Studies in Detoxication

15. ON THE GLUCURONIDES OF STILBOESTROL, HEXOESTROL AND DIENOESTROL

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The importance of stilboestrol, hexoestrol and dien-oestrol as chemotherapeutic substances of value as oestrogens and as anticancer compounds has now been established (cf. Dodds, 1945; Haddow, 1947). Their fate in the body, however, has not been fully worked out. In 1939 Stroud (1939*a*) showed that stilboestrol, on injection into rabbits, was excreted both in a free and combined state and, in 1942, Mazur & Shorr isolated stilboestrol monoglucuronide from the urine of rabbits receiving small doses of stilboestrol by injection. The glucuronide isolated corresponded to 30% of the dose. Since stilboestrol is a phenol it would be expected to form an ethereal sulphate. Teague (1945), however, has reported that it causes no significant change in the organic sulphate output of rabbits or rats. The same worker found no change in the neutral sulphur output. These experiments with intact animals therefore suggest that stilboestrol undergoes but one change in the body, namely conjugation of one of its hydroxyl groups with glucuronic acid. *In vitro* experiments carried out by Zimmerberg (1946) (cf. Zondek, Sulman & Sklow, 1943) showed that stilboestrol disappeared when added to rat-liver slices in a Ringer-bicarbonate medium at 37°. The stilboestrol was believed to be conjugated or oxidized, only one hydroxyl being attacked. Zimmerberg suggested that conjugation could take place with sulphuric or another acid, probably glucuronic acid, depending on which is available.

In the case of hexoestrol and dien-oestrol Stroud (1939*a*) showed that both these compounds were excreted in a combined form by rabbits. Up to the present time no reliable data have been available on the quantitative aspects of the conjugation of these three oestrogens. In the present paper quantitative determinations of the outputs of glucuronic acid and ethereal sulphate in rabbits following the feeding of these compounds will be presented. A description is also given of the isolation and characterization of the glucuronides. Further work is in progress on these glucuronides, for stereochemical considerations suggest that the nature of the glucuronides from hexoestrol and dien-oestrol may be more complicated than is apparent at first sight.

METHODS

Animals. Rabbits (giant chinchilla), kept on a constant diet of 50 g. of Lever's cubes and 100 g. of cabbage *per diem*, were used throughout these experiments. Compounds were administered in all cases in aqueous suspension by means of a stomach tube.

Glucuronic acid was determined according to the method of Hanson, Mills & Williams (1944).

Ethereal sulphate and neutral sulphur were determined gravimetrically by the Folin method (cf. Peters & Van Slyke, 1932).

Spectroscopic observations were made with a Hilger E3 quartz spectrograph.

RESULTS

Experiments with stilboestrol

Quantitative results. These are given in Table 1, and a typical experiment is illustrated in Fig. 1. The results show that at a dose level of 150 mg./kg. an average of 71% of the dose of stilboestrol can be accounted for as a monoglucuronide excreted in the urine. The glucuronic acid output is raised above normal for about 4 days, maximum excretion of the glucuronide occurring on the second day after dosing. Neither the ethereal sulphate nor the neutral sulphur output appears to be disturbed.

Isolation of stilboestrol mono-β-D-glucuronide. Stilboestrol (0.8 g.) (British Drug Houses Ltd., m.p. 173°) was fed to each of 6 rabbits and the urines were collected each day during the next 4 days. Mazur & Shorr (1942) have reported that the sodium salt of stilboestrol glucuronide is relatively insoluble in water. Since rabbit urine is normally alkaline (pH > 8), it is reasonable to expect that the urinary deposit would contain appreciable amounts of this sodium salt. The urines were therefore centrifuged and the deposits obtained worked up separately. The deposits were triturated with small amounts of dilute H₂SO₄, and then anhydrous Na₂SO₄ was added to give a thick pasty mass. The latter was now triturated with ether (5 × 100 ml. portions). The ether extracts were reduced to 100 ml. and the insoluble sodium salt was precipitated on adding 30 ml. saturated NaHCO₃ solution. After keeping at 0° for 1–2 hr. the salt was filtered and dried *in vacuo*. The centrifuged urines were acidified with dilute H₂SO₄ and extracted with 4 or 5 portions of ether. The ethereal extracts were then treated as in the case of the deposits. The yields in a good

Table 1. *Excretion of glucuronic acid, ethereal sulphate and neutral sulphur by rabbits receiving stilboestrol orally*

