./l. These values give a rough average of 50 i.u. of oestrogenic activity per 24 hr. From the data given by Emmens (1939) it may be calculated that this amount of oestrogenic activity would be given by 0·05 mg. of oestrone or by 0·35 mg. of oestriol. Accordingly if it were assumed that most of the oestrogen in human male urine is oestriol the average value for the 'apparent oestrogen' content determined colorimetrically is in reasonable agreement with the average value determined by bioassay. However, although there are indications that oestriol normally occurs in human male urine (Pincus, 1945), there is no reason to believe that it is the predominant oestrogen present.

Recovery of oestrone and oestriol added to hydrolyzed human male urine

In order to assess the value of the 'fading' technique as a method of correcting for the non-oestrogen brown colour, a series of recovery experiments was made in which oestrone and/or oestriol in known amounts were added to suitable measured portions of acid-hydrolyzed 24 hr. specimens of human male urine. The oestrogen was added to the urine after hydrolysis rather than before in order to avoid complicating the issue by the losses due to destruction which are known to occur during the hydrolysis (cf. Cohen & Marrian, 1935). Since the object of these experiments was to compare the recoveries obtained by the 'fading' technique

with those obtained by the direct Kober method, rather than to determine the overall error of the whole procedure, this course seemed to be justifiable.

With increasing amounts of oestrogen the corrected and uncorrected recovery figures become closer to one another, and it will be apparent from the results that for urines containing more than

Table 2. *Recovery of oestrogen added to acid-hydrolyzed human male urine*

Added oestrogen (mg./24 hr.)			Fraction of 24 hr. urine sample used (%)	Absorptiometer readings		Recovery of oestrogen (%)	
Oestrone	Oestriol	Total as oestriol		Before fading (*A*)	After fading (*B*)	From (*A* − *B*)	From (*A*)
0·00	1·00	1·00	2	0·168	0·108	80	210
0·00	1·00	1·00	2	0·165	0·107	78	205
1·05	0·00	1·16	2	0·248	0·162	100	275
1·05	0·00	1·16	2	0·224	0·140	97	266
0·70	0·30	1·07	2	0·220	0·161	78	264
0·50	1·58	2·13	1	0·145	0·074	92	178
0·50	1·58	2·13	1	0·151	0·070	104	185
1·60	0·53	2·30	1	0·154	0·073	94	172
1·60	0·53	2·30	1	0·147	0·070	91	164
0·00	5·30	5·30	1	0·253	0·067	95	123
0·00	5·30	5·30	1	0·248	0·067	93	121
3·13	0·99	4·44	1	0·250	0·072	116	152
3·13	0·99	4·44	1	0·255	0·077	116	157
4·00	0·00	4·40	1	0·231	0·066	104	130
4·00	0·00	4·40	1	0·234	0·068	105	131
2·09	4·95	7·25	0·4	0·140	0·028	112	129
2·09	4·95	7·25	0·4	0·139	0·027	112	129
2·50	10·0	12·8	0·4	0·206	0·037	89	98
2·50	10·0	12·8	0·4	0·206	0·038	88	98
0·00	15·9	15·9	0·4	0·248	0·029	94	101
0·00	15·9	15·9	0·4	0·232	0·030	87	94
15·0	0·00	16·5	0·4	0·254	0·033	102	104
15·0	0·00	16·5	0·4	0·267	0·036	107	110
10·0	25·0	36·0	0·1	0·128	0·014	85	86
10·0	25·0	36·0	0·1	0·131	0·011	89	88
26·1	9·90	38·6	0·1	0·151	0·014	103	107
26·1	9·90	38·6	0·1	0·169	0·013	116	118
0·00	64·5	64·5	0·1	0·211	0·018	85	81
0·00	64·5	64·5	0·1	0·228	0·022	89	87
68·3	0·00	75·1	0·1	0·270	0·021	90	93
68·3	0·00	75·1	0·1	0·281	0·018	95	96

The results of these recovery experiments are shown in Table 2. It will be seen that when less than *c.* 10 mg. of oestrogen/24 hr. was present, necessitating the carrying out of the colour reaction on the equivalent of 1 % or more of the 24 hr. urine specimen, the recoveries obtained by the uncorrected Kober method were grossly high, whereas those obtained by the 'fading' technique were mostly within the range of 80–110 %. It is justifiable, therefore, to conclude that this new method of correcting for the non-oestrogen brown colour will be of some value in the analysis of urine obtained during early and middle pregnancy.

c. 15 mg. oestrogen/24 hr. a correction is hardly necessary.

