CLXXXIX. THE HYDROLYSIS OF THE COMBINED FORMS OF OESTRONE AND OESTRIOL PRESENT IN HUMAN PREGNANCY URINE.

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For several years it has been recognised that the yields of oestrogenic material which can be obtained from human pregnancy urine by extraction with immiscible fat-solvents may be considerably increased by preliminary acidification of the urine [Marrian, 1930; 1933; Doisy *et al.*, 1930]. Zondek [1934, 1] did, indeed, state that such preliminary treatment was unnecessary to ensure complete extraction of the hormones, but later [1934, 2] revised his opinions as a result of work carried out by Borchardt *et al.* [1934]. The latter authors were responsible for the first systematic attempt to determine the optimum conditions for the liberation of oestrin from its combined state in human pregnancy urine. They showed that the amount of hormone that could be extracted by prolonged boiling with benzene was greatly increased by acidification of the urine to p_H 3·0, and still further increased by the addition of hydrochloric acid to a concentration of 3·6 %.

The present authors [1934] developed a colorimetric method, based on the Kober test [1931] for oestrin, for the separate quantitative estimation of oestrone and oestriol in ethereal extracts of human pregnancy urine. This method enabled a more complete study of the factors influencing the hydrolysis by acids of the "oestrin-esters" in urine to be made than would have been possible if only the biological test for oestrin had been available. It was shown that when pregnancy urine was acidified to p_H 1–2 and then heated at 100°, there occurred a slow liberation of ether-soluble oestrin from the combined form, which reached a maximum after 16 hours. Heating for longer periods resulted in a decrease in the amount of ether-extractable oestrin, indicating that destruction of the liberated hormone had occurred. By heating the urine acidified to p_H 1–2 in an autoclave at 15 lbs. pressure for 2–4 hours, a larger amount of ether-soluble oestrin was liberated than when the urine was heated at 100°. Autoclaving for longer periods destroyed the liberated oestrin. This work clearly showed that, as might be expected, the rate of hydrolysis of the "oestrin esters" in urine is considerably increased by an increase in temperature, and that destruction of the hormone may occur on prolonged heating in an acid aqueous medium.

Few attempts have been made to effect liberation of the combined oestrin in urine by means other than that of hydrolysis with acids. Marrian [1933] found that keeping the urine until considerable bacterial decomposition had occurred was extremely effective as a means of liberating the combined oestrin, but that heating to 100° at an alkaline reaction for short periods was ineffective. Both these findings were confirmed by Cohen and Marrian [1934].

For two reasons it was clearly necessary to conduct a more detailed investigation of the factors involved in the hydrolysis of the "oestrin esters" in human pregnancy urine. In the first place, estimation of the oestrin content of urine

either by direct biological assays on the whole urine[1] or by colorimetric or biological assays on ethereal extracts, must necessarily be extremely inaccurate unless the conditions for optimum hydrolysis with minimum destruction are known. In the second place, data on the stability of the "oestrin esters" to mild treatment with acids and alkalis were required in order to devise methods for their isolation in a state of purity. To these ends therefore the effects of temperature and p_H (both acid and alkaline) on the rates of hydrolysis of the "esters" and on the rates of the destruction of the liberated oestrins have been studied in greater detail. Experiments have also been conducted to determine the nature of the destruction caused by prolonged hydrolysis.

Hydrolysis of the "oestrin esters" by acid.

(1) *The effect of temperature.* A finding of some practical importance has been that acid hydrolysis is almost undetectable at temperatures at or near 0°. Urines acidified to p_H 1·0 and stored for several weeks in the ice-box showed no appreciable increase in ether-soluble oestrin. The present experiments confirm the fact that for rapid and effective acid hydrolysis of the "oestrin esters" heating at about 120° in an autoclave is preferable to heating at 100°.