Rabbit no.	Wt. (kg.)	Dose (mg./kg.)	'Extra' glucuronic acid* (% of dose)	'Extra' ethereal sulphate (% of dose)	'Extra' neutral sulphur (% of dose)
81	2.6	150	77.3	—	—
87	2.85	150	73.9	—	—
89	2.75	150	67.1	—	—
90	2.9	150	66.2	—	—
97	2.35	250	—	0	—
69	2.45	250	—	0	0
82	3.0	100	—	0	—
86	2.75	100	—	0	0
80	3.1	250	—	—	0

* Calculated as a monoglucuronide.

experiment are given in Table 2. For analysis the salt (plates) was purified by recrystallization from methanol. (Found: Na, 4.7%; calc. for $C_{24}H_{27}O_8Na$, Na, 4.7%.) To obtain the free glucuronide, the sodium salt was triturated

Fig. 1. The glucuronic acid and ethereal sulphate output in rabbits receiving stilboestrol orally. ●—●, glucuronic acid curve for rabbit no. 90 that received 440 mg. of stilboestrol on day 2. ○—○, ethereal sulphate output in rabbit no. 86 that received 275 mg. stilboestrol on day 2.

Table 2. *Yields of sodium stilboestrol monoglucuronide from rabbit urine*

(Amount of stilboestrol fed was 4.8 g. (6 rabbits). Total Na salt recovered was 5.37 g.)

Day	Sodium salt from	
	Centrifuged urine (g.)	Urinary deposit (g.)
1	0.48	0.65
2	0.70	1.88
3	0.40	0.61
4	0.25	0.40
Totals	1.83	3.54

with dilute H_2SO_4 , and then anhydrous Na_2SO_4 added to form a thick pasty mass. The glucuronide was extracted from this by stirring with several portions of ether. The ethereal extracts were taken to dryness and the residue dissolved in a small amount of acetone, from which stilboestrol monoglucuronide *dihydrate* crystallizes on adding

benzene in excess. On recrystallization from benzene-acetone mixtures it forms masses of long needles, m.p. 179° (decomp.), sintering at $65-75^\circ$ and showing $[\alpha]_D^{20} = -56.5^\circ$ ($c=1$ in ethanol). (Found: C, 59.8; H, 6.8%; equiv. by titration, 489; glucuronic acid, 39%. $C_{24}H_{28}O_8 \cdot 2H_2O$ requires C, 60.0; H, 6.7%; equiv. 481; glucuronic acid, 40.3%.) The determination of water of crystallization was not satisfactory since the compound decomposed on prolonged heating at 110° . It was sparingly soluble in cold water and benzene, but readily soluble in ethanol, acetone, ether and hot water. It gave an intense naphthoresorcinol reaction and a deep cherry-red colour in the Dingemans $SbCl_5$ reaction (see Dingemans, 1940).

Benzylamine salt of stilboestrol glucuronide. Pure stilboestrol glucuronide (200 mg.) was dissolved in 20 ml. ethanol followed by 20 ml. ethyl acetate. Benzylamine (10 drops) was then added to the mixture with stirring. After a few seconds the salt crystallized out. After 15 min. at 0° , the mixture was filtered, and the salt washed with ethanol and dried in the desiccator (yield 210 mg., m.p. 218° decomp.). On recrystallization from 10% ethanol, the salt formed long white needles, m.p. 223° (decomp.), and showed $[\alpha]_D^{20} = -55.3^\circ$ ($c=0.172$ in 50% aqueous acetone). (Found: C, 63.75; H, 6.9; N, 2.55%. $C_{31}H_{37}O_8N \cdot 2H_2O$ requires C, 63.4; H, 7.0; N, 2.4%.) The salt was sparingly soluble in all the usual solvents.

Detection of free stilboestrol. (a) In urine. Urine from rabbits receiving stilboestrol orally was centrifuged and the deposit removed. It was then acidified and extracted with ether in a continuous extractor for several hours. The glucuronide in the ether extract was removed by six treatments with saturated sodium bicarbonate solution. The ether was now extracted with 10% (w/v) Na_2CO_3 to remove free stilboestrol. Acidification of this latter extract, followed by extraction with ether, yielded a solution giving a red colour in the Dingemans reaction thus indicating the presence of free stilboestrol. A separate experiment showed that stilboestrol glucuronide could be completely removed, as indicated by the Dingemans reaction, from an ethereal solution by saturated $NaHCO_3$. By this method small amounts of free stilboestrol were found in the urine for 4 days after dosing.

