Metabolic Fates of Diethylstilboestrol Sulphates in the Rat

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(Received 12 October 1976)

The metabolic fates and modes of excretion of diethylstilboestrol mono[^{35}S]sulphate and diethylstilboestrol di[^{35}S]sulphate were studied in the rat. Both of the esters were desulphated to some extent *in vivo*. In addition, significant amounts of radioactivity appeared in the bile as diethylstilboestrol mono[^{35}S]sulphate monoglucuronide. The percentage of the dose appearing in bile as the diconjugate was substantially greater in experiments with diethylstilboestrol mono[^{35}S]sulphate than with diethylstilboestrol di[^{35}S]sulphate. Whole-body radioautography and studies with isolated perfused liver confirmed the liver as the major metabolic organ for both esters. When the metabolite diethylstilboestrol mono[^{35}S]sulphate monoglucuronide isolated from the bile was reinjected, it was excreted in the bile unchanged. Studies *in vitro* demonstrated that both esters were substrates for arylsulphatase C with K_m values in the range 52–76 μM . The metabolic fates and modes of excretion of the esters are discussed in relation to the enzyme complement of rat liver.

Diethylstilboestrol has been used as a synthetic oestrogen in humans and in food-producing animals. It has been shown to have carcinogenic properties in a number of animal species (Umberger, 1975) and its use has been associated with the occurrence of tumours in humans (Herbst *et al.*, 1972).

The metabolic fate of diethylstilboestrol has been studied in several animal species (Abou-El-Makarem *et al.*, 1967) and it has been shown that biliary excretion as a glucuronic acid conjugate represents a major pathway for elimination (Klaasen, 1973). The disulphate ester of diethylstilboestrol, which is approximately half as active as diethylstilboestrol itself with respect to oestrogenic activity (Bishop *et al.*, 1951), has been used as a water-soluble form of the synthetic oestrogen. Nevertheless, the metabolism of sulphated forms of diethylstilboestrol has not been reported. The present paper describes investigations on the metabolic fate and modes for excretion of diethylstilboestrol di[^{35}S]sulphate and diethylstilboestrol mono[^{35}S]sulphate in the rat.

Materials and Methods

Potassium diethylstilboestrol di[^{35}S]sulphate

To a well-stirred mixture of *NN*-dimethylaniline (2.9 ml) and carbon disulphide (4 ml) at -5°C was added dropwise 0.27 ml of chloro[^{35}S]sulphonic

acid (specific radioactivity 11 mCi/mmol; obtained from The Radiochemical Centre, Amersham, Bucks., U.K.). The mixture was stirred for 30 min, after which time it was allowed to reach room temperature. Diethylstilboestrol [0.536 g; Sigma (London) Chemical Co., Kingston, Surrey, U.K.] was then added slowly, with stirring; the mixture was stirred continuously for 1 h, and then stored at room temperature for a further 16 h. After centrifuging (2000 g), the supernatant was discarded and the pellet extracted with ether (3 $\times$ 20 ml) to remove any remaining diethylstilboestrol. Aq. KOH (1.2 ml; 50%, w/v) was added to neutralize any remaining acid, and the potassium salt of diethylstilboestrol di[^{35}S]sulphate was crystallized from hot water (80 $^\circ\text{C}$). The product was dried *in vacuo* over P_2O_5 at room temperature. Pure potassium diethylstilboestrol di[^{35}S]sulphate was prepared from the crude product by preparative t.l.c. The crude product was applied as streaks and the plates were developed in solvent A. The appropriate area of silica gel was then removed and extracted (3 $\times$ 30 ml) with acetone/water (3:2, v/v). After filtering to remove silica gel the solution was evaporated to dryness at 40 $^\circ\text{C}$ *in vacuo*. The solid was dissolved in a minimum volume of hot water (80 $^\circ\text{C}$), neutralized with aq. KOH (50%, w/v) and cooled to 4 $^\circ\text{C}$. The product was isolated and recrystallized twice from water. The product (250 mg) gave analysis values for K^+ and SO_4^{2-} agreeing with the structure of anhydrous potassium diethylstilboestrol di[^{35}S]sulphate (Found: K, 15.3; SO_4^{2-} , 12.3. $\text{C}_{18}\text{H}_{18}\text{O}_8\text{S}_2\text{K}_2$ requires K, 15.5; SO_4^{2-} , 12.7%).

