The Excretion of Synthetic Oestrogens

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In 1939 Stroud investigated the excretion of hexoestrol (*meso*-3:4-di(*p*-hydroxyphenyl)-hexane), stilboestrol (*trans*-3:4-di(*p*-hydroxyphenyl)-hex-3-ene), and dienoestrol (3:4-di(*p*-hydroxyphenyl)-hexa-2:4-diene) when large amounts were administered to rabbits, and later Mazur & Schorr (1942) and Simpson & Wilder Smith (1948), isolated and characterized the corresponding glucuronides.

This was followed by quantitative studies on their urinary excretion at high dose levels (Dodgson, Garton, Stubbs & Williams, 1948; Simpson & Wilder Smith, 1949), while more recently Twombly & Schoenewaldt (1951) and Hanahan, Daskalakis, Edwards & Dauben (1953) using [¹⁴C]stilboestrol have demonstrated the importance of the bile and faeces in the excretion of this oestrogen.

It was therefore decided to determine the biological activity of the faeces after administering hexoestrol and stilboestrol to rabbits, and also to carry out a systematic investigation of the effect of dose and route of administration on the ratio of free to conjugated oestrogen eliminated in the urine.

The fate in the rabbit of 17 α -ethinyloestradiol, triphenylchloroethylene (1-chloro-1:2:2-triphenyl-ethylene), dipropyldibenzanthracenediol (9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene) and 4-*tert*-amyl phenol has also been investigated; except for the last compound (which was only weakly oestrogenic; Dodds & Lawson, 1938) the amounts present in the urine and faeces have been determined by biological assay. Whenever possible the active compounds were isolated or examined by paper chromatography.

Among these oestrogens only the metabolism of ethinyloestradiol has been previously studied (Stimmel & May, 1951) and these workers could find no detectable amounts of oestrone, oestradiol or oestriol, possible *in vivo* transformation products, in the urine of men following its ingestion. There was evidence, however, of a small amount (not exceeding 3%) of the administered compound in the urine.

EXPERIMENTAL AND RESULTS

General methods

The oestrogens were dissolved or suspended in aqueous ethanol (20%, v/v) for oral administration, and in arachis oil for subcutaneous injection.

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A single rabbit was used for each dose with the exception of triphenylchloroethylene where two animals were used.

Single doses were administered up to quantities of 100 mg.; above this, the doses were divided. Thus 1 g. of hexoestrol and stilboestrol were given as 4 $\times$ 250 mg. on consecutive days, 750 mg. of dipropyldibenzanthracenediol also (as four equal doses) over 4 days and 3 g. triphenylchloroethylene as 2 $\times$ 750 mg. to each of two rabbits on consecutive days. 4-*tert*-Amyl phenol (5 g.) was administered in six equal doses over 12 days.

The animals weighed between 2.5 and 3.5 kg. All were males except for one female (see Table 2). The urine was collected for 3 days and the faeces for 7 days after the last injection; toluene was added to both as a preservative. The urine was stored at 0–5° and the faeces were dried at room temperature and finally *in vacuo* over CaCl₂.

Extraction and separation. The urine was made just acid to congo red and continuously extracted with ether for 24 hr. It was assayed as total extract or divided into acidic, phenolic and neutral fractions by repeated hand extraction, first with saturated NaHCO₃ solution and then with 2*N*-NaOH. Both the NaHCO₃ and NaOH solutions were acidified to pH 2–3, extracted with ether, the solvent was removed at room temperature *in vacuo* and the residue dissolved in a known volume of absolute ethanol for biological estimation. In some instances the neutralized aqueous solutions were assayed directly.

The dried faeces were crushed, extracted with ether in a Soxhlet extractor for 24 hr. and assayed in 3% aqueous ethanol.

Bioassay. The pure oestrogens in 3% aqueous ethanol (water containing 3%, v/v, ethanol) or arachis oil were administered to groups of five ovariectomized rats, the procedure of subcutaneous injection and vaginal smearing being the standard one used at this Institute (Wilder Smith & Williams, 1948).

This was in order to determine the amount of each substance required to give 50% of positive responses (ED₅₀) (Table 1), which was calculated from the bioassay results by the 'moving averages' statistical method of Thompson (1947).