(b) *In faeces.* By a similar method free stilboestrol in small amounts was detected in the faeces of rabbits on all 4 days after dosing.

Experiments with hexoestrol

Quantitative results. These are given in Table 3, and Fig. 2 illustrates a typical experiment. These figures indicate that about 71% of hexoestrol is excreted as a glucuronide, the excretion persisting for 4–5 days after dosing. The ethereal sulphate and neutral sulphur outputs are not raised.

Table 3. *The excretion of glucuronic acid, ethereal sulphate and neutral sulphur by rabbits receiving hexoestrol orally*

Rabbit no.	Dose (mg./kg.)	Extra glucuronic acid (% of dose)	Extra ethereal sulphate (% of dose)	Extra neutral S (% of dose)
85	200	77.1	—	—
87	200	73.1	—	—
97	200	63.4	—	—
89	250	—	0	0
90	250	—	0	0

Fig. 2. The glucuronic acid and ethereal sulphate output in rabbits receiving hexoestrol orally. ●—●, glucuronic acid curve for rabbit no. 85 that received 558 mg. of hexoestrol on day 4. ○—○, ethereal sulphate curve for rabbit no. 90 that received 770 mg. of hexoestrol on day 4.

Isolation of *p*- β -D-glucuronosidodiphenyl. Preliminary experiments on the isolation of hexoestrol glucuronide indicated that its properties were not similar to those of stilboestrol glucuronide. In order to avoid undue wastage of hexoestrol, model experiments were first carried out on *p*-hydroxydiphenyl since it was thought that its glucuronide might be similar to that of saturated hexoestrol.

p-Hydroxydiphenyl (1 g.) (m.p. 164–165°; British Drug Houses Ltd.) suspended in water was fed to a rabbit by stomach tube. The urine (150 ml.) was collected after 24 hr. It gave a strong naphthoresorcinol reaction. On acidifying with HCl a fine white precipitate separated which could not be easily filtered. Extraction of the urine with ether yielded no glucuronide. The urine was therefore extracted three times in a separating funnel with a 1:3 ethanol-ether

mixture. The extract was evaporated and a crystalline residue (0.75 g.) was left. In a similar manner, the urine for the second day yielded 155 mg. of the material. The whole was recrystallized from a large volume of hot water and *p*- β -D-glucuronosidodiphenyl (*p*-phenylphenylglucuronide) crystallized as needles, m.p. 189°, showing $[\alpha]_D^{20} = -90.6^\circ$ ($c=1$ in 0.1N-NaOH). (Found: C, 58.1; H, 5.6%. $C_{18}H_{18}O_7 \cdot 1\frac{1}{2}H_2O$ requires C, 57.9; H, 5.7%.) It gives a strong naphthoresorcinol reaction, but only after heating with HCl and naphthoresorcinol for 2 min., i.e. twice the usual heating time (cf. dienoestrol glucuronide).

Benzylamine salt of *p*-glucuronosidodiphenyl. Ten drops of benzylamine were added gradually with stirring to 100 mg. of glucuronosidodiphenyl dissolved in 30 ml. of an equal mixture of ethanol and ethyl acetate. After 2 hr. at 0° the separated benzylamine salt was filtered and washed with a little ethanol (yield, 100 mg.). On crystallization from dilute aqueous ethanol, it formed colourless needles, m.p. 205° (decomp.) and showed $[\alpha]_D^{20} = -69.7^\circ$ ($c=1$ in 0.1N-NaOH). (Found: C, 65.5; H, 6.15; N, 2.9%. $C_{25}H_{27}O_7N \cdot \frac{1}{2}H_2O$ requires C, 64.9; H, 6.2; N, 3.0%.) This salt is more soluble in cold water than the corresponding salts of the oestrogens.

Hexoestrol monoglucuronide. Hexoestrol (1 g.) (*meso*-4:4'-dihydroxy- $\gamma\delta$ -diphenyl hexane, m.p. 184°; British Drug Houses Ltd.) was fed by stomach tube to each of five rabbits. The urine was collected daily for 4 days, each day's urine being worked up separately. Extraction with ether yielded very little material and the urine gave a strong naphthoresorcinol reaction only after prolonged boiling. Each day's urine was worked up as follows.