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Potassium diethylstilboestrol mono[³⁵S]sulphate

Diethylstilboestrol (0.808 g) was dissolved in dry pyridine (15 ml) and the mixture was cooled to 0°C. Chloro[³⁵S]sulphonic acid (0.4 g, 8.9 mCi/mmol) was added dropwise with constant stirring and the mixture was further stirred for 30 min. The mixture was subsequently stored at room temperature for 16 h, the solution was diluted with ether (2 vol.) and the white precipitate was removed by centrifuging (2000 g). The precipitate was washed with ether (2 × 20 ml) to remove any unchanged diethylstilboestrol, dissolved in a minimum volume of hot (80°C) water and the pH adjusted to 7.0 with aq. KOH (20%, w/v). The solution was cooled (4°C) and the precipitate, isolated by centrifuging (2000 g), was dissolved in a minimum volume of acetone/water (1:1, v/v) and applied as streaks to preparative chromatography plates. The chromatograms were developed in solvent A. The appropriate areas of silica gel were located, removed and extracted with acetone/water (1:1, v/v; 3 × 20 ml). The resulting solution was evaporated to dryness *in vacuo* at 40°C and the product was recrystallized from hot (80°C) aq. 1 M-KOH. The product was recrystallized twice from hot water and dried *in vacuo* over P₂O₅ at room temperature. The product gave analysis values for K⁺ and SO₄²⁻ agreeing with the structure of anhydrous potassium diethylstilboestrol mono[³⁵S]sulphate (Found: K, 9.84; SO₄²⁻, 8.2. C₁₈H₁₉O₅SK requires K, 10.1; SO₄²⁻, 8.3%).

Potassium diethylstilboestrol di[³⁵S]sulphate and potassium diethylstilboestrol mono[³⁵S]sulphate were stored as frozen aqueous solutions (5 mg/ml) to minimize any breakdown by self-irradiation processes. Under these conditions the esters were stable.

Chromatography and paper electrophoresis

Thin-layer chromatograms were run on silica gel (Kieselgel G; Merck A. G., Darmstadt, W. Germany). All plates were activated by heating at 110°C for 45 min before use. Solvent systems used were: A, butan-1-ol/acetic acid/water (50:12:25, by vol.); B, benzene/ethyl methyl ketone/ethanol/water (3:3:3:1, by vol.). Horizontal paper electrophoresis was carried out Whatman no. 1 paper for 2 h at a potential gradient of 10 V/cm. Buffers used were 0.1 M-sodium acetate/acetic acid, pH 5.0, 0.1 M-sodium phosphate, pH 7.0, or 0.1 M-Tris/HCl, pH 8.5. Radioactive areas on chromatograms and electrophoretograms were detected by radioautography for suitable periods on Kodak Industrex B X-ray film or by scanning. The relative amount of radioactivity associated with each spot was estimated from the record of the scanner as described by Jones & Dodgson (1965).

Experimental animals

Adult male and female MRC Hooded rats were used throughout. Intraperitoneal injections of the esters were carried out while the animals were under ether anaesthesia. The animals were placed in metabolism cages designed for the separate collection of urine and faeces and were allowed food and water *ad libitum*.

In experiments on rats with bile-duct and ureter cannulae, animals were anaesthetized with ether, and Nembutal (15 mg/kg body wt.) was administered via the cannulated jugular vein. The bile duct and ureters were cannulated and animals were maintained under Nembutal anaesthesia throughout the experiments.

Isolated-liver perfusion

Isolated rat livers were perfused as described by Curtis *et al.* (1971).

Whole-body radioautography

The method used was that described by Powell *et al.* (1967).

Measurement of radioactivity in biological materials

The measurement of radioactivity in urine, bile and blood samples was as described by Flynn *et al.* (1967). Total [³⁵S]sulphate content was determined after hydrolysis of samples for 16 h. Time-hydrolysis curves for the ³⁵S-labelled sulphate esters of diethylstilboestrol showed that hydrolysis was complete after this time. The total ³⁵S contents of faeces and carcasses were precipitated and counted for radioactivity as Ba³⁵SO₄ after oxidation with fuming HNO₃ by the method of Young *et al.* (1949).

