An Investigation into the Chemical Estimation of the Oestrogens in Urine

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In view of the great difficulties inherent in the biological estimation of the oestrogens, the introduction of a satisfactory chemical method would be a notable advance. Until recently no chemical method was claimed to be suitable for the estimation of the oestrogens in normal urine. The method of Kober (1931) and that of Bachman (1939) and others can be used only in the assay of pregnancy urine, of which the oestrogen content is nearly a thousandfold greater than that of normal urine. When, therefore, Talbot, Wolfe, MacLachlan, Karush & Butler (1940) introduced a new chemical method by which they claimed it was possible to estimate oestrone and oestradiol in normal urine, it was considered advisable to investigate the method, because the authors had not verified it by comparison with biological assay.

METHODS

The method of Talbot *et al.* consists essentially of the isolation of the weakly phenolic ketones in urine, coupling with diazotized dianisidine, and the measurement of the colour produced.

Extraction of urine. The method used is based upon that of Smith, Smith & Schiller (1939). The process initially used in the present series of investigations was carried out as described by Talbot *et al.* Details in the example of rat urine were as follows.

Urine was collected from a group of rats into a flask containing 1 ml. of conc. HCl and a sample taken, equivalent to the daily output of 10 rats (usually of the order of 70 ml., including rinsings). Conc. HCl (10 ml.) was added and the solution boiled under reflux for 10 min. After cooling it was extracted with ether (4 × 25 ml.). The ether extract was washed with 20% Na₂CO₃ (3 × 20 ml.) (all the coloured impurity is not removed by the single washing recommended by Talbot), and then extracted alternately with 0.1N-NaOH (3 × 25 ml.) and 0.1N-NaOH containing 10% Na₂S₂O₄ (2 × 25 ml.). (The extract was shaken with the hydrosulphite on each occasion for 3 min.) The aqueous residue containing strongly acidic and phenolic material (including oestriol) was discarded. The ether extract was washed with 0.5N-HCl (2 × 25 ml.) and water (3 × 25 ml.), evaporated to dryness and redissolved in 50 ml. of toluene. This solution was extracted with N-NaOH (4 × 20 ml.) and the toluene residue, containing neutral ketosteroids, discarded. The alkaline extract was acidified to litmus but not to Congo red with conc. HCl and extracted with ether (5 × 30 ml.). The ether was washed alternately with

0.1N-NaOH (2 × 20 ml.) and 0.1N-NaOH-Na₂S₂O₄ (1 × 20 ml.), then with 0.5N-HCl (2 × 20 ml.) and finally with water (3 × 25 ml.). The ether was evaporated and the residue taken up in 4 ml. ethanol and refluxed for 0.5 hr. with 0.5 ml. of glacial acetic acid and 0.5 g. Girard's reagent T. After the addition of 40 ml. of ice-water and 3 ml. of 10% NaOH, the non-ketonic fraction was extracted with ether (4 × 40 ml.). This ethereal extract contained the oestradiol and after washing with 0.1N-NaOH (2 × 25 ml.) and water (2 × 25 ml.), was dried and ready for colorimetric assay.

The aqueous residue was acidified with 1 ml. of conc. H₂SO₄ and, after the addition of 60 ml. of water and 40 ml. of ether, left for 1.25 hr. at room temp. It was then extracted with ether (4 × 25 ml.) and the ether extract washed with 25 ml. of 0.1N-NaOH and 3 × 25 ml. of water. This ether-soluble fraction contained the oestrone. The ether solutions were dried with anhydrous Na₂SO₄, the ether distilled off and 0.8 ml. of redistilled ethanol added. Two 0.3 ml. portions were used for the colour reaction.

It was found possible to shorten the extraction process as follows, without affecting the final assay figures: (1) The hydrosulphite washings were omitted. (2) The Girard reaction was completed in 15 min. instead of 30 min. (3) The Girard complex was hydrolyzed by boiling for 5 min., instead of standing at room temp. for 1.25 hr.

When the evidence (shown later) indicated that the shortened process gave results identical with those obtained by using Talbot's full extraction procedure, the shortened process was used for all the subsequent extractions.

The reagent was prepared and the colour reaction carried out as described by Talbot *et al.* The colour was estimated in a Spekker Photoelectric Absorptiometer, using a No. 6 blue filter (λ of max. transmission 4800–5000 Å.).