The urine (600–800 ml.) was acidified with 10N- H_2SO_4 and then shaken with 200 ml. of a 1:5 ethanol-ether mixture. A copious white precipitate of inorganic material separated and this was filtered off at the pump. The filtrate was saturated with $(NH_4)_2SO_4$ thus causing an effective separation of the ethanol-ether layer which was removed. The urine layer was extracted with two further 200 ml. portions of ethanol-ether. The combined extracts (dried by anhydrous Na_2SO_4) were taken to dryness *in vacuo* at 60°. A clear brown oil remained (which crystallized in some experiments). This was boiled with 30 ml. water and, after adding charcoal, filtered. On cooling, the glucuronide separated as an oil. The oil was redissolved in the water which was then saturated with $(NH_4)_2SO_4$ and the glucuronide extracted with 1:5 ethanol-ether. Removal of the solvents left a partially crystalline mass which further crystallized on triturating with ether. The crystals were filtered off and the mother liquor evaporated to a gum which was used for making the benzylamine salt.

The total yield of crystalline material was 0.5 g. On recrystallization from water or benzene-acetone, *hexoestrol mono- β -D-glucuronide trihydrate* was obtained as colourless needles, m.p. 183–184° (decomp.) after sintering at 100° and showing $[\alpha]_D^{20} = -66.3^\circ$ ($c=2$ in 0.1N-NaOH). (Found: C, 57.8;

* This rotation is quoted tentatively, since we suspect resolution during recrystallization (see discussion).

H, 6.95 %; glucuronic acid, 39.3 %. $C_{24}H_{30}O_8 \cdot 3H_2O$ requires C, 57.6; H, 7.25 %; glucuronic acid, 38.8 %.) The pure compound was insoluble in ether, benzene, light petroleum and chloroform and sparingly soluble (< 1 %) in ethanol, acetone and cold water. It gives a strong naphthoresorcinol test on prolonged boiling. Since it is insoluble in chloroform, the Dingemans $SbCl_5$ test cannot be carried out in the ordinary way. On suspending the glucuronide in dry chloroform and then warming the solution with an excess of 25 % $SbCl_5$ in chloroform a pale carmine colour is produced similar to that obtained with hexoestrol itself.

The benzylamine salt of hexoestrol monoglucuronide. Some of the residual gum obtained above after separation of crystalline hexoestrol glucuronide was dissolved in a mixture of ethanol and ethyl acetate (1:1). A slight excess of benzylamine was added drop by drop with stirring. No crystals separated. The mixture was therefore warmed on a water bath and on cooling and stirring, a crystalline solid separated. The crystals (m.p. 218°) were filtered and washed with absolute ethanol and dried in a desiccator. The salt on recrystallization from dilute aqueous ethanol formed colourless needles, m.p. 222°, and showed $[\alpha]_D^{20} = -48.7^\circ$ ($c=0.5$ in 2N-HCl in 50 % aqueous ethanol). (Found: C, 62.8; H, 7.0; N, 2.3 %. $C_{31}H_{30}O_8N \cdot 2H_2O$ requires C, 63.1; H, 7.35; N, 2.4 %.) It gave a strong naphthoresorcinol reaction on prolonged boiling, and a pale carmine colour in the Dingemans reaction carried out as for hexoestrol glucuronide. It was insoluble in ethanol, ethyl acetate and cold water, but soluble in hot water.

Table 4. *The glucuronic acid output in rabbits receiving dienioestrol orally*

Rabbit no.	Dose (mg./kg.)	Glucuronic acid output		
		Average normal (mg./day)	Average output/day for 3 days after dosing (mg./day)	Increase (mg.)
81	200	127	132	15
82	200	154	150	—
83	200	135	146	33
84	200	160	224	192

Table 5. *Ethereal sulphate and neutral sulphur output in rabbits receiving dienioestrol orally*

Rabbit no.	Dose (mg./kg.)	Ethereal sulphate increase	Neutral S increase
98	250	0	—
68	250	0	—
89	250	0	0
90	250	0	0
87	250	0	0
81	250	0	0

