LXXXII. SOME NOTES ON THE ISOLATION OF OESTRONE AND EQUILIN FROM THE URINE OF PREGNANT MARES.

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LAST year a method was reported [Beall and Marrian, 1934] for the isolation of oestrone from the urine of pregnant mares. By means of a solvent-partition process a crude toluene extract of the acid-hydrolysed pregnancy urine was purified to give a "weak phenol" concentrate containing the extracted oestrone. The ketonic material in this fraction was precipitated from alkaline solution by the use of mercuric hydroxide and the oestrone separated by high-vacuum distillation.

Several modifications in the process have since been introduced. Hydrolysis conditions have been improved, thus increasing greatly the yields of active material which can be removed by the initial toluene extraction; the solvent process has been shortened and the mercuric hydroxide-ketone reaction has been modified so that it is more easily conducted. By this modified process it is possible to obtain high yields of pure oestrone (M.P. 254–256° uncorr.) and also considerable amounts of equilin.

Acid hydrolysis of the urine.

The urine was collected in weekly batches and was concentrated *in vacuo* to about one-quarter of its original volume, all subsequent steps being carried out with such concentrates.

In the previous paper it was pointed out that it is more satisfactory to carry out the acid hydrolysis of mare's urine at room temperature than to boil or to autoclave the acidified material, since the liberated oestrone is readily destroyed in an acid medium at high temperatures. Extremely high yields of active material have been obtained by acidifying the urine concentrates to p_H 0·8–1·0 and keeping them at room temperature for 7–10 days before commencing the toluene extractions. These conditions are the same as those recommended by Curtis [1933] but much higher yields of oestrogenic material have been obtained than previously reported in the literature.

The toluene extracts were processed by the method already described and the oestrogenic materials in the final "weak phenol" concentrates were assayed by the colorimetric method of Cohen and Marrian [1934].

The "weak phenol" concentrates would be expected to contain other phenolic oestrogenic compounds such as equilin, hippulin and equilenin [Girard *et al.*, 1932, 1, 2, 3]. Whilst it was realised that these might also act as chromogens, the colorimetric assays were, for convenience, evaluated in terms of oestrone. It was recognised that such values might not be absolute, but it was

considered that they would, at least, be relative and serve as a basis of comparison for the different batches of urine. In cases where the "weak phenols" were assayed in terms of oestrone by the biological method the values obtained were, within experimental error, in agreement with the colorimetric figures, thus justifying this procedure.

Excretion of oestrin by the pregnant mare.

Urine was collected from a mare throughout the term of pregnancy and, after hydrolysis by the method described in the previous section, colorimetric assays were carried out on the "weak phenol" fraction. The concentration of oestrogenic substances in the urine was too low during the early stages of pregnancy to warrant their isolation. Collections were therefore limited to a period of one week at monthly intervals. As soon as the amounts of active material had risen sufficiently to make their isolation possible, all the urine excreted was collected. The results of the experiment are given in Table I.

Table I. *Oestrin excreted by a mare (number 439).*

Served Aug. 15, 1934; foaled July 14, 1935.

Volume of urine l.	Period of collection	Month of pregnancy	Chromogenic material in "weak phenol" conc. (assayed as oestrone) g.	Chromogenic material per litre unconc. urine mg.
23	Sept. 10–17	1	0·002	Trace
27	Oct. 9–18	2	0·020	0·7
45	Nov. 12–18	3	0·25	5·5
18	Dec. 10–17	4	0·59	33
32	Jan. 7–14	5	2·68	84
118	Jan. 14–Feb. 11	6	9·41	80
100	Feb. 11–Mar. 11	7	10·42	104
77*	Mar. 11–Apr. 22	8	7·98	104
77	Apr. 22–May 20	9	3·52	46
104	May 20–June 15	10	1·97	19
45	June 15–July 1	11	0·22	4·9

* Part of collection lost.

This table shows that the amount of oestrogenic material excreted in the urine starts to rise during the 3rd month of pregnancy, reaches a maximum during the 7th and 8th months and then falls off rapidly so that at term only a relatively small quantity is present. These findings are in agreement with those reported by other workers [Cole and Saunders, 1935; Kober, 1935].