(2) *The effect of acidity.* As would be expected, the rate of hydrolysis of the "oestrin esters" is greatly increased with an increase in hydrogen ion concentration. Quantitative study of the effect of variations in p_H was however complicated by the fact that during hydrolysis the p_H of the urine increased very considerably owing to the formation of ammonia by the decomposition of urea. For instance a urine acidified to p_H 1·0 and then heated at 120° for 1 hour, may have a final p_H as high as 7. Since the extent of this change in p_H depends upon the urea content of the urine, which may be extremely variable, it has not been possible to determine the exact initial p_H of the urine necessary for optimum hydrolysis. It has been found however that maximum hydrolysis results after 2 hours' heating at 120°, when the final p_H of the urine is 1·0. In order to attain the conditions necessary to arrive at this final p_H, it is sufficient for the majority of urine samples to acidify to p_H 1·0 and then to add 3·3 ml. of 12*N* HCl per 100 ml. of urine. The concentration of urea may be so high in some specimens however that this excess of acid is insufficient to keep the final p_H to 1·0 or less. It is therefore essential to determine the p_H of the urine after hydrolysis, and if necessary to repeat the hydrolysis with a larger excess of acid. It must be emphasised however that the addition of too large an excess of acid is to be avoided, since the rate of destruction of the liberated oestrin is greatly increased with increased acidity (see following section).

(3) *Destruction of the oestrins by heating in acid solution.* Destruction of liberated oestrone and oestriol in urine by prolonged heating in acid solution was found to be considerably increased by an increase in temperature. A urine completely hydrolysed with respect to its "oestrin esters" was found to suffer little loss in liberated oestrin when stored in the ice-box at p_H 1·0 for several days. At 100° the rate of destruction was significantly more rapid. In one case 35 % of the total oestrin was destroyed by heating at p_H 1·0 for 20 hours longer than was necessary for complete hydrolysis of the esters. The rate of destruction was still further increased by heating at 120°.

In an early experiment it had been noticed that destruction of oestrin in a sample of acidified urine heated at 120° was greater when the autoclave was

[1] Preliminary experiments have shown that the combined forms of oestrone and oestriol in urine are much less active than the free forms in inducing vaginal cornification in ovariectomised mice.

opened at intervals during the hydrolysis than when the hydrolysis was allowed to proceed uninterruptedly for the same total time. This suggested that the oxygen absorbed by the urine from the air might be a factor in the destruction of the oestrin. Experiments were therefore conducted to test this possibility. It was found that the rate of destruction of oestrin during hydrolysis could be greatly increased by shaking the urine with air at intervals and still further increased by periodic saturation with oxygen. Thus it seems extremely probable that the destruction of oestrone and oestriol which occurs in prolonged heating of acidified urines is oxidative in character. It should be pointed out that destruction of oestriol in acid solution has invariably been found to be more rapid than destruction of oestrone under the same conditions. The possibility remained that this destruction might be caused through the agency of some constituent of the urine rather than by direct oxidation of oestrone and oestriol. It was shown however that marked destruction of pure oestrone and oestriol resulted when they were heated in aqueous acid solution. In this case destruction of oestriol was again observed to be more rapid than that of oestrone.

The practical significance of these findings is considerable. In order to carry out an estimation of the oestrin content of urine with any degree of accuracy, the conditions for acid hydrolysis must be such that maximum hydrolysis of the "esters" and minimum destruction of the liberated oestrin results. Actually it is difficult to obtain complete hydrolysis of the "esters" without some destruction of the free oestrin even when precautions against access of oxygen to the urine are observed. It has been found however that if the urines are heated to 120° for 2 hours after proper acidification (see previous section) the hydrolysis of the "esters" is complete as far as can be determined, whilst the destruction is not sufficiently great to introduce a serious error into the estimation.

Hydrolysis of the "oestrin esters" by alkali.

As has been previously mentioned, earlier work showed that heating alkaline urine to 100° for short periods was ineffective in causing hydrolysis of the combined forms of the oestrins. In the present work it has been shown that under more vigorous conditions slow hydrolysis of the "esters" does occur. After heating at 100° for 8 hours a urine sample containing NaOH at *N* concentration, a slight but perceptible degree of hydrolysis of the "esters" occurred. When such alkaline urines were heated at 120° in an autoclave the hydrolysis was considerably more rapid. During the first few hours a fairly rapid hydrolysis of both oestrone and oestriol "esters" was observed, so that at the end of 4 hours of heating approximately half of the total oestriol was liberated, while the proportion of oestrone "ester" hydrolysed under these conditions varied considerably with different urine samples, being complete in some cases and only about half complete in others. The oestrone and oestriol "esters" remaining unhydrolysed after 4 hours were found to be remarkably resistant to further hydrolysis even after the addition of more alkali. This curious state of affairs could not be explained on the grounds that the apparently unhydrolysed esters were destroyed, since by subsequent acid hydrolysis the remaining oestrone and oestriol were nearly quantitatively recovered. A possible explanation seemed to be that there were present in the urine two different types of oestrin esters, one of which was readily hydrolysed by alkali while the other was alkali-stable. This explanation would have been acceptable if it had not been for the fact that on more than one occasion it was observed that when a second sample of the urine was worked up some time after the first, there was a significant