Experiments with dienioestrol

Quantitative results. These are given in Tables 4 and 5 and single experiments are illustrated in Fig. 3. These tables show that dienioestrol does not

disturb the ethereal sulphate and neutral sulphur output. In the case of glucuronic acid, Table 4 shows that in three cases there is apparently no disturbance of the normal glucuronic acid output. In the fourth case there is an appreciable rise corresponding to nearly 50 % of the dose. Qualitative experiments (see below) have shown that a glucuronide is actually excreted and the negative results in most of our quantitative glucuronic acid estimations

Fig. 3. The glucuronic acid and ethereal sulphate output in rabbits receiving dienioestrol orally. ●—●, glucuronic acid curve for rabbit no. 84 that received 556 mg. dienioestrol on day 3. ○—○, ethereal sulphate curve for rabbit no. 87 that received 930 mg. of dienioestrol on day 3.

can be ascribed to the stability of the glucuronide to acid hydrolysis. Stable glucuronides which cannot be quantitatively estimated by the naphthoresorcinol method have been described on previous occasions, e.g. euxanthic acid, *o*-aminophenyl-glucuronide and *p*-sulphonamido-*o*-aminophenyl-glucuronide (Hanson *et al.* 1944). The behaviour of dienioestrol monoglucuronide in the naphthoresorcinol procedure is being further investigated.

Dienoestrol monoglucuronide. Preliminary experiments showed that a single oral dose of 1 g. of dienioestrol (m.p. 238–239°) was not readily absorbed by rabbits and much of the compound appeared unchanged in the faeces. Therefore six rabbits were each given four separate doses of 0.25 g. during 2 days (at 11 a.m. and 4 p.m. each day). The urines were worked up daily for 5 days after the first dose, by the same method as was used for hexoestrol glucuronide. From the ether-ethanol extracts dienioestrol glucuronide was obtained as an oil which partially crystallized on triturating with ether. The residual urines after ether-ethanol extractions were then subjected to prolonged extraction (30 hr.) with ether in continuous extractors. In this way a total of 3.5 g. of crystalline glucuronide was obtained and 0.32 g. of the benzylamine salt was prepared from residues (cf. hexoestrol glucuronide). On repeated recrystallization from acidulated water *dienoestrol mono-β-D-glucuronide monohydrate* separated as

colourless needles, m.p. 181–183° (decomp.) after sintering at 120–135° and showing $[\alpha]_D^{20} -45.9^\circ$ ($c=1$ in 0.1N-NaOH). (Found: C, 62.65; H, 6.15%. $C_{24}H_{28}O_8 \cdot H_2O$ requires C, 62.6; H, 6.1%.)

The glucuronide is insoluble in ether, benzene and light petroleum, sparingly soluble in cold water, acetone and ethanol, but fairly soluble in the latter solvents when hot. A suspension of the glucuronide in chloroform gives the same magenta colour with $SbCl_5$ (Dingemans reaction) as does the parent oestrogen. It is remarkably stable to acid hydrolysis

C, 63.6; H, 6.7; N, 2.4%.) It behaves in the same way as the parent glucuronide in the Dingemans and naphthoresorcinol reactions.

Spectroscopic observations

Stilboestrol. A number of workers have studied the ultraviolet absorption spectrum of stilboestrol. Elvidge (1939) reported a single maximum in the absorption curve at 240 m μ . with ϵ_{max} 15,500–15,800. In a later paper (Elvidge, 1940) λ_{max} is given

Fig. 4. Ultraviolet absorption spectra of stilboestrol and stilboestrol glucuronide. $\times-\times$ stilboestrol in absolute ethanol. $\circ-\circ$ stilboestrol in 50% (v/v) aqueous ethanol + 1 equiv. KOH. $\bullet-\bullet$ stilboestrol in 50% (v/v) aqueous ethanol + 5 equiv. KOH. $\times-\times$ stilboestrol in 50% (v/v) aqueous ethanol + excess 0.005M-KOH. $\circ-\circ$ stilboestrol glucuronide in absolute ethanol.

for it can be recrystallized from acidulated water without hydrolysis. In the qualitative naphthoresorcinol test in which HCl of at least 5N strength is used, heating for 5 min. is necessary to get a positive reaction. If the naphthoresorcinol test is carried out on the glucuronide in the normal manner, i.e. 1 min. heating, the test is negative.

