acetone followed by an acid solution containing *p*-dimethylaminobenzaldehyde, and as further confirmation the presence of galactose and rhamnose was shown by carrying out a two-dimensional chromatogram according to the technique described for amino-acids by Consden, Gordon & Martin (1944).

With the blood group A substance, the analysis was complicated by the fact that only some 70 % of the material was carbohydrate in nature, and an interfering substance was found which gave rise to a spot at R_F 0.26 in collidine after spraying with the ammoniacal silver nitrate reagent. This substance was removed by treatment with a cation-exchange resin, and was therefore considered to be basic in nature. The analysis showed clearly the presence of glucosamine, galactose and fucose, but, in spite of indications in the literature of the presence of mannose in allied blood group A substances, this sugar was not found in the hydrolysis products of the polysaccharide under investigation.

The analysis of these two trial substances indicates clearly the importance of control of the hydrolysis conditions, and in assessing the results of such analyses it should be borne in mind that the hydrolysis of complex polysaccharides may proceed

in stages, some destruction of the less stable sugar residues taking place before complete liberation of residues constituting a resistant core.

SUMMARY

1. The method of separating reducing sugars on filter-paper partition chromatograms described in this volume p. 238 has been applied to the analysis of the products of hydrolysis of two carbohydrate-containing materials: (a) the specific polysaccharide of *Bact. dysenteriae* (Shiga), and (b) the blood group A substance from hog gastric mucin.

2. The results of the analysis of (a) agree with results in the literature in showing the presence of glucosamine, galactose and rhamnose.

3. In the hydrolysis products of (b) glucosamine, galactose and fucose were found. Mannose, which has been observed in active polysaccharide fractions from other sources, was not found in the specific substance from hog gastric mucin.

The author wishes to express his thanks to Dr W. T. J. Morgan for his interest and for the gift of the polysaccharides used in this investigation.

This work forms part of the programme of the Food Investigation Board of the Department of Scientific and Industrial Research.

REFERENCES

- Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1946). *Nature, Lond.*, **158**, 879.
 Bray, H. G., Henry, H. & Stacey, M. (1946a). *Biochem. J.* **40**, 124.
 Bray, H. G., Henry, H. & Stacey, M. (1946b). *Biochem. J.* **40**, 130.
 Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
 Landsteiner, K. & Hart, R. A. (1940). *J. exp. Med.* **71**, 551.
 Meyer, K., Smyth, E. & Palmer, J. (1937). *J. biol. Chem.* **119**, 73.
 Morgan, W. T. J. (1936). *Biochem. J.* **30**, 909.
 Morgan, W. T. J. (1937). *Biochem. J.* **31**, 2003.
 Morgan, W. T. J. (1938). *Helv. chim. Acta*, **21**, 469.
 Morgan, W. T. J. (1944). *Brit. med. Bull.* **2**, 165.
 Morgan, W. T. J. (1945). *Rep. chem. Soc.* **42**, 231.
 Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
 Wiener, A. S. (1943). *Blood Groups and Transfusion*, 3rd ed. Baltimore: C. Thomas.

Urinary Elimination of Synthetic Oestrogens and Stilboestrol Glucuronide in Animals

By A. E. WILDER SMITH (Countess of Lisburne Memorial Fellow)
 AND P. C. WILLIAMS (Late Proffit Research Student)
Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1

(Received 25 June 1947)

Clinical experience has shown that oestrogen treatment of carcinoma of the prostate fails after a varying lapse of time in many cases. Although a similar delayed recurrence of symptoms also frequently follows treatment of prostatic carcinoma by castration (Angrist & Khoury, 1944; Nesbit, Pazzos & Cummings, 1944; Huggins, 1946) there is a slight possibility that the failure of continued

oestrogen treatment (in spite of increased dosage) is due to some bodily adaptation involving an altered metabolism of the drug. Hence it was thought worth while to study the metabolism of synthetic oestrogens in patients and animals; at the same time the opportunity has been taken to collect the mono-glucuronides of dienoestrol (4:4'-dihydroxy- γ : δ -diphenylhexa- β : δ -diene), hexoestrol (4:4'-dihydroxy-

γ : δ -diphenyl-*n*-hexane), and stilboestrol (4:4'-dihydroxy- α : β -diethylstilbene) for chemical and biological investigation (Simpson & Wilder Smith, 1948).