Particular attention must be drawn to the recoveries obtained when *c.* 1 mg. of oestrogen/24 hr. was present. In view of the magnitude of the male urine 'blanks' these apparently good recoveries must be regarded as being fortuitous, and it must be assumed that the small unavoidable losses which occur during the extraction process were largely offset by the urine 'blank'. In this connexion the authors are of the opinion that little quantitative significance should be placed on results indicating less than *c.* 2 mg. oestrogen/24 hr.

SUMMARY

1. The pink solutions yielded by oestrone or oestriol in the Kober reaction with the phenolsulphonic acid reagent are completely decolorized after heating at 100° for 1·5 hr.

2. The brown solutions yielded by ether-soluble phenolic fractions of human male urine in the Kober reaction undergo little change, as judged by absorption at *c.* 520 mμ., when similarly treated.

3. On the basis of these observations a simple method has been developed of correcting for the interfering brown colour which is developed from non-oestrogenic substances in the determination of oestrogens in human pregnancy urine.

The authors are indebted to the Medical Research Council for a grant from which the expenses of this work were defrayed. They also wish to express their gratitude to their colleagues who co-operated in the collection of urine samples, and to Messrs Parke Davis and Co. for a gift of 'theelol'.

REFERENCES

Bachman, C. & Pettit, D. S. (1941). *J. biol. Chem.* **138**, 689.
Callow, N. H., Callow, R. K., Emmens, C. W. & Stroud, S. W. (1939). *J. Endocrinol.* **1**, 76.
Cohen, S. L. & Marrian, G. F. (1934). *Biochem. J.* **28**, 1603.
Cohen, S. L. & Marrian, G. F. (1935). *Biochem. J.* **29**, 1577.
Emmens, C. W. (1939). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 234. London: H.M. Stationery Office.
Jayle, M., Crépy, O. & Judas, O. (1943). *Bull. Soc. Chim. biol., Paris*, **25**, 301.
Kober, S. (1931). *Biochem. Z.* **239**, 209.
Kober, S. (1938). *Biochem. J.* **32**, 357.
Pincus, G. (1945). *J. clin. Endocrinol.* **5**, 291.
Smith, G. van S. & Smith, O. W. (1935). *Amer. J. Physiol.* **112**, 340.
Smith, G. van S., Smith, O. W. & Pincus, G. (1938). *Amer. J. Physiol.* **121**, 98.
Stimmel, B. F. (1946). *J. biol. Chem.* **162**, 99.
Venning, E. H., Evelyn, K. A., Harkness, E. V. & Browne, J. S. L. (1937). *J. biol. Chem.* **120**, 225.

The Influence of *l*-Ascorbic Acid on the Rupture of the Benzene Ring of *l*-Tyrosine Consumed in High Doses by Guinea-pigs

By H. A. PAINTER and S. S. ZILVA (Member of the Scientific Staff, Medical Research Council), *Lister Institute, London*

(*Received* 7 *February* 1947)

Kotake, Matsuoka & Okagawa (1922) observed that when a massive dose (10 g.) of *l*-tyrosine was administered to adult rabbits the aromatic part of the *l*-tyrosine molecule was not fully metabolized, since *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids were excreted in the urine. Recent work by Sealock & Silberstein (1939, 1940) and Levine, Marples & Gordon (1939, 1941*a*, 1941*b*) threw a new light on the rupture of the benzene ring of *l*-tyrosine by the animal organism. The former investigators found that when 0·5 g. or more of *l*-tyrosine was administered to guinea-pigs on a scorbutic diet, homogentisic, *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids were excreted in the urine. When, however, *l*-ascorbic acid was administered in sufficiently high doses the excretion of these aromatic compounds ceased. Levine and his co-workers made a similar observation on infants. They found that premature infants on diets containing 5 g./kg. body weight/day or more of protein excreted *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic, but not homogentisic acids. On the other hand, full-term infants on a similar diet only excreted these aromatic amino-acids when they consumed 1 g./kg. body weight of either *l*-tyrosine or *l*-phenylalanine. In both cases the excretion of the aromatic amino-acids could be abolished by the consumption of *l*-ascorbic acid. These observations led to a tentative suggestion that *l*-ascorbic acid participated directly or indirectly in protein metabolism. The experiments described in this communication were primarily devised to test this hypothesis.

EXPERIMENTAL

Technique

Experimental animals and diet (see Penney & Zilva, 1946).

Collection of urine. The experimental animals were kept in metabolism cages and the urines were collected in tubes which, except in experiments of short duration, contained sufficient HCl to keep the urines acid to congo red. The urines to be tested for homogentisic acid, were collected under liquid paraffin.