The benzylamine salt of dienoestrol glucuronide. This salt was prepared from the gummy residues after crystallization of the free glucuronide as described for hexoestrol. It was recrystallized from dilute aqueous ethanol and formed colourless needles, m.p. 223° (decomp.), and showed $[\alpha]_D^{20} = -35.4$ ($c=0.5$ in 0.1N-NaOH). (Found: C, 63.2; H, 6.7; N, 2.6%. $C_{31}H_{35}O_8N \cdot 2H_2O$ requires

as 239 m μ . with ϵ_{max} 16,000. Kharasch & Kleiman (1943) reported λ_{max} 250 m μ . Solmsen (1943) reported a maximum at 240 m μ . with ϵ_{max} 15,700 and an inflexion near 280 m μ , $\epsilon=7000$. The occurrence of this inflexion was confirmed by Kaiser & Koenig (1946), but they recorded the maximum at 250 m μ .

The sample of stilboestrol used in the present work showed in ethanol solution λ_{max} 240 m μ , ϵ_{max} 16,400, with inflexions near 245 m μ , $\epsilon=15,000$; and 270–280 m μ , $\epsilon=5000$ (Fig. 4).

The monoglucuronide shows λ_{max} 235 m μ , ϵ_{max} 14,600 with an inflexion 270–280 m μ , $\epsilon=5000$ (Fig. 4).

The ultraviolet absorption spectrum has also been studied in aqueous ethanol containing various

amounts of potassium hydroxide, in an attempt to obtain information regarding the ionization of the phenolic hydroxyl groups which might be correlated with the ease of biological conjugation (Fig. 4).

In 50% (v/v) aqueous ethanol containing one equivalent of KOH, the main absorption band of stilboestrol splits into two bands: $\lambda_{\max}$ 235 m μ ., $\epsilon_{\max}$ 15,000; $\lambda_{\max}$ 244 m μ ., $\epsilon_{\max}$ 14,500. The inflexion near 280 m μ . remains unchanged. Further

Hexoestrol. Elvidge (1939) reported two maxima in the absorption spectrum of hexoestrol $\lambda_{\max}$ 229 and 281 m μ . Kaiser & Koenig (1946) recorded $\lambda_{\max}$ 230 and 280 m μ .

It has been found, however, that the actual position of $\lambda_{\max}$ varies with the solvent. In 50% (v/v) aqueous ethanol the maxima occur at 240 and 295 m μ . with $\epsilon_{\max}$ 18,000 and 4700 respectively (Fig. 5). Addition of one equivalent of KOH to this solution shifts the spectrum to shorter wave lengths,

Fig. 5. Ultraviolet absorption spectra of hexoestrol and hexoestrol glucuronide. $\times-\times$ hexoestrol in 50% (v/v) aqueous ethanol. $\circ-\circ$ hexoestrol in 50% (v/v) aqueous ethanol + 1 equiv. KOH. $\bullet-\bullet$ hexoestrol in 50% (v/v) aqueous ethanol + excess 0.005M-KOH. $\otimes-\otimes$ hexoestrol glucuronide in absolute ethanol.

addition of alkali results in the longer wave length component becoming predominant together with the disappearance of the 280 m μ . inflexion. This variation in the position of $\lambda_{\max}$ with solvent between 235 and 250 m μ . may account for the lack of agreement between different workers regarding the actual position of the band. Furthermore, if the shift towards longer wave lengths with increasing pH of the solvent is ascribed to increased ionization of the stilboestrol, then the presence of an inflexion near 245 m μ . in ethanol solution and in a solution buffered at pH 7.4 is indicative of some ionization under these conditions.

$\lambda_{\max}$ 227.5 and 278 m μ . with $\epsilon_{\max}$ 20,200 and 3200 respectively (Fig. 5). Further addition of alkali up to five equivalents has little effect, but in excess of alkali (0.005M in a solution $M \times 10^{-4}$ with respect to hexoestrol) the spectrum reverts to that of hexoestrol in aqueous ethanol, showing $\lambda_{\max}$ 243.5 and 293.5 m μ . with $\epsilon_{\max}$ 21,200 and 3700 respectively (Fig. 5).

Hexoestrol glucuronide in ethanol shows $\lambda_{\max}$ 227 and 276 m μ . with $\epsilon_{\max}$ 22,000 and 2400 respectively (Fig. 5).