Rats (45–145) were used for each determination, and it can be seen from Table 1 that the sensitivity of the method depended on the solution medium, lower results being obtained when 3% ethanol instead of arachis oil was used.

Knowing the ED₅₀ of each oestrogen, it was now possible to determine their percentage excretion in the rabbit by a process of 'bracketing'. On the assumption that the recovered material contained 10, 25, 50, 75 and 100% of the administered oestrogen, samples calculated to contain the ED₅₀ were injected into the rats (five at each dose level) and the approximate range of recovery thus obtained. This range was later narrowed by intermediate dosing. In the results of Tables 2 and 3, quantities corresponding to the lower percentages quoted gave 80–100% response in five

rats; these percentages are therefore the minimum possible values for recovery. Quantities corresponding to the higher figures gave 0% response in five rats; these percentages therefore represented the maximum possible value for recovery.

In all cases, the extracted urine residue was tested for activity to ensure that extraction was complete, and an extract of 200 ml. of normal male and female rabbit urine was also shown to have no activity.

Examination of compounds and metabolites. All the administered phenolic oestrogens and metabolites with the exception of 4-*tert*.-amyl phenol were examined by paper chromatography (Jellinck, 1953), the system *C* of Bush (1952) being used for oestriol.

Hexoestrol

The glucuronide was separated from the free oestrogen by extracting the ethereal solution, once with 0.5 vol. and twice with 0.25 vol. of saturated NaHCO_3 and then twice with 0.25 vol. of water. The pooled aqueous fraction was backwashed twice with 0.25 vol. of ether, which in turn was backwashed twice with 0.25 vol. of bicarbonate solution and once with 0.25 vol. of water. The pooled aqueous fraction was extracted yet once more with twice 0.2 vol. of ether, before acidifying and ether extracting.

As hexoestrol is about 35 times more active than its glucuronide (Table 1), it was essential that the separation should be complete, and two control experiments with a mixture of 50 μg . hexoestrol and 300 μg . hexoestrol

Table 1. *Median effective dose of synthetic oestrogens in ovariectomized rats*

In 3% aqueous ethanol except for values marked *A* which are in arachis oil.

Compound	ED ₅₀ (μg .)	No. of rats used
Hexoestrol	{ 0.30 0.51 (<i>A</i>)	145 50
Hexoestrol glucuronide	11	65
Stilboestrol	0.27	85
Ethinylloestradiol	{ 0.10 0.25 (<i>A</i>)	45 85
Triphenylchloroethylene	16	90
Dipropyldibenzanthracenediol	11	55

glucuronide in 3.5 ml. of absolute ethanol added to a 100 ml. of rabbit urine were carried out to ensure that both the method of extraction and separation were quantitative. The recoveries were 80–110% for hexoestrol and 110–150% for the conjugated oestrogen. The glucuronide of hexoestrol was assayed directly because it was found that refluxing 2 mg. of this conjugate for 1.5 hr. with 15% (v/v) HCl resulted in 60–70% destruction when assayed as free hexoestrol. The addition of zinc dust as used by Smith & Smith (1941) for the natural oestrogens, did not protect it.

Any disulphate of hexoestrol would remain behind in the original aqueous layer at the pH that the continuous extraction had been carried out, as found by Simpson & Wilder Smith (1949), and their method of hydrolysis was adopted. The hydrolysed solution was extracted with ether and assayed in 3% aqueous ethanol. The glucuronide of hexoestrol was isolated by the method of Simpson & Wilder Smith (1948).

Results of recovery experiments are shown in Table 2.

Stilboestrol

This oestrogen was separated from its glucuronide by the process used for hexoestrol, and the percentage excreted in the free form in the urine and faeces determined (Table 3).

Stilboestrol (4 mg.) was obtained by crystallization from benzene from the phenolic and neutral fraction of the urine of the rabbit dosed with 1 g. of the oestrogen. The isolated compound was examined by paper chromatography and was shown to have the same infrared spectrum at 600–2000 cm^{-1} as pure stilboestrol.

Stilboestrol glucuronide was isolated by the method of Simpson & Wilder Smith (1948).