The metabolism of the natural oestrogens has been widely studied, but that of the synthetic oestrogens comparatively little. Stroud (1939) found that in the rabbit 21.3% of a high dose of hexoestrol was excreted in the urine, 15.7% in the free state and 5.6% in a conjugated form. The corresponding figures for stilboestrol were 14.2 and 4.2%, and for dienoestrol 11.0 and 3.0%. In view of the instability of dienoestrol (Wilder Smith & Williams, 1945; 1947) this low figure is not surprising as the vigorous conditions probably caused some decomposition. No attempt was made to isolate the conjugated oestrogen as such, and in no case was more than 25% of the total administered oestrogen accounted for.

Mazur & Schorr (1942) dosed rabbits with stilboestrol and isolated from the urine 30% of the administered oestrogen as the monoglucuronide. No other synthetic oestrogens were investigated and no other metabolic products were reported.

We have found that in the rabbit a high percentage of the synthetic oestrogen given is excreted as glucuronide or other derivative soluble in sodium bicarbonate, while quite a small amount is excreted as free oestrogen. Much lower total excretion was found in the cat.

METHODS

Urine collection and extraction. The urines were treated as described by Simpson & Wilder Smith (1948), care being taken to avoid hydrolysis of glucuronide by heat or by bacterial growth. After acidification of the urine to Congo red with dilute HCl in the cold, ether extraction (by hand and continuous) yielded an extract which gave a fraction, soluble in bicarbonate, containing glucuronide, which was characterized as described by Simpson & Wilder Smith (1948), and a fraction soluble in caustic soda, containing free oestrogen. The latter extract was made acid to Congo red, extracted with ether and the extract assayed biologically, giving the yield of free oestrogen excreted. As an additional check, a sample of the original unextracted urine was tested for oestrogenic activity: this gave an indication of the combined activity due to free and conjugated oestrogen. After the sodium oestrogen glucuronide had crystallized from the bicarbonate solution (where this was used) the mother liquor was acidified, extracted with ether, and the ether extract assayed biologically; this eliminated errors due to incomplete crystallization. That the ether extraction of the acidified but unhydrolyzed urine was complete was proved in several experiments by hydrolyzing afterwards, and extracting the urine with ether in a continuous extractor. No oestrogens could be detected either in the bicarbonate or caustic soda fractions from the ether extract. Thus complete extraction of both free and conjugated oestrogens had been attained (see below, p. 256).

Biological assay and expression of results. Usually the amount of oestrogen present in the urine was so great that the crude urine could be assayed without any preliminary extraction. In other cases the various fractions of the urine during extraction were assayed to serve as a check on the completeness of the chemical separations.

The method of assay was the one usual in this Institute: Spayed adult rats were injected twice daily for 3 days with the extract or urine to be tested and the vaginae of the rats were smeared twice daily during two following days. Smears consisting of epithelial and/or cornified cells only were taken to represent positive responses. The number of samples to be tested and the uncertainty of their activity made it impossible for more than 5–10 rats to be used at each dose level and the doses used were in the proportions 1, 2, 5, 10, 20, 50, 100, etc., so the results have only a very low degree of accuracy.

The content of the samples assayed, biologically was estimated (on the assumption that all the activity was due to free oestrogen or to oestrogen glucuronide) by reference to dose-response curves for the substances concerned (Simpson & Wilder Smith, 1948). In either case and also where chemical separations were effected the content of free oestrogen was calculated and is expressed throughout as a percentage of the total dose given.