It should be borne in mind that the values in Table I do not represent the total oestrogenic potency of the original urine since 20–30 % of the activity is removed in the "strong phenol" fraction. Since the oestrone is concentrated in the "weak phenol" fraction this loss of active material was not investigated.

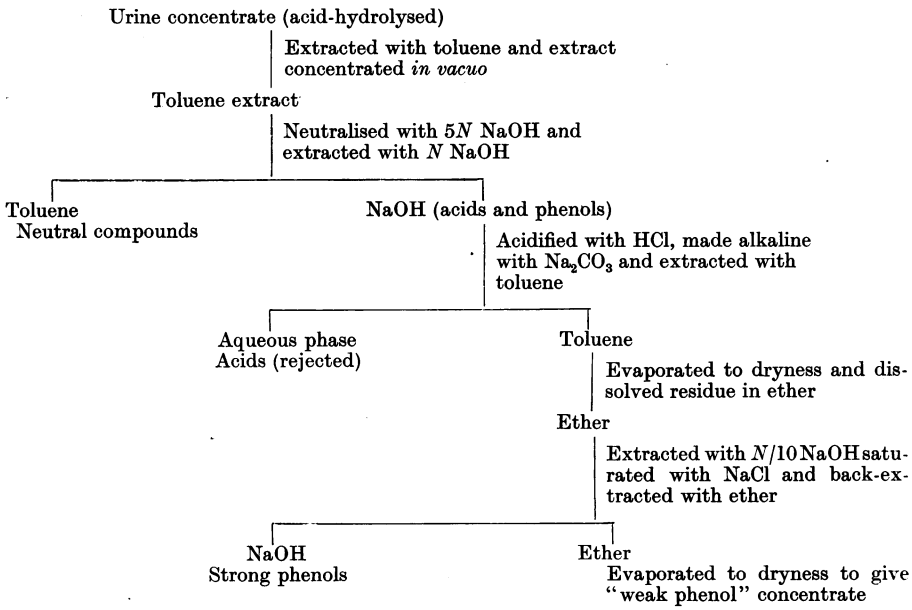
Modified solvent partition process.

The purification of the crude toluene extract of the acid-hydrolysed urine has been greatly shortened by the adoption of the following procedure.

After concentration of the initial toluene extract to a reasonable volume, sufficient 5*N* NaOH was added to neutralise the acidic substances present, the aqueous phase was removed and the toluene extracted with several portions of *N* NaOH. The combined aqueous extracts were acidified with HCl, made

alkaline with an excess of saturated Na_2CO_3 and re-extracted with toluene. This removed the greater part of the phenolic materials, including the oestrogenic compounds, while the acids remained in the aqueous phase as their sodium salts. The toluene was washed with water, evaporated to dryness and, as in the previously described procedure, the "strong" and "weak" phenols were separated from ether solution by washing with *N*/10 NaOH. It was found that saturation of the NaOH with NaCl prevented the retention of oestrone by the alkaline solution. The extraction process is summarised in Table II.

Table II. *Modified solvent purification process.*



A further purification of the "weak phenol" concentrates has been introduced before the mercury reaction. Vacuum-distillation of the concentrates at 90–100°/0·02–0·03 mm. removed 45–55 % of the weight without any loss of oestrogenic material. The residues obtained by this treatment were, in general, dark brown crystalline masses. Some typical results obtained by this procedure are given in Table III.

Table III. *Effect of distillation of "weak phenol" concentrates.*

Original weight of concentrate g.	Weight after distillation g.	% loss of weight	Colorimetric assay of residue (as oestrone) g.	% chromogenic material in distilled residue
3·90	2·07	47	0·72	35
11·01	5·80	47	2·82	49
4·43	2·17	49	1·26	58
4·45	2·09	53	1·50	71
2·93	1·49	49	0·90	60

Modified mercuric hydroxide-ketone reaction.

Interaction of the ketonic material and mercuric hydroxide was brought about, as previously described, by heating in aqueous alkaline solution. After cooling the mercury-ketone complex was precipitated by the addition of ammonium hydroxide and, after standing overnight, the solids were centrifuged out and washed. This procedure was found to be much more satisfactory than that previously described.

The washed complex was hydrolysed by boiling for 15 min. in 15% (by volume) alcoholic HCl and the hot solution was filtered to remove any insoluble mercury salts.