Preparation of subcellular fractions

Livers from adult male MRC Hooded rats were used throughout. Rats were killed by a blow on the back of the head and the livers were quickly excised into a chilled (0°C) beaker. All the subsequent manipulations were carried out at 0°C. Livers (10 g) were homogenized in unbuffered 0.25 M-sucrose (50 ml) in a Potter-Elvehjem-type homogenizer. The volume of homogenate was adjusted to 100 ml with 0.25 M-sucrose. A portion was retained for protein assay, and the remainder was fractionated by the method of de Duve *et al.* (1955). Fractions were assayed for appropriate marker enzymes (Milsom *et al.*, 1972), and protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin [Sigma (London) Chemical Co., Kingston, Surrey, U.K.] as standard. Individual fractions were resuspended in 0.25 M-sucrose so that 5 ml of the suspension was equivalent to 1 g of liver.

Assay conditions for the measurement of hydrolytic activity towards the [^{35}S]sulphate esters of diethylstilboestrol

Incubation mixtures contained 300 μl of the appropriate subcellular fraction and 900 μl of 0.1 M-Tris/HCl buffer, pH 7.5, containing either potassium diethylstilboestrol di[^{35}S]sulphate (100 μM) or potassium diethylstilboestrol mono[^{35}S]sulphate (100 μM). The mixtures were incubated at 37°C and after 10 min the reaction was stopped by addition of an equal volume of ethanol. The precipitated protein was removed by centrifuging (2000g) and portions (0.5 ml) of the supernatant were assayed for total [^{35}S]sulphate and inorganic [^{35}S]sulphate. Ester sulphate was obtained by difference. The following control incubations were also assayed: (a) those in which the subcellular fraction and substrate were separately incubated and mixed immediately before the addition of ethanol; (b) those in which boiled subcellular fractions were incubated with substrate.

Experimental and Results

Experiments with diethylstilboestrol di[^{35}S]sulphate

Whole-body radioautography. Male and female rats (30–50g body wt.) received intraperitoneal injections of potassium diethylstilboestrol di[^{35}S]sulphate (1 mg/animal). Animals were killed after 10, 20, 30 min, 1, 6, 12 and 24 h, and whole-body radioautograms were prepared (see Plate 1). In radioautograms obtained 30 min after injection only trace amounts of radioactivity remained in the intraperitoneal cavity. The radioactivity accumulated rapidly in the liver,

and 10 min after administration of the ester the amount of radioactivity in the liver greatly exceeded that associated with the blood system. Radioautograms of animals killed after 30 min showed accumulation of radioisotope in areas corresponding to the kidney and bladder. There was also evidence of considerable accumulation of radioactivity in cartilaginous tissue 6 h after injection. In all radioautograms, only trace amounts of ^{35}S appeared in the gastrointestinal tract, and radioautograms of animals killed after 24 h showed only minor amounts of radioisotope remaining.

Intraperitoneal administration. In preliminary experiments rats (three male and three female) received intraperitoneal injections of potassium diethylstilboestrol di[^{35}S]sulphate (1 mg/0.2 ml of water per 200 g body wt.). Urines were collected at intervals of 6, 12, 24 and 48 h after administration of the ester and assayed for total and inorganic [^{35}S]sulphate content. Faeces were collected over 48 h, and at this time animals were killed. Total ^{35}S present in faeces and carcasses was determined. The results are shown in Table 1. Most of the radioactivity was recovered in the urine in 48 h (average recovery in males 58.3% of the dose, in females 68.5% of the dose) almost entirely as inorganic [^{35}S]sulphate (55.5% in males, 62.5% in females). Between 11.2% and 44% of the administered radioactivity was recovered in the faeces and a significant amount of the injected radioactivity remained in carcasses (4.1–28.6%).

Qualitative analysis of urine. Urines obtained from rats receiving intraperitoneal injections of potassium diethylstilboestrol di[^{35}S]sulphate were subjected to

Table 1. Distribution of ^{35}S in urine, faeces and carcasses of male and female rats injected intraperitoneally with diethylstilboestrol di[^{35}S]sulphate (1 mg/200g body wt.)

For experimental details see the text. Results are mean values for three animals, with ranges in parentheses.