difference in the relative proportions of the alkali-labile and alkali-stable forms. At present no explanation of this curious phenomenon can be given.

After heating alkaline urines at 120° for periods longer than were necessary to give optimum hydrolysis of the "esters" it was observed that there was a slow decrease in the amounts of ether-extractable oestrone and oestriol. Experiments carried out by heating solutions of pure oestrone and oestriol in *N* NaOH showed that destruction of the hormones occurred. It may be noted that the oestrin destroyed in this way cannot be recovered by subsequent acid hydrolysis, so that this phenomenon of slow destruction by heating with alkali is entirely distinct from the phenomenon of the incomplete hydrolysis of the "esters" by alkali referred to above. It was also shown that this alkaline destruction, like that caused by acids, is oxidative in nature.

Experimental.

(1) *The effect of temperature on the rate of hydrolysis of "oestrin esters" in human pregnancy urine.*

300 ml. samples of urine were adjusted to the desired p_H by addition of 12*N* HCl. The samples to be hydrolysed at 6° were placed in the ice-box; those to be hydrolysed at 100° and 120° were heated in a boiling water-bath and in an autoclave under 15 lbs. pressure respectively. The oestrone and oestriol in the hydrolysed urines were extracted, separated and estimated colorimetrically in the manner previously described [Cohen and Marrian, 1934]. Determinations of the amounts of free ether-soluble oestrone and oestriol were made in both batches of urine. The results are shown in Table I.

Table I.

Urine batch	Hydrolytic treatment			mg. oestrin per 100 ml. urine			
				Before hydrolysis		After hydrolysis	
	p_H	Temp. °C.	Time hours	Oestriol	Oestrone	Oestriol	Oestrone
P.U. 11	3·0	6	336	0·005	0·007	0·008	0·003
	1·0	6	336	0·005	0·007	0·008	0·002
	1·0	120	4	0·005	0·007	0·642	0·083
P.U. 8	1·0	100	4	0·085	0·019	0·750	0·100
	1·0	120	4	0·085	0·019	1·000	0·125

Table II.

Urine batch	p_H		mg. oestrin liberated per 100 ml. urine	
	Initial	Final	Oestriol	Oestrone
P.U. 11	3·0	6·0	0·008	0·006
	1·0	4·5	0·220	0·045
I.P.U. 6 B	1·0	4·0	0·400	0·060
	1·0 +10 ml. HCl	<1·0	1·420	0·100
P.U. 12	1·0	4·5	0·163	0·030
	1·0 + 3 ml. HCl	1·0	0·290	0·045
	1·0 + 5 ml. HCl	<1·0	0·420	0·060
	1·0 +10 ml. HCl	<1·0	0·550	0·070
	1·0 +20 ml. HCl	<1·0	0·600	0·080

(2) *The effect of acidity on the rate of hydrolysis of "oestrin esters".*

300 ml. samples of urine were acidified to p_H 1·0 (in one case to p_H 3·0) by the addition of 12*N* HCl. To certain of these samples different excesses of 12*N* acid were added. All the samples were then heated for 1 hour in the autoclave at 120°. The final p_H of the hydrolysed urine was measured and the liberated oestrone and oestriol were determined as usual. In each case control estimations on unheated acidified urines were carried out in order to determine the amounts of ether-soluble oestrone and oestriol initially present. The results are shown in Table II.