Ethinylloestradiol

It was shown by paper chromatography that the unchanged oestrogen was present in the faeces, but no oestrone, α -oestradiol or oestriol could be detected there. The urine showed less than 1% activity even when assayed as oestriol, which is the least active member of the natural oestrogen series (ED₅₀, 2.7 μg .), and hydrolysis by refluxing with 15% (v/v) HCl did not increase the activity.

The bioassay results are shown in Table 3.

Table 2. *Hexoestrol recovery values from urine and faeces in the rabbit*

All values as percentage of oestrogen administered; calculated as described on p. 262.

Route of administration	Dose (mg.)	Excreted in the urine			Excreted in the faeces as free oestrogen (%)	Total recovered (%)
		As ethereal sulphate (%)	As mono-glucuronide (%)	As free oestrogen (%)		
Oral	1	—	18–24	15–25	10–15	43–64
	1	<2	35–45	2–5	4–10	41–60
	1*	<2	40–60	1–2	5–10	46–72
	10	—	6–9	8–13	8–15	22–37
	100	—	4–10	8–15	5–10	17–35
	1000	—	25–40	2–5	<5	27–45
Subcutaneous	1	—	<20	5–12	<10	—
	10	—	25–40	10–15	7–10	42–65
	100	—	5–8	8–12	25–35	38–55
	1000	—	18–25	3–7	4–7	25–39

* Female rabbit.

Table 3. *Recovery of activity of synthetic oestrogens from urine and faeces in the rabbit*

All values as percentage of oestrogen administered; calculated as described on p. 262.

Compound	Route of administration	Dose (mg.)	Activity in urine			Activity in faeces (%)	Total activity recovered (%)
			Total extract (%)	Acid fraction (%)	Phenolic and neutral fraction (%)		
Stilboestrol	Subcutaneous	1	—	—	8-13	35-50	—
		10	—	—	12-20	10-15	—
		100	—	—	12-14	2-5	—
		1000	—	27*	<1	<1	27
Ethinyl-oestradiol	Subcutaneous	40	<1	—	—	12-17	12-17
Triphenyl-chloroethylene	Oral	3000	—	0.75-1.5	<1	16-24	17-25
Dipropyldibenz-anthracenediol	Subcutaneous	750	<1	—	—	3-5	3-5

* Isolated in crystalline form.

Triphenylchloroethylene

Most of the oestrogenic activity was found in the faeces and a small amount in the acid fraction of the urine (Table 3).

All acids and phenols were removed from the faeces extract by repeated extraction with 2*N*-NaOH, and the neutral ethereal residue was well washed with water and dried over anhydrous Na₂SO₄ before evaporating to dryness. The residue was dissolved in warm absolute ethanol and the impure crystals (198 mg.) which appeared on cooling were filtered and recrystallized from absolute ethanol. A pure crystalline compound (93 mg.) was thus obtained, which did not depress the m.p. of triphenylchloroethylene and which had the same characteristic infrared spectrum in the 'finger print' region (600-2000 cm.⁻¹).

The NaOH-soluble fraction of the faeces extract showed less than 5% activity, and the acid fraction of the urine did not give a positive Tollens naphthoresorcinol reaction for glucuronide.

Dipropyldibenzanthracenediol

A small amount of activity was present in the faeces but there was less than 1% activity in the urine (Table 3) which was not increased by hydrolysis for 10 min. with 15% (v/v) HCl. The urine extract did not give a positive Tollens naphthoresorcinol reaction for glucuronide, and all attempts to isolate crystalline material from it were without success.

4-tert.-Amyl phenol

This oestrogen was not assayed because it was only weakly active (80 mg./rat) but its *glucuronide* was isolated from the acid fraction of an ether extract by the following method. The NaHCO₃ fraction was acidified, extracted with ether and the solvent removed. The tar, which gave a strong positive Tollens naphthoresorcinol reaction for glucuronide, was dissolved in 50 ml. absolute ethanol, boiled with animal charcoal, filtered, and ethanolic KOH (3 g. KOH/100 ml. absolute ethanol) added until no more solid was precipitated. This solid was filtered, dissolved in 50 ml. water, acidified to pH 2-3 and extracted with ether until the aqueous residue gave no colour in the Tollens test. The ether was removed and the syrupy residue crystallized from benzene-acetone (12:1, v/v). Yield: 1.44 g. (13.9% recovery). White crystals

m.p. (decomp.) 167-168° after sintering at 70-75° and then gradually darkening in colour.