Calibration curve. We had difficulty at first in constructing a calibration curve for oestrone, because we were unable to produce a consistent depth of colour with crystalline oestrone when the reaction was carried out as directed by Talbot *et al.* This difficulty was overcome by carrying out the reaction at about 0°. The ethanolic solution of the hormone containing the added Na₂CO₃ was immersed in ice and salt for a few minutes before addition of the reagent. Consistent results were then obtained.

As an example, four samples of urine extract were treated with diazotized dianisidine at room temperature and the absorptiometer readings were 0.320, 0.180, 0.185 and 0.145 respectively. When the reaction was carried out at 0° the readings were 0.135, 0.140, 0.135 and 0.138 respectively. The calibration curve using known amounts of oestrone (within the range of 5–50 µg.) was then very similar to that constructed from Talbot's data and identical with that for oestradiol.

RESULTS

Extraction process

A number of assays of urine was carried out using the full extraction method of Talbot and the modified procedure as described above. Both methods gave identical results as indicated in Table 1.

Table 1. *Comparison of Talbot's full extraction method and the modified procedure (rat urine)*

	Talbot method ($\mu\text{g.}/10$ rat-days)	Modified procedure ($\mu\text{g.}/10$ rat-days)
Ketonic fraction	27	29
Non-ketonic fraction	63	55

Assay of urine samples

Urine from three different sources, human male and female, and female rats (three batches), was extracted and the oestrogen estimated chemically. The results are given in Table 2.

Table 2. *Chemical estimation of oestrogens in urine*

	Ketonic ($\mu\text{g.}/100$ ml.)	Non-ketonic ($\mu\text{g.}/100$ ml.)
Human urine (male)	38	73
Human urine (female)	93	77
	($\mu\text{g.}/10$ rat-days)	
Rat urine (female) 1	31	83
2	44	123
3	40	58

The values obtained for human urine are considerably higher than the levels usually accepted (male urine 5–15 $\mu\text{g.}/\text{day}$, female 10–36 $\mu\text{g.}/\text{day}$ total oestrogen). We are unable to find figures in the literature for the oestrogen content of rat urine.

Comparison of biological and chemical assay

We decided to compare the results obtained by biological estimation of the oestrone in rat urine with those obtained by chemical estimation. The equivalent output of 200 rat-days of urine was extracted by the modified procedure and the ketonic fraction isolated. Three groups each of 10 mice were used for the urine assay and two control groups of 10 mice given crystalline oestrone. The mice were ovariectomized one week before injection. The material to be tested was dissolved in arachis oil and injected intramuscularly in three portions (0.25 ml. each) on the morning, evening and the following morning, and the vaginal smear examined on the

3rd, 4th and 5th day after the first injection. The results are shown in Table 3, from which it is evident that the biological estimation shows a content of 3 $\mu\text{g.}$ of oestrone in the equivalent of approx. 360 rat-days of urine. This is in marked contrast with the chemical estimation of 3 $\mu\text{g.}$ per rat per day.

It was clear that the chemical method measured material other than oestrogens. Evidence for this assertion was sought by carrying out procedures which could be expected to produce a marked change in the oestrogen content of urine.

Chemical assay of rat urine of varied oestrogen content

Urine from a group (A) of 14 rats was collected daily, before and after bilateral ovariectomy and the oestrogen content determined by the chemical method. It was anticipated that as a result of ovariectomy there would be a decreased output of oestrogen in the urine. The results in Table 4 indicate that there is no substantial difference in the oestrogen content of normal and ovariectomized rats' urine when assayed by the chemical method.

Table 4. *Chemical estimation of rat urine of varied oestrogen content*

	Ketonic ($\mu\text{g.}/10$ rat-days)	Non-ketonic ($\mu\text{g.}/10$ rat-days)
Before ovariectomy	30–35	60–100
2 days after ovariectomy	24	60
2 weeks after ovariectomy	26	114

It is well established that only 1–10 % of injected oestrogen can be recovered in the urine of women and experimental animals. The remainder cannot be traced in a biologically active form, and it is possible that it is excreted in the urine as a biologically inactive derivative of the oestrogens. We investigated the possibility that such an intermediate breakdown product may have been contributing to the colour reaction. Oestrone (150 $\mu\text{g.}$) was injected into each of a group (B) of 9 ovariectomized rats and a chemical assay of the urine excreted during the 48 hr. following the injection was carried out. Table 5 shows that the oestrone injection did not affect the excretion of material which reacts with diazotized dianisidine.

