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INVESTIGATIONS INTO THE HYDROLYSIS OF CONJUGATED OESTROGENS

I. THE VALUE OF VARIOUS PURIFICATION PROCEDURES PRIOR TO HYDROLYSIS

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SUMMARY

Oestrone, 2-methoxyoestrone, 17β-oestradiol and oestriol were estimated after either acid or enzyme hydrolysis in pregnancy urines which had been subjected to gel-filtration or conjugate extraction prior to the hydrolysis. The results were compared with those obtained after hydrolysis performed on untreated urine. Neither gel-filtration nor conjugate extraction seem to offer any significant advantage when a method of high specificity is used. Enzyme hydrolysis gave higher values for 17β-oestratiol and oestriol than did acid hydrolysis, whereas the opposite was found for oestrone; concerning 2-methoxyoestrone no difference could be found between acid and enzyme hydrolysis.

INTRODUCTION

IN METHODOLOGY the separation of specific from non-specific substances occurring in biological fluids is a difficult problem of tremendous importance. The number of purification and separation procedures proposed and sometimes used is therefore enormous.

A tempting purification step is the removal of impurities through isolation of the steroid conjugates prior to hydrolysis. This could be valuable for two reasons, firstly through removal of substances interfering with the estimation and secondly, through improving the conditions for the hydrolysis of the conjugate.

Various procedures to this purpose have been proposed, one of the most popular being the extraction of the conjugate with organic solvents, sometimes as a so-called forced extraction after increasing the salt concentration of the urine (for review see Jayle[7]). A more recent procedure is gel-filtration on Sephadex columns, which is claimed to give purer extracts and to remove inhibitors of enzyme hydrolysis [2] and also to improve the acid hydrolysis [1].

Conjugate extraction as well as gel-filtration are, however, rather complicated procedures and we have therefore investigated whether the advantages of these methods are in reasonable proportion to the efforts involved.

EXPERIMENTAL

Pregnancy urines with a high content of oestriol, but without glucose or protein, were selected for the experiments.

Trivial names. Oestrone: 3-hydroxy-1,3,5(1o) oestratriene 17-one. Oestradiol: 1,3,5(1o)-oestratriene 3,17β diol. Oestriol: 1,3,5(1o)-oestra triene-3,16α,17β-triol. 2-methoxyoestrone: 3-hydroxy-1,3,5(1o) oestratriene, 17-one, 2-methylether.

Gel-filtration. Swollen Sephadex G-25 medium was packed in a 40 × 2·5 cm column. 1/50 of a 24-hr urine was applied to the column and eluted with distilled water with a flow-rate of 3–4 ml per min. For each urine investigated a pilot experiment was performed to find out the elution pattern of oestriol which corresponded closely to that described by Beling[2]. The conjugated oestriol was

usually eluted in the fraction from 180 to 270 ml. When the fraction was collected for further studies, a safety margin of 20 ml to each side was included. Preliminary experiments indicated that the conjugates of oestrone and 17β-oestradiol were eluted in the same fraction as the conjugated oestriol.

The free oestrogens appeared much later over a very broad zone (420–590 ml). After use the columns were therefore eluted with 400 ml distilled water to remove the free oestrogens.

Conjugate extraction. In earlier experiments the butanol extractions did not give satisfactory results and we therefore used the forced extraction described by Edwards *et al.*[4] in a modification described by Frandsen *et al.*[6]. The urine samples were diluted to the same volume as obtained after the gel-filtration. After acidification to pH 1 with sulphuric acid and addition of 50 per cent (w/v) ammonium sulphate, the urine was extracted three times with 1 vol. of ethyl–acetate–ethanol (3 + 1). The combined organic phases were washed with 1/10 vol. of 50 per cent ammonium sulphate and evaporated.

Untreated urine was diluted with distilled water to the same volume as obtained after gel-filtration.

Enzyme hydrolysis was performed as described by Jayle [7], pH was adjusted to 5·2 and 1/10 vol of 3M acetate buffer pH 5·2 and 10 drops of chloroform were added. After addition of 1000 units of β-glucuronidase and 8000 units of sulphatase (suc gastrique d'helix pomatia, Industrie biologique française) per ml of diluted urine, the specimens were incubated at 37°C for 24 hr.

Acid hydrolysis was performed as described by Frandsen [6]. After addition of 3 per cent sulphuric acid the urine was autoclaved at 127°C for 1 hr.

The hydrolysed urine was extracted with 3 vol. of diethylether and the ether extract purified according to Brown [3]. The residue was dissolved in 1 ml ethanol, 25 ml benzene and 25 ml *n*-hexane and then extracted twice with 25 ml distilled water. In the aqueous phase oestriol was estimated as described by Brown [3], but due to the large amounts only 1/25 was subjected to chromatography. The organic phase was evaporated and oestrone, 2-methoxyoestrone and 17β-oestradiol were estimated according to the method of Svenstrup [8] involving Girard-separation, methylation, chromatography on columns of aluminium oxide and Kober colour reaction. All estimations were performed in duplicate.

RESULTS

The results are given in Tables 1–4. In Table 1 we have the values found in the 2-methoxyoestrone fraction and it is seen that there was no difference between the values found after acid hydrolysis or enzyme hydrolysis. The values found after conjugate extraction were comparable to those found in the untreated urines, whereas the values after gel-filtration were lower. It should be noted that the values in urine no. 18920 are below the sensitivity of the method.

The oestrone fraction (Table 2) presented higher values after acid hydrolysis than after enzyme hydrolysis, and here again no difference was observed between the conjugate-extracted and the untreated urines, whereas the values found after gel-filtration were definitely lower.