		Distribution of ^{35}S (% of ^{35}S injected)			
		Male rats		Female rats	
	Time (h)	Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate
^{35}S content of urine samples	6	24.0 (19.4–28.6)	0.2 (0.0–0.3)	28.7 (18.3–37.1)	0.8 (0.0–2.3)
	12	16.4 (13.9–20.3)	0.6 (0.0–1.7)	15.3 (12.7–19.8)	3.0 (2.8–3.2)
	24	10.7 (9.3–11.6)	1.3 (0.0–3.9)	12.4 (9.0–15.5)	0.4 (0.0–1.1)
	48	4.4 (2.3–5.8)	0.7 (0.3–1.1)	6.1 (5.0–7.5)	1.8 (0.5–4.0)
	Total	55.5	2.8	62.5	6.0
Total ^{35}S content of faeces		23.9 (13.6–44.0)		13.8 (11.2–16.2)	
Total ^{35}S content of carcasses		19.1 (4.1–28.6)		18.4 (16.0–20.4)	
Recovery (% of injected dose)		100.6 (96.8–103.7)		100.6 (98.7–103.1)	

t.l.c. in solvents A and B and electrophoresis, together with appropriate controls, i.e. normal rat urine to which potassium diethylstilboestrol di[³⁵S]sulphate had been added and normal rat urine to which inorganic [³⁵S]sulphate had been added. One major radioactive component only was detected and its mobility corresponded to that of inorganic [³⁵S]sulphate. No parent ester sulphate was detected in any urine sample. Occasionally, traces of ³⁵S-labelled compounds appeared in some urine samples (12h and 24h).

Intravenous administration. Potassium diethylstilboestrol di[³⁵S]sulphate was administered intravenously (1 mg/0.2 ml of water per 200 g body wt.) to rats (three male and three female) with bile-duct and ureter cannulae. Urine and bile were collected at 15 min intervals for 2 h and then at hourly intervals for a further 4 h. Urine and bile samples were assayed for total and inorganic [³⁵S]sulphate. The results are shown in Table 2. Most of the excreted radioactivity appeared in the urine (average recovery in males 31.2% of the dose, in females 32.7% of the dose) as inorganic [³⁵S]sulphate. However, appreciable amounts of the injected radioactive dose appeared in the bile, largely as ester [³⁵S]sulphate (average values for males 16.5% of the dose, for females 17.8%).

Further experiments were carried out in which rats received the ester at dosages of 5 mg/200 g body wt. and 10 mg/200 g body wt. The results of these experiments showed no significant deviation from the results obtained at a dose of 1 mg/200 g body wt. Thus between 49.8% and 69.2% of the radioactivity was recovered in bile and urine in 6 h at both dosages. The radioactivity was recovered mainly in the urine (36.7–51%), but some also appeared in the bile (13–19.6%), almost entirely as ester [³⁵S]sulphate.

Qualitative analysis of bile and urine samples. Urine and bile samples from cannulated animals receiving intravenous injections of diethylstilboestrol di[³⁵S]sulphate (1, 5 and 10 mg/100 g body wt.) were subjected to t.l.c. and electrophoresis. Appropriate controls were also subjected to t.l.c. and electrophoresis, namely normal bile and urine to which diethylstilboestrol di[³⁵S]sulphate or inorganic [³⁵S]sulphate had been added. The major radioactive component in urine was chromatographically and electrophoretically identical with inorganic [³⁵S]sulphate. Trace amounts (less than 1% of radioactivity in urine) of other ³⁵S-labelled materials were detected, but one of these could be identified with authentic diethylstilboestrol di[³⁵S]sulphate.

Bile contained one major radioactive component (metabolite A, approx. 90% of radioactivity in bile). In addition, traces only of three further components were observed on some chromatograms. These were chromatographically identical with unchanged diethylstilboestrol di[³⁵S]sulphate, diethylstilboestrol mono[³⁵S]sulphate and inorganic [³⁵S]sulphate.

Table 2. Distribution of ³⁵S in urine and bile of male and female rats injected intravenously with either diethylstilboestrol di[³⁵S]sulphate or diethylstilboestrol mono[³⁵S]sulphate at a dose of 1 mg/200 g body wt.

For details see the text. Results are mean values for three animals with ranges in parentheses.

	Distribution of ³⁵ S (% of ³⁵ S injected)					
	Male rats			Female rats		
	Urine		Bile	Urine		Bile
	Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate
Diethylstilboestrol di[³⁵ S]sulphate	31.2 (21.6–50.4)	4.1 (0.0–10.7)	5.2 (1.9–7.4)	32.7 (23.5–50.6)	4.4 (0.2–9.4)	17.8 (10.5–22.8)
Diethylstilboestrol mono[³⁵ S]sulphate	22.4 (16.9–25.8)	2.7 (1.0–4.6)	9.3 (6.7–13.2)	31.7 (29.1–33.9)	0.8 (0.0–1.6)	44.5 (39.1–47.5)

EXPLANATION OF PLATE I

Whole-body radioautograms showing the distribution of ^{35}S in young rats: (a) 20 min after intraperitoneal injection of 1 mg of potassium diethylstilboestrol di[^{35}S]sulphate, (b) 6 h after intraperitoneal injection of potassium diethylstilboestrol mono[^{35}S]sulphate.