RESULTS

Elimination after subcutaneous injection into rabbits

The results summarized in Table 1 are derived from an experiment where four female rabbits were injected daily (except Sundays) with 30 mg. of dienoestrol or stilboestrol for 4 weeks.

The total proportion of the administered oestrogen eliminated is somewhat higher for stilboestrol than for dienoestrol. In this particular experiment the difference is not statistically significant but Stroud (1939) found the same difference, and in other experiments we have recovered as much as 46% of administered stilboestrol as the glucuronide.

During the injection period the total elimination remained fairly constant. There was a slight tendency for the proportion of free oestrogen to decline after the first week (though the third week in rabbit 3/46 is exceptional) and with stilboestrol there may be a slight delay before the glucuronide elimination process becomes fully effective. It may be noted that the crystallization of the glucuronide from the sodium bicarbonate was rather more complete with dienoestrol than with stilboestrol (cf. columns 4 and 5, Table 1).

One of the most striking facts revealed is that the elimination of these high doses is so rapid. Although the injections were made in oily solution and oil remained unabsorbed at the end of the sixth week of the experiment, yet even in the week after the injections had stopped the excretion had dropped to low levels.

Table 1. *Urinary elimination of subcutaneously injected oestrogens*

(Six injections of 30 mg. of oestrogen each week for 4 weeks.)

Percentage of total weekly dose eliminated					
Rabbit	Week	(ii) As glucuronide			
		(i) Free	Gravimetric	Biological*	Total
Dienoestrol injections					
No. 1/46, ♀	1	5	16	1	22
	2	1	—	—	—
	3	1	14	5	20
	4	1	15	5	21
	5	0.1	3	<1	3
	6	—	—	<1	<1
No. 2/46, ♀	1	5	21	1	27
	2	1	18	4	23
	3	1	17	4	22
	4	1	22	3	26
	5	0.1	—	<1	<1
	6	—	—	<1	<1
Stilboestrol injections					
No. 3/46, ♀	1	10	1	10	21
	2	5	19	10	34
	3	15	7	10	32
	4	1	10	10	21
	5	0.1	—	>1	>1
	6	—	—	<1	<1
No. 4/46, ♀	1	10	2	10	22
	2	10	—	10	20
	3	5	10	10	25
	4	5	13	10	28
	5	0.1	—	4	4
	6	—	—	<1	<1

* Biological assay of mother liquids after removal of gravimetrically estimated compound.

Elimination after subcutaneous injection into cats

Two female cats each received 1.8 g. of dienioestrol (dissolved in sesame oil with a little added ethanol) by subcutaneous injection during 1 week. Their urine was collected during the week of the injections and the following week.

About 1 % of the injected oestrogen was excreted in the free form, but no glucuronide could be isolated. However, the fraction soluble in bicarbonate showed oestrogenic activity on biological assay, and if this activity were all due to glucuronide it would account for 4 % of the administered dienioestrol.

During the injection period the daily urine volume in the cats was abnormally low. A similar decrease had been noticed in the injected rabbits: in their case the decrease was due to the smaller consumption of cabbage, which was the chief component of their diet. It is well known that oestrogens in high doses depress the appetite and, although the fluid intake of the cats was not measured, we have little doubt that it was this which was responsible for the small output in the cats also.

The toxic effects of dienioestrol were much more marked in the cats than in the rabbits. Both the cats in this experiment died; one 2 weeks, and the other 3 weeks, after the first injection.

Effect of urinary pH on elimination of injected dienioestrol by female rabbits

A possible reason for the different rates of elimination of oestrogen glucuronide in cats and rabbits might lie in the difference in the reaction of their urine. That in the normally fed rabbit is alkaline while cats' urine is strongly acid.

To investigate this possibility two mature female rabbits were fed on a diet of oatmeal, bread and water until their urine was consistently acid to litmus (pH 5) and then injected subcutaneously with 1.5 g. of dienioestrol (dissolved in sesame oil) as before.