For the *monohydrate*, found: C, 57.0; H, 7.6. C₁₇H₂₄O₇, H₂O requires: C, 57.0; H, 7.3%. Equiv. wt., found: 355; required, 358. Loss in wt. on drying at 110° *in vacuo*: found, 5.2; required, 5.03%.

For the *anhydrous glucuronide*, C₁₇H₂₄O₇, found: C, 59.5; H, 6.8. C₁₇H₂₄O₇ requires C, 60.0; H, 7.1%.

DISCUSSION

Hexoestrol

The results (Table 2) show that there are very large variations in the amounts excreted as the free and conjugated compounds in the urine and as 'total' oestrogen in the faeces. They also show that neither the dose nor the route of administration seems to have a significant effect on the excretion pattern.

It has been assumed that the amount of activity present in the acid fraction of the extract is solely due to the glucuronide, since on no occasion has more than a trace of ethereal sulphate been found (Dodgson *et al.* 1948; Starnes & Teague, 1949; Simpson & Wilder Smith, 1949), and in any case the latter would not be extracted from the urine by the procedure used (Simpson & Wilder Smith, 1949; Teague & Brown, 1951).

The rabbit is not generally considered suitable for recovery experiments from faeces but hexoestrol appears to be resistant to bacterial attack, as unchanged hexoestrol could be detected in the faeces and high recovery percentages were obtained even with very small amounts of administered oestrogen. This seemed to be also true for the other compounds investigated.

Ethinyl-oestradiol

The results are similar to those found for men by Stimmel & May (1951) and in addition it has been shown that the faeces are a major route of excretion for this oestrogen in the rabbit.

Triphenylchloroethylene

This compound had been fed in the hope that it would be hydroxylated and excreted in the urine in either the free or conjugated forms or both, as this might have settled the controversy about whether the compound was an oestrogen or pro-oestrogen. All the urinary activity was found in the acid fraction and as no glucuronide could be detected, either a mercapturic acid conjugate of the unchanged oestrogen or an ethereal sulphate, if hydroxylation had taken place, could account for it. Work is in progress at the present to isolate and characterize this compound. The high activity in the faeces is in agreement with the results of Paterson, Gilbert & Gallagher (1949), who on injecting mice intravenously with the radioactive bromo analogue (triphenylbromoethylene) found that most of the organic bromide was in the faeces.

4-tert.-Amyl phenol

The fate of 4-tert.-amyl phenol in the rabbit was investigated because it is the simplest known compound which has oestrogenic activity, but the only metabolite isolated has been its glucuronide, which as far as is known has not been characterized before.

In conclusion it should be added that in most cases the doses given were far in excess of those needed to show physiological activity but it had been hoped that some metabolites would be isolated and the experiment could then have been repeated with considerably less oestrogen and those metabolites detected and estimated by a micro method.

SUMMARY

1. The excretion pattern of hexoestrol in the rabbit has been determined but it has not been possible to show that either the dose or the route of administration had any marked effect on the ratio of free to conjugated oestrogen in the urine. The percentage excreted as disulphate in the urine is low, even when small doses are administered.

2. Ethinyloestradiol was excreted unchanged mainly in the faeces and no trace of oestrone, α -oestradiol or oestriol could be detected in either the urine or faeces.

3. The percentage of administered stilboestrol, triphenylchloroethylene and dipropyldibenzanthracenediol excreted in the urine and faeces has also been determined. Stilboestrol and its glucuronide were isolated from urine and triphenylchloroethylene recovered unchanged from the faeces.

4. The glucuronide of 4-tert.-amyl phenol has been isolated from urine and characterized.

5. It has been shown for hexoestrol and ethinyloestradiol that their ED_{50} as oestrogens varies according to whether they are assayed biologically in arachis oil or 3 % aqueous ethanol, the sensitivity being greater when the latter solvent is used.

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