Concerning the oestradiol fraction (Table 3), higher values were found after

Table 1. *2-Methoxyoestrone.* Values in microgram per 24 hr

Urine no.	Acid			Enzyme			Free
	gel-filtr.	untreated	conj. extr.	gel-filtr.	untreated	conj. extr.	
11329	95	158	165	97	163	163	63
18920	4	10	8	7	11	13	0
20762	905	937	920	890	937	937	7
19165	417	424	401	399	418	408	0
11317	38	45	47	41	48	42	0
Mean	298	315	309	287	315	313	14
% of untreated acid	95	100	98	91	100	100	

Table 2. *Oestrone*. Values in microgram per 24 hr

Urine no.	Acid			Enzyme			Free
	gel-filtr.	untreated	conj. extr.	gel-filtr.	untreated	conj. extr.	
11329	270	639	627	200	530	515	450
18920	400	510	402	279	370	369	97
20762	980	1045	1060	782	870	855	57
19165	1883	2085	2145	1853	1923	1943	137
11317	471	516	515	401	440	427	53
Mean	800	958	948	703	825	823	158
% of untreated acid	84	100	99	74	86	86	

Table 3. *17β-oestradiol*. Values in microgram per 24 hr

Urine no.	Acid			Enzyme			Free
	gel-filtr.	untreated	conj-extr.	gel-filtr.	untreated	conj-extr.	
11329	123	239	229	135	220	235	120
18920	160	289	204	280	352	297	39
20762	356	377	383	406	422	414	14
19165	723	711	658	798	833	755	22
11317	143	141	143	158	167	167	20
Mean	301	351	323	355	399	373	43
% of untreated acid	86	100	92	101	114	106	

Table 4. *Oestriol*. Values in microgram per 24 hr

Urine no.	Acid			Enzyme			Free
	gel-filtr.	untreated	conj-extr.	gel-filtr.	untreated	conj-extr.	
11329	18·7	20·5	21·0	25·9	27·3	26·0	3·0
18920	28·8	31·4	29·2	31·4	33·3	32·8	1·3
20762	30·0	29·4	30·5	31·6	34·1	32·0	1·7
19165	28·5	28·8	28·4	32·0	31·1	31·1	0·3
11317	22·7	22·8	20·7	24·7	25·5	22·3	0·5
Mean	25·8	26·6	25·9	29·1	30·3	28·9	1·4
% of untreated acid	97	100	97	110	114	109	

enzyme hydrolysis than after acid hydrolysis. Conjugate extraction gave slightly lower values and gel-filtration definitely lower values.

In the oestriol fraction (Table 4) higher values were found in the enzyme hydrolysed specimens than in the acid hydrolysed ones. Gel-filtration and conjugate extraction gave slightly lower values, but the difference was small.

The purification obtained through the procedures prior to hydrolysis is illustrated in Table 5 where the density of the Kober colour at 400 mμ, which is outside the absorption region of the oestrogen Kober colour, was used as a measure for the degree of purity. It is seen that neither conjugate extraction nor gel-filtration resulted in significant purification of the extracts. The extracts were definitely more pure after enzyme hydrolysis than after acid hydrolysis, apart from the oestriol fraction, where the opposite was found, but apparently this difference was eliminated by the Allen-correction.

Table 5. Density of the Kober reaction at 400 mμ. Mean values of the urine samples investigated based on 1/50 of a 24 hr urine, except for oestriol where a 1/1250 of a 24 hr urine was used

	Acid			Enzyme		
	gel-filtr.	untreated	conj. extr.	gel-filter.	untreated	conj. extr.
2-methoxyoestrone	0·113	0·130	0·142	0·059	0·061	0·062
Oestrone	0·163	0·180	0·184	0·098	0·107	0·103
17β-oestradiol	0·182	0·203	0·190	0·096	0·089	0·091
Oestriol	0·126	0·127	0·121	0·170	0·158	0·151

DISCUSSION

Although the series only comprises five urine specimens, the uniformity of the results allow certain conclusions to be made, and the negative outcome does not warrant the investment of further time-consuming efforts. The gel-filtration or conjugate extraction prior to hydrolysis seems to be of little value since the yield of oestriol, oestrone, 17β-oestradiol and 2-methoxyoestrone was not increased by these procedures, and no significant purification was obtained through these rather laborious procedures. Beling[2] found that the enzyme hydrolysis gave higher results after gel-filtration of the urine because the gel-filtration removed some inhibitors. Our experiments did not confirm this, but it should be noted that we used ten times more enzyme than recommended by Beling[2]. If the inhibition of the enzyme hydrolysis could be overcome by an increase in the amount of enzyme, this procedure must certainly be preferable to that of gel-filtration.

Adlercreutz[1] has found that acid hydrolysis also gives higher yields after gel-filtration. Our experiments did not confirm this observation, probably because we diluted the untreated urine to the same volume as obtained after gel-filtration. It is a common observation that higher values are found after dilution of urine prior to acid hydrolysis, and as a matter of fact simple dilution of urine is more economic in time and materials than gel-filtration.

The higher oestriol and 17β-oestradiol values found after enzyme hydrolysis are probably due to a less pronounced destruction, but the possibility of incomplete acid hydrolysis cannot be ruled out entirely. The higher oestrone values found after acid hydrolysis were rather unexpected and could be due to the fact that some of the oestrone is excreted as oestrone sulphate, which is difficult to hydrolyse enzymatically. At present we are investigating these problems.

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