The results given in Table 2 show clearly that an acid urine does not prevent the elimination of large proportions of injected dienioestrol in the form of the glucuronide. Again, as with the experiments in rabbits with alkaline urine, the variations in glucuronide yield are wide.

Table 2. *Elimination of dienoestrol glucuronide in rabbits with acid urine (pH 5) after subcutaneous injection of dienoestrol*

Rabbit	Dosage		Urine collection	
	Time (weeks)	Amount (mg.)	Time (weeks)	Percentage of dienoestrol excreted as glucuronide
R 24/46	1-3	1500	1-7	24
R 25/46	1-3	1500	1-7	48

Table 3. *Elimination of hexoestrol glucuronide in rabbits after oral administration of hexoestrol*

Rabbit	Total dose (mg.)	Days of		Percentage of hexoestrol excreted as glucuronide
		(i) Dosing	(ii) Collection	
R 27/46	1000	1	1-9	30
R 31/46	700	1	1-4	46

Elimination after oral administration in rabbits

Single doses of hexoestrol, dissolved in aqueous ethanol or in sesame oil, were given by mouth to two female rabbits and their urine was collected (see Table 3).

The urine from R 27/46 was also assayed biologically. The activity found was equivalent to 5% of the administered dose if it were all free hexoestrol, or to 90% of the administered dose if it were all glucuronide. As this last figure is so much greater than the glucuronide isolated chemically it appears that some of the hexoestrol was, in fact, eliminated in the free form.

Similar experiments where two doses of stilboestrol, dienoestrol or hexoestrol were given 6 days apart gave similar results (Table 4).

of the oestrogen given). This proves that all the conjugated and free oestrogen had been extracted from the unhydrolyzed urine in the first place. The results again show that the oestrogen eliminated in the urine is predominantly conjugated.

Urinary elimination of stilboestrol glucuronide fed to the rabbit

Stilboestrol glucuronide (250 mg.) dissolved in sesame oil was fed by stomach tube to a female rabbit, the urine being collected and worked up as in the previous experiments. The striking feature of the results (Table 5) is that very low yields of glucuronide were obtained, showing that the glucuronide is certainly not the end product of metabolism. A second experiment in which 100 mg. of

Table 4. *Elimination of oestrogen after oral administration during 6 days of stilboestrol (S), hexoestrol (H) or dienoestrol (D)*

Rabbit	Total dosage (mg.)	Collection period (days)	Percentage of total dose eliminated			
			(i) Free Biological	(ii) As glucuronide		
				Gravimetric	Biological*	Total
R 32/47	1800S	14	5	9	23	32
	Control†	14	<1	0	<1	<1
R 33/47	2000H	11	<1	14	26	40
	Control	11	<1	0	<1	<1
R 30/47	1450D	14	5	7	25	32
	Control	14	<1	0	<1	<1

* As in Table 1.

† Control collections were made after a lapse of 10 days from the end of the experimental collection.

In this experiment all the urine samples after ether extraction were hydrolyzed with hydrochloric acid under reflux and re-extracted with ether. These ether extracts were then worked up in the usual manner and biologically assayed. No oestrogenic activity was detectable in any of the extracts at the highest dose level tested (equivalent to a 1% recovery

stilboestrol glucuronide dissolved in sesame oil were injected subcutaneously yielded similar results (Table 5).

The results of this experiment demonstrate that the glucuronide formed after the administration of stilboestrol is not a stable end product but is further metabolized in the body.

Table 5. *Urinary elimination of oestrogen after single dose of stilboestrol glucuronide*

Rabbit	Dose (mg.)	Collection period (days)	Percentage of total dose eliminated			
			(i) Free Biological	(ii) As glucuronide		
				Gravimetric	Biological*	Total
R 33/47†	250	4	1	12	1	13
R 7/47‡	100	4	3	0	25	25

* As in Table 1.