It is difficult to interpret these results on the basis of ionization of hexoestrol, particularly since the

shift to shorter wave lengths on addition of one equivalent of KOH is contrary to the usual behaviour of phenolic compounds. (Phenol itself shows $\lambda_{\max}$ 215 and 273 m μ . in ethanol solution and $\lambda_{\max}$ 230 and 285 m μ . in alkali.) It seems more likely that the variations are to be ascribed to a solvent effect only, the effects of any ionization changes being subsidiary.

measurements show that at least 70 % of the drugs is conjugated with glucuronic acid and this is probably true also for dienioestrol. This conjugation probably results in a detoxication and an inactivation of these compounds. Not one of the three compounds appears, from quantitative data, to form a mercapturic acid or an ethereal sulphate. The absence of mercapturic acid formation is not

Fig. 6. Ultraviolet absorption spectra of dienioestrol and dienioestrol glucuronide. $\times$ — $\times$ dienioestrol in absolute ethanol. $\circ$ — $\circ$ dienioestrol in 50% (v/v) aqueous ethanol+1 equiv. KOH. $\otimes$ — $\otimes$ dienioestrol glucuronide in absolute ethanol.

Dienioestrol. The sample of dienioestrol shows an absorption spectrum similar in general shape to that of stilboestrol, with $\lambda_{\max}$ 229 m μ ., $\epsilon_{\max}$ 30,000. There is also an inflexion in the curve between 275 and 280 m μ . with $\epsilon = 4000$ (Fig. 6). Elvidge (1940) gives $\lambda_{\max}$ 228 m μ ., $\epsilon_{\max}$ 26,600. There is little change in spectrum with variation in solvent pH, whilst the glucuronide shows $\lambda_{\max}$ 228 m μ ., $\epsilon_{\max}$ 34,700, with an inflexion near 275 m μ ., $\epsilon = 4000$ (Fig. 6).

These spectra give little information regarding any possible ionization of the dienioestrol molecule. It is probable that the absorption maximum at 229 m μ . has its origin in the ethylenic double bonds, and is little affected by ionization of the phenolic hydroxyls.

DISCUSSION

The present results indicate that in the rabbit the main metabolic change undergone by the three synthetic oestrogens studied is conjugation with glucuronic acid to form monoglucuronides. In the cases of stilboestrol and hexoestrol quantitative

surprising for all three are para-substituted compounds and therefore the position in the aromatic ring where acetylcysteine conjugation normally occurs is blocked. Furthermore there is no known case of an aromatic hydroxy compound forming a mercapturic acid. However, the apparent absence of ethereal sulphate conjugation is surprising, for all three compounds contain phenolic hydroxyl groups, the prerequisites for sulphate conjugation. There is of course the possibility that ethereal sulphates are slowly formed and excreted, but that their concentration in the urine is too low to be detected by the quantitative method used. This possibility is being investigated. If the absence of ethereal sulphates is confirmed, then we must look to the nature of the hydroxyl groups for the reason, for phenols are known which, when fed to animals, do not conjugate as ethereal sulphate, e.g. salicylic acid (Williams, 1938). In the case of salicylic acid the phenolic hydroxyl is not free as a result of hydrogen bonding. It is possible that ethereal sulphate formation is related to the ionization of phenolic hydroxyl groups

for it takes place only with phenols. Etheral sulphate formation with other types of hydroxyl groups, such as those found in alcohols, has never been recorded. In the alcohols it is known that the OH groups are extremely feebly ionized (e.g. glycol, $K_a = 6 \times 10^{-15}$; glycerol, $K_a = 7 \times 10^{-15}$) whereas in phenols the ionization constants vary between $K_a = 5.6 \times 10^{-8}$ for *p*-nitrophenol to about 1×10^{-10} for phenol (see Britton, 1942). This suggests a small optimum ionization of the OH group for etheral sulphate formation. The ionization constants of the synthetic oestrogens have not been recorded. Our spectroscopic data indicate that stilboestrol will ionize in alkaline solution, but the data for hexoestrol and dienoestrol do not permit of any conclusion in this respect. The relation between ionization constants and etheral sulphate formation is being further investigated.

That stilboestrol has a *trans*-structure has, however, been questioned on spectroscopic grounds by Solmssen (1943).