Eight duplicate estimations on the same batch of mixed human male urine gave values for the ketonic fraction ranging between 20 and 39, mean 30 $\mu\text{g.}/100$ ml.; and for the non-ketonic fraction, 51 and 83,

Table 3. *Comparison of biological and chemical estimation of oestrogen content of rat urine*

Biological method	Control—cryst. oestrone	0.085 $\mu\text{g.}/\text{mouse}$	7 animals in pro-oestrus
		0.135 $\mu\text{g.}/\text{mouse}$	5 in oestrus: 5 in pro-oestrus
	Urine extract	16 rat-days/mouse	5 in oestrus: 4 in pro-oestrus. 1 death
Chemical method	Urine extract	16 rat-day sample	48 $\mu\text{g.}$

Table 5. *Chemical estimation of oestrogens in rat urine*(Ovariectomized rats—group B—treated with 150 μ g. oestrone.)

	Before injection (μ g./10 rat-days)	After injection
Ketonic	24	20
Non-ketonic	57	60

mean 68 μ g./100 ml. Thus it is to be expected that a 50 % change in the oestrogen content of urine would be detectable by the chemical method. The administration of a total of 1350 μ g. of oestrone to a group of rats would, therefore, be expected to result in a significant rise in the urinary content of colour-reacting material if this were due to a breakdown product of oestrone.

Diazotized dianisidine reacts with all phenolic groupings and the extremely high values obtained in these urine assays are probably due to non-oestrogenic phenolic compounds. This possibility was tested by removing the phenols from the urine.

Removal of phenols

The phenols were extracted from the urine according to the method of Schmulovitz & Wylie (1935-6). In this method, the volatile phenols (phenol and cresol) are removed by steam volatilization. The non-volatile phenols (pyrocatechol) and the aromatic hydroxy acids (*p*-hydroxybenzoic, phenylacetic and phenylpropionic acids) are removed by exhaustive extraction from ethereal solution by sodium carbonate, while oestrone and oestradiol are not affected. This stage was included in the extraction of a sample of mixed male urine prior to the first ether extraction.

The ketonic fraction of the urine according to the chemical estimation was 500 μ g. in a 24 hr. sample before phenol removal and 42 μ g. in a 24 hr. sample after treatment. This last figure is approximately within the range of the accepted values obtained by biological methods. (The non-ketonic—oestradiol—fraction was too strongly coloured after this extraction procedure to be estimated colorimetrically.)

The excessively high values obtained by Talbot's method thus appear to be due to the incidental assay of phenols other than the oestrogens.

Recovery of added oestrone

A sample of mixed human male urine was extracted and after removing the phenols was assayed in duplicate. A portion of this urine was assayed after the addition of 200 μ g. of oestrone. None of the added hormone was detected.

Hence despite this modification of the process the chemical estimation of oestrogens in urine still remains unsatisfactory.

DISCUSSION

The results reported in the present paper show that Talbot's method for the chemical estimation of the oestrogens in urine gives results many times higher than those obtained by the biological method. If the chemical method gave a true measure of the oestrogen content of urine, the values obtained for the urine of totally ovariectomized animals should be considerably lower than in the case of normal animals. Such results were not obtained. This indicates that it is not oestrogenic material which is producing colour reaction.

The theory that the colour developed might be due to a biologically inactive metabolic product of oestrogen became untenable as the result of injecting a large dose of oestrone into a group of rats. If any appreciable proportion of the large dose of oestrone had been thus excreted it would have been readily detected, as the method has been shown to be capable of demonstrating a 50 % change in the urine content of material which reacts with diazotized dianisidine.

When the phenols are removed the results of the chemical estimation of the oestrogens are of the same order as those obtained by the biological method. Thus Talbot's method, relying as it does upon the isolation, by means of Girard's reagent, of weakly phenolic ketones (of which oestrone is said to be the only example present in urine) does not in fact effect this isolation and estimates other phenolic compounds.

It is clear that no chemical method is yet available for the estimation of oestrogens in normal urine and the biological assay remains the only reliable method.

SUMMARY

An investigation of Talbot's method (Talbot *et al.* 1940) of estimating oestrogenic substances in urine indicates that the results so obtained do not represent quantitatively the amount of oestrogen excreted in the urine.

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REFERENCES

- Bachman, C. (1939). *J. biol. Chem.* **131**, 455.
 Kober, S. (1931). *Biochem. Z.* **239**, 209.
 Schmulovitz, M. J. & Wylie, H. B. (1935-6). *J. Lab. clin. Med.* **21**, 210.
 Smith, O. W., Smith, G. V. & Schiller, S. (1939). *Endocrinology*, **25**, 509.
 Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F. & Butler, A. M. (1940). *J. biol. Chem.* **134**, 319.