† Oral administration.

‡ Subcutaneous injection.

DISCUSSION

Our results confirm those of Mazur & Schorr (1942) in demonstrating that rabbits excrete a high proportion of administered stilboestrol in the urine as glucuronide, but do not agree with the finding of Stroud (1939) that high proportions are excreted in the free form. This discrepancy is probably due to the method of working up used by Stroud. We have also found that dienestrol and hexoestrol behave in the same way as stilboestrol, all yielding about 35 % of the administered dose as monoglucuronide in the urine. We have assumed that all the conjugated oestrogen is monoglucuronide as, in those cases where only 1 % remained for biological assay, the 99 % was in fact glucuronide. These conclusions relate to the very large doses we have used and are not necessarily applicable to lower, therapeutic doses.

The yields obtained in our earlier experiments were somewhat lower than those realized later; this is probably due to increased experience in handling these rather intractable substances. The main difficulty lies in the isolation of the glucuronides, either free or as sodium salts. This may be seen for example by the variable proportions of glucuronide remaining for biological assay in Tables 1, 4 and 5. Perhaps this can be attributed to the accumulation of various bicarbonate-soluble substances in the urine. If so, the time during which the urine is collected after dosage should be kept as short as possible, consistent with the recovery of the greater proportion of administered oestrogen; indeed the rapidity with which the dose is eliminated (see Table 1) renders any prolonged collection after dosage unnecessary.

The only stage so far identified in the metabolism of the synthetic oestrogens is that of glucuronide formation. We were unable to isolate any sulphate esters of the administered oestrogens and therefore

have no direct evidence of their production in the rabbit. In man this form of elimination of natural oestrogens is well known and there is some indirect evidence of a small excretion of synthetic oestrogen sulphates (unpublished data).

It is very doubtful whether such metabolism studies can shed any light on the mechanism of oestrogenic action. Glucuronide and sulphate formation are too widespread to be specific for such action, while the complex degradation products of the natural oestrogens cannot be vital for oestrogenic action since the synthetic oestrogens produce the same effect without undergoing the same chemical changes. For closer study of oestrogenic action it would probably be advantageous to use the most chemically stable oestrogens available, e.g. hexoestrol (Wilder Smith & Williams, 1945, 1947) in order to avoid the complications of side reactions which are irrelevant to oestrogenicity.

SUMMARY

1. The urinary excretion of oestrogen after the administration of stilboestrol, hexoestrol, dienestrol, and stilboestrol glucuronide has been examined in the rabbit and cat.

2. In the rabbit about 35 % of the administered oestrogen can be accounted for in the urine. In the cat much lower recoveries are obtained.

3. In the rabbit the chief metabolic products excreted are the monoglucuronides, but these substances are metabolized further to inactive products.

We are indebted to the Council of the Middlesex Hospital for the provision of laboratory facilities for this work and to Mrs Culliford and to Mr B. D. Shepherd for technical assistance. The investigation was aided by a grant from the Medical Research Council and by an apparatus grant from the Central Research Fund, University of London.

REFERENCES

- Angrist, A. & Khoury, E. N. (1944). *Urol. cutan. Rev.* **48**, 577.
 Huggins, C. (1946). *J. Amer. med. Ass.* **131**, 576.
 Mazur, A. & Schorr, E. (1942). *J. biol. Chem.* **144**, 283.
 Nesbit, R. M., Pazzos, R. & Cummings, R. M. (1944). *J. Urol.* **52**, 570.
 Simpson, S. A. & Wilder Smith, A. E. (1948). *Biochem. J.* **42**, 258.
 Stroud, S. W. (1939). *J. Endocrinol.* **1**, 201.
 Wilder Smith, A. E. & Williams, P. C. (1945). *Nature, Lond.*, **156**, 718.
 Wilder Smith, A. E. & Williams, P. C. (1947). *J. Endocrinol.* **5**, 152.