(3) *The effect of acidity on the rate of destruction of oestrin liberated by hydrolysis from the combined form in pregnancy urine.*

1200 ml. of urine (batch P.U. 12) were adjusted to p_H 1·0 and then divided into four 300 ml. samples. To three of these 5, 10 and 20 ml. excess of 12*N* HCl were added respectively. All four samples were then autoclaved at 120° for 4 hours. The samples were removed from the autoclave after 1 and 2 hours for readjustment of the p_H to 1·0; this readjustment was necessary only for the sample to which no excess of acid had been added. The oestrone and oestriol in each sample were then estimated in the usual manner. The results are shown in Table III. It will be seen that the sample heated for 4 hours at an approximately constant p_H of 1·0 gave nearly the same oestrone and oestriol values as were obtained by heating the urine for 1 hour with a larger excess of acid (see Table II). The 4 hours' heating with a large excess of acid caused considerable destruction of the oestrin, the amount of destruction being greater in the more strongly acidified samples.

Table III.

ml. 12*N* HCl added to 300 ml. urine at p_H 1·0	mg. oestrin per 100 ml. urine after 4 hours' heating		% destruction	
	Oestriol	Oestrone	Oestriol	Oestrone
0	0·625	0·090	0	0
5	0·583	0·087	7·0	3·3
10	0·508	0·082	20·0	9·0
20	0·416	0·072	34·0	20·0

(4) *The effect of oxygen on the rate of destruction of oestrin in urine by acid.*

Three 300 ml. samples of urine (batch P.U. 13) were strongly acidified by the addition of 10 ml. of 12*N* HCl after adjusting to p_H 1·0. One sample was heated continuously in the autoclave at 120° for 4 hours. A second sample was heated to 120° for four separate periods of 1 hour, being shaken thoroughly with air before each period. A third sample was also heated for four separate periods of 1 hour, but before each period it was thoroughly saturated with oxygen. Ether-soluble oestrone and oestriol were estimated in each after the hydrolyses. As a control, a fourth sample of urine was hydrolysed under conditions which give maximum liberation of free oestrin. A similar experiment was conducted on another batch of urine (P.U. 14) after a greater degree of acidification. The results are shown in Table IV.

Table IV.

Urine batch	ml. 12N HCl added to 300 ml. urine at p_H 1·0	Hydrolytic treatment at 120°	mg. oestrin per 100 ml. urine after hydrolysis		% loss	
			Oestriol	Oestrone	Oestriol	Oestrone
P.U. 13	*12	2 hours continuously	0·650	0·082	—	—
	10	4 hours continuously	0·675	0·083	0	0
	10	4 periods of 1 hour, shaking with air	0·600	0·080	11·0	4·0
	10	4 periods of 1 hour, saturation with oxygen	0·550	0·078	19·0	6·0
P.U. 14	*10	2 hours continuously	1·000	0·090	—	—
	20	4 hours continuously	0·865	0·090	13·5	0
	20	4 periods of 1 hour, shaking with air	0·642	0·085	35·8	5·6
	20	4 periods of 1 hour, saturation with oxygen	0·517	0·068	48·3	24·4

* Control experiments upon which the % losses in the last two columns are calculated.

(5) *The destruction of pure oestrone and oestriol by heating in acid solution.*

Aqueous solutions containing known amounts of oestrone and oestriol were prepared by the addition to water of small volumes of strong alcoholic solutions of the two compounds. The oestrone and oestriol in one such solution of 200 ml. were estimated directly without any further treatment. The losses due to the extractions were within the range of experimental error of the method of assay. To each of five other 200 ml. volumes of such solutions were added 20 ml. of 12N HCl. Two of these solutions were then heated at 120° in the autoclave for 3 hours continuously, three were autoclaved for three periods of 1 hour each, one being shaken with air before each period, while the remaining two were saturated with oxygen before each period of autoclaving. The oestrone and oestriol in each were then determined as usual. The results are shown in Table V.