In the case of hexoestrol, the position is not quite so clear, since hexoestrol is an internally compensated compound, i.e. *meso*-3:4-di(*p*-hydroxyphenyl)-hexane. Conjugation of one of the hydroxyls of hexoestrol with glucuronic acid destroys this internal compensation and therefore two enantiomorphous hexoestrol monoglucuronides (II and III)

Table 6. *The properties of the monoglucuronides of synthetic oestrogens*

Property	Stilboestrol glucuronide	Hexoestrol glucuronide	Dienoestrol glucuronide
Hydration	2H ₂ O	3H ₂ O	1H ₂ O
M.p.	179°	183–184°	181–183°
[α] _D	–56.5° (in ethanol)	–66.3°* (in 0.1 N-NaOH)	–45.9° (in 0.1 N-NaOH)
Ultraviolet absorption spectra in ethanol	λ _{max.} 235 mμ. ε _{max.} 14,600 ~270–280 mμ. 5,000	λ _{max.} 227 mμ. ε _{max.} 22,000 276 mμ. 2,400	λ _{max.} 228 mμ. ε _{max.} 34,700 ~275 mμ. 4,000
Stability to acid hydrolysis	Easily hydrolyzed	Hydrolyzed with some difficulty	Hydrolyzed with much difficulty
M.p. of benzylamine salt	223°	222°	223°

* See discussion.

The nature of the glucuronides of the synthetic oestrogens also calls for some comment (see Table 6). They vary in their stability to acid hydrolysis, for whereas stilboestrol glucuronide is relatively easily hydrolyzed, dienoestrol glucuronide is hydrolyzed only with difficulty, hexoestrol glucuronide taking an intermediate position. All three are hydrated which is a common feature of many glucuronides. It has been frequently observed in this laboratory that a large number of glucuronides do not crystallize without water of crystallization and removal of this water usually results in the production of a non-crystalline glass. The negative rotations of the three glucuronides indicate that, like all biosynthetic glucuronides, they are β-glycosides.

The exact structures of these glucuronides have yet to be determined for a consideration of the stereochemistry of hexoestrol and dienoestrol indicates that the biosynthesis of more than one monoglucuronide is possible in these cases. The structure of stilboestrol glucuronide is straightforward because, if we accept a *trans*-stilbene structure for stilboestrol, then the glucuronide can be formulated as 4-(β'-D-glucuronido)-4'-hydroxy-α:β-diethyl-*trans*-stilbene (I).

should arise. This point is being further investigated, for it is quite possible that the glucuronide isolated is a racemate.

where —PhOG = —C₆H₄OC₆H₄O₆.

Similar difficulties arise with dienoestrol, but for slightly different reasons. As far as we are aware, the configuration of dienoestrol has not been established, but we know that it is a 3:4-di(*p*-hydroxyphenyl)-hexa-2:4-diene.

If it has structure (IV), then two monoglucuronides are possible depending upon which hydroxyl is conjugated. With structures (V) and (VI) only one monoglucuronide is possible in each case. These points are being further investigated.

Stroud (1939b) has shown that stilbene when injected into rabbits gives rise to hippuric acid, which indicated splitting and oxidation at the double bond.

IV

V

VI

A similar change is therefore possible with stilboestrol, the product in this case being *p*-hydroxybenzoic acid. We have examined stilboestrol urine for *p*-hydroxybenzoic acid, but were unable to find it. We conclude therefore that, in the rabbit, the double bond in stilboestrol is resistant to biological attack.

SUMMARY

1. A preliminary study has been made of the fate of stilboestrol, hexoestrol and dienooestrol in the rabbit.

2. The preparation and properties of the monoglucuronides of stilboestrol, hexoestrol and dienooestrol are described.

oestrol prepared by feeding these oestrogens to rabbits are described.

3. The benzylamine salts of the above glucuronides have been prepared and are described.

4. The isolation of *p*-glucuronosidodiphenyl from the urine of a rabbit receiving *p*-hydroxydiphenyl is described, together with its benzylamine salt.

5. Some 70 % of stilboestrol and of hexoestrol is excreted by rabbits combined with glucuronic acid. In the case of dienooestrol the amount conjugated is uncertain because dienooestrol monoglucuronide is highly resistant to acid hydrolysis and hence its determination by the method of Hanson, Mills & Williams (1944) is uncertain.

6. None of the three oestrogens affects the ethereal sulphate and neutral sulphur level of rabbit urine. These results are discussed and the possible reasons for them indicated.

7. The absorption spectra of the three oestrogens and their glucuronides under various conditions have been studied.

8. There is no indication that stilboestrol is degraded *in vivo* to single ring compounds.

9. The stereochemistry of hexoestrol and dienooestrol glucuronides is discussed.

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