The tedious chloroform-potassium chloride separation for the removal of the mercuric salts from the ketonic materials has been dispensed with. Instead, the excess of HCl in the filtered hydrolysate was neutralised with NH_4OH, the solution being kept just acid to prevent mercuric hydroxide from precipitating. The alcohol was distilled off *in vacuo*, the residue diluted with water to dissolve the inorganic salts, and the granular ketonic material filtered off and washed well with water. To ensure the complete removal of mercuric compounds the washed solid was dissolved in alcohol and treated with H_2S. After filtering out any HgS, the alcohol was removed and the oestrogenic material in the residue was separated by sublimation at 160–170°/0·02–0·03 mm.

Attempts to recrystallise pure oestrone directly from this sublimate met with failure. However, utilising the quinoline reaction described by Butenandt and Westphal [1934] and recrystallising the purified product from 95% alcohol, the pure hormone was obtained.

Isolation of equilin.

In the previous paper it was pointed out that only part of the oestrogenic material of the "weak phenol" concentrates appeared in the mercury-ketone complex. This loss was found to be due mainly to equilin which occurs in the urine in relatively large amounts. It forms a mercury complex precipitated by NH_4OH, which differs from the oestrone complex in that it is soluble in alcohol and so is removed to a great extent in the alcoholic washings.

In order to recover the equilin from these washings they were concentrated to a small volume and the equilin complex was hydrolysed by adding 15% (by volume) HCl and boiling for 15 min. The hydrolysate was treated in a similar manner to the hydrolysate of the mercury-ketone complex and the crude equilin was filtered off. This material was distilled at 160–170°/0·02–0·03 mm. and the equilin (M.P. 238–239°) recrystallised from the distillate by the use of 80% alcohol.

Difficulty was encountered in certain batches of urine where the equilin content was higher than usual since in such cases it was not removed completely by the alcoholic washings of the mercury-ammonia precipitate and interfered with the final purification of the oestrone. This trouble was overcome by heating the distilled "weak phenol" concentrate with benzene (10 ml. benzene per g. of concentrate) before carrying out the mercury reaction. On cooling a crystalline precipitate was formed containing 80–90% of the oestrone together with so little impurity that it could be isolated directly by use of the quinoline reaction and subsequent recrystallisation from alcohol. The benzene mother-liquors were taken to dryness, the ketonic and non-ketonic compounds separated by the mercury reaction and the equilin and oestrone isolated from the alcoholic washings and mercury-ketone complex respectively in the manner already outlined.

Summary of the method finally adopted.

The acid-hydrolysed urine was extracted with toluene and the "weak phenols" separated by partition with solvents as outlined in Table II. After vacuum distillation this concentrate was divided into benzene-soluble and benzene-insoluble fractions. The ketonic compounds in the benzene-soluble fraction were precipitated by the mercuric hydroxide-ammonia reaction, the equilin and oestrone in this precipitate being separated by the use of alcohol. By acid hydrolysis of the equilin complex in the alcoholic washings, removal of the alcohol and vacuum-distillation of the residue, a crude equilin-containing distillate was obtained from which pure equilin was crystallised by the use of 80% alcohol. In a similar way acid hydrolysis of the mercury-ketone complex yielded a residue from which the crude oestrone-containing material was concentrated by sublimation. Purification of this sublimate was accomplished by the use of the quinoline reaction (Butenandt) and subsequent crystallisation of the oestrone from alcohol.

In the case of the benzene-insoluble material the oestrone was isolated directly by use of the quinoline reaction and subsequent crystallisation from alcohol, the mercury reaction being unnecessary for this fraction. Some typical results of the amounts of oestrone (M.P. 254–256°) isolated from these benzene-insoluble fractions are given in Table IV.

Table IV. *Oestrone isolated from benzene-insoluble fraction.*

Volume unconcentrated urine	Colorimetric assay of "weak phenols"	Weight benzene-insoluble material	Crude oestrone (from quinoline)	Recrystallised oestrone
l.	g.	g.	g.	g.
72	3·45	2·66	1·68	1·41
18	2·40	1·67	0·97	0·76
86	4·40	3·78	—	1·10
67	3·83	—	1·48	—
72	6·45	3·85	1·78	—

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