Table V.

mg. oestrin initially present in aqueous solution		ml. 12N HCl added	Hydrolytic treatment at 120°	mg. oestrin recovered		% loss	
Oestriol	Oestrone			Oestriol	Oestrone	Oestriol	Oestrone
1·000	0·100	0	None	0·870	0·106	13·0	0
0·808	0·132	20·0	3 hours continuously	0·510	0·120	37·0	9
0·808	0·132	20·0	3 periods of 1 hour, shaking with air	0·390	0·110	52·0	17·0
0·808	0·132	20·0	3 periods of 1 hour, saturation with oxygen	0·210	0·065	74·0	50·0
—	0·200	20·0	3 hours continuously	—	0·157	—	17·2
—	0·200	20·0	3 periods of 1 hour, saturation with oxygen	—	0·083	—	58·5

(6) *The effect of temperature on the rate of hydrolysis by alkali of "oestrin esters".*

To 300 ml. samples of pregnancy urine were added 35 ml. of 40 % NaOH. The samples were then heated at 100° or 120° for the required periods. After acidification to litmus, the mixtures were extracted and the oestrone and oestriol liberated by the hydrolysis determined as usual. Control samples of each batch

of urine so treated were acid-hydrolysed under optimum conditions in order to determine the total amounts of oestrone and oestriol present. The results are shown in Table VI.

Table VI.

Urine batch	Heating conditions: Temp. °C.	Heating conditions: Time hours	mg. liberated per 100 ml. urine by alkaline hydrolysis: Oestriol	mg. liberated per 100 ml. urine by alkaline hydrolysis: Oestrone	Total oestrin present in combined form per 100 ml. urine: Oestriol	Total oestrin present in combined form per 100 ml. urine: Oestrone	% hydrolysis by alkali: Oestriol	% hydrolysis by alkali: Oestrone
P.U. 7	100	8	0·086	0·013	0·677	0·058	12·7	22·4
	100	2	0·022	0·003	0·677	0·058	3·2	5·2
P.U. 12	120	2	0·110	0·047	0·610	0·090	18·0	52·2

(7) *The effect of concentration of alkali on the rate of hydrolysis of "oestrin esters".*

To each of four 200 ml. samples of pregnancy urine (P.U. 16) were added 10, 25, 50 and 100 ml. respectively of 40 % NaOH. The samples were autoclaved in nickel crucibles[1] at 120° for 11 hours, acidified to litmus with HCl and assayed for oestrone and oestriol as usual. The maximum amounts of oestrone and oestriol obtainable by acid hydrolysis under optimum conditions were determined in a separate sample of the same urine. From these figures the percentage hydrolysis of the "ester" by alkali in each experiment was determined. It will be seen from the results shown in Table VII that increasing the amount of alkali from 10 to 50 ml. per sample caused increased hydrolysis of the esters. Further increase in the alkali concentration however did not increase the hydrolysis. It will be further seen that the maximum hydrolysis obtainable by alkali is considerably less than the maximum hydrolysis obtained by acid.

Table VII.

ml. 40 % NaOH added to 200 ml. of urine	mg. total oestrin per 100 ml. determined by acid hydrolysis: Oestriol	mg. total oestrin per 100 ml. determined by acid hydrolysis: Oestrone	mg. oestrin per 100 ml. liberated by alkaline hydrolysis: Oestriol	mg. oestrin per 100 ml. liberated by alkaline hydrolysis: Oestrone	% hydrolysis by NaOH: Oestriol	% hydrolysis by NaOH: Oestrone
10	1·06	0·170	0·140	0·015	13·1	8·8
25	,,	,,	0·440	0·087	41·5	51·2
50	,,	,,	0·538	0·097	50·8	57·0
100	,,	,,	0·538	0·067	50·8	39·4

(8) *The inability of alkali to effect complete hydrolysis of "oestrin esters".*

To each of three 150 ml. samples of urine (P.U. 17) were added 50 ml. of 40 % NaOH. The samples were then autoclaved at 120° for 4, 8 and 13 hours respectively. After acidification with HCl, the liberated oestrin was extracted and estimated as usual. The acidified urines after extraction were adjusted to p_H 1·0 and further autoclaved for 2 hours in order to hydrolyse the esters which were unattacked by the alkali. An acid hydrolysis of the original urine was also carried out in order to determine the total oestriol and oestrone present. It will be seen from Table VIII that the total amounts of oestrin liberated by alkaline hydrolysis and subsequent acid hydrolysis are identical within the limits of experimental error in each case with the amount of total oestrin as determined in the original urine by acid hydrolysis.

[1] Nickel crucibles were preferable for these alkaline hydrolyses owing to the fact that the strong alkali in the urines rapidly attacked glass.

Table VIII.

mg. total oestrin determined by acid hydrolysis		Period of alkaline hydrolysis	mg. oestrin liberated by alkali (A)		mg. oestrin liberated by subsequent acid hydrolysis (B)		(A) + (B)	
Oestriol	Oestrone	hours	Oestriol	Oestrone	Oestriol	Oestrone	Oestriol	Oestrone
1·17	0·060	4	0·43	0·029	0·60	0·064	1·03	0·093
,,	,,	8	0·55	0·035	0·47	0·058	1·02	0·093
,,	,,	13	0·59	0·035	0·45	0·056	1·04	0·091

In order to confirm the fact that complete hydrolysis of "oestrin esters" cannot be attained by alkali, a 200 ml. sample of P.U. 16 which had been previously hydrolysed for 11 hours with alkali (see Table VII) and from which the liberated oestrin had been removed by ether extraction, was re-hydrolysed for 4 hours after the addition of 25 ml. of 40 % NaOH. This treatment liberated only 0·029 mg. of oestriol and 0·004 mg. of oestrone. Subsequent acid hydrolysis liberated 0·28 mg. of oestriol and 0·03 mg. of oestrone.

(9) *The destruction of pure oestrone and oestriol by heating with alkali in presence of oxygen.*

Two solutions containing 1 mg. of oestriol in 100 ml. of water were prepared. To each were added 30 ml. of 40 % NaOH. One sample was autoclaved at 120° for an uninterrupted 3-hour period. The other was saturated with oxygen and autoclaved for three periods of one hour, the solution being resaturated with oxygen at each interval. Two solutions containing 0·2 mg. of oestrone in 100 ml. of water were similarly treated. After hydrolysis the samples were acidified, and the oestrin was extracted and estimated as usual.

Table IX.

	Hydrolytic treatment	mg. estimated after heating	% destruction
1·0 mg. oestriol	3 hours continuously	0·51	49·0
,, ,,	3 periods of 1 hour, saturation with oxygen	0·06	99·4
0·2 mg. oestrone	3 hours continuously	0·114	42·0
,, ,,	3 periods of 1 hour, saturation with oxygen	0·010	95·0

Summary.

1. A study has been made of the factors which influence the rate of hydrolysis of the ether-insoluble oestrone and oestriol "esters" present in human pregnancy urine.

2. When urine is adjusted to p_H 1·0, the hydrolysis is negligible at 6°. When the final p_H of the urine is about 1·0, hydrolysis is complete in about 16 hours at 100° and in 2 hours at 200°. Owing to utilisation of the acid by the hydrolysis of urea, the initial p_H of the urine must be below 1·0. The rate of hydrolysis of the "esters" is increased by further increasing the acidity of the urine.

3. Destruction of both oestrone and oestriol occurs when they are heated in acid solution in the presence of oxygen. The rate of destruction is increased by increasing the acid concentration and by frequent introduction of oxygen. The rate of such destruction of oestrone is less than that of oestriol.

4. In order to make possible the accurate assay of oestrone and oestriol in pregnancy urine it is necessary to hydrolyse the urine under conditions which

combine maximum hydrolysis of the esters with minimum destruction of the liberated oestrone and oestriol. These conditions are generally fulfilled when the urine is adjusted to p_H 1·0, further acidified by the addition of 3·3 ml. of 12N HCl per 100 ml. of urine and autoclaved at 120° for 2 hours. The final p_H of the urine must however be determined in every case and if it is found to be greater than 1·0, the hydrolysis must be repeated with a larger excess of acid.

5. Hydrolysis of about 50 % of the oestriol "ester" in pregnancy urine can be effected by heating the urine made 2N with NaOH to 120° for 6–8 hours. In some, but not in all urines this treatment hydrolyses all the oestrone "ester". Further hydrolysis in the presence of higher concentrations of alkali or for longer periods is ineffective in breaking down the alkali-resistant "ester" fraction. These alkali-resistant "esters" can be subsequently completely hydrolysed by acid. The significance of the incomplete hydrolysis of the "esters" by alkali is not yet understood.

6. Prolonged heating in alkaline solution in the presence of oxygen results in destruction of both oestrone and oestriol.

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