After radioautography, thin-layer plates were sprayed with phosphoric acid/ethanolic 0.2% naphtharesorcinol (1:22, v/v), and heated at 105°C for 10 min (Randerath, 1963). Each bile sample contained a component which gave a characteristic blue colour typical of glucuronic acid conjugates and which coincided on chromatography with the major radioactive component.

Confirmatory evidence for the presence of glucuronic acid conjugates was obtained in experiments in which bile was incubated with β -glucuronidase. Bile (0.5 ml) was mixed with an equal volume of a solution of β -glucuronidase [Sigma type B1 from bovine liver, 2000 Fishman units (Talalay *et al.*, 1946)/ml in 0.1 M-sodium phosphate buffer, pH 6.5] and incubated at 37°C for 4 h. Portions (40 μ l) of the incubation mixture, together with untreated bile and appropriate markers, were subjected to t.l.c. in solvent B. Scans from these chromatograms demonstrated a marked decrease (approx. 50%) in the concentration of metabolite A and a concomitant enhancement in the concentration of a radioactive component chromatographically identical with diethylstilboestrol mono[35 S]sulphate. The decrease in radioactivity associated with metabolite A was equivalent to the increase in radioactivity associated with diethylstilboestrol mono[35 S]sulphate.

The positive reaction obtained with the naphtharesorcinol reagent, coupled with the results obtained with β -glucuronidase, allowed the identifi-

cation of metabolite A as diethylstilboestrol mono[35 S]sulphate monoglucuronide.

Metabolic fate of metabolite A. Metabolite A was prepared from bile obtained from rats receiving diethylstilboestrol di[35 S]sulphate at dosages of 5 and 10 mg/200 g body wt. Bile samples were combined and concentrated to approx. 1 ml at 40°C *in vacuo*. The concentrate was diluted to 40 ml with acetone/water (1:1, v/v). After centrifuging (2000g), the supernatant was applied as streaks to preparative thin-layer plates, which were developed in solvent B. The position of the major metabolite in the bile was located by short-wave u.v. light (254 nm); the appropriate area of silica gel was removed and eluted with acetone/water (1:1, v/v; 3 $\times$ 100 ml). The eluate was evaporated to dryness at 40°C *in vacuo* and the residue was crystallized from the minimum volume of water. The yield of radioactive material was 5 mg (as estimated by radioactivity content) and was shown to be chromatographically homogeneous and chromatographically identical with metabolite A.

Aqueous solutions of metabolite A (1 ml, containing approx. 0.3 mg) were injected intravenously into anaesthetized male rats. Bile was collected 1 h and 2 h after injection and radioactivity measured. Of the injected radioactivity, 96.4% (animal I) and 88.4% (animal II) of the dose was present in the bile after 2 h. When bile samples were subjected to t.l.c. in solvent A one radioactive spot, identical in mobility with metabolite A, was detected.

Fig. 1. Time course of the biliary excretion of radioactivity and the appearance of inorganic [35 S]sulphate after the addition of potassium diethylstilboestrol di[35 S]sulphate or potassium diethylstilboestrol mono[35 S]sulphate to isolated perfused rat livers (a) Inorganic [35 S]sulphate in perfusate and (b) radioactivity in bile after the addition of potassium diethylstilboestrol di[35 S]sulphate; (c) inorganic [35 S]sulphate in perfusate and (d) radioactivity in bile after the addition of potassium diethylstilboestrol mono[35 S]sulphate. Results are means of four experiments with each ester (1 mg/10 ml of perfusate).

Isolated-liver-perfusion studies with potassium diethylstilboestrol di[³⁵S]sulphate. To assess the contribution of the liver to the metabolic degradation of diethylstilboestrol disulphate the metabolic fate of the ³⁵S-labelled ester was studied in the isolated perfused liver system.

Preliminary experiments were carried out to investigate possible hydrolysis in blood in the absence of the liver. Diethylstilboestrol di[³⁵S]sulphate (1 mg) was added to heparinized whole blood (10 ml) and incubated at 37°C for 6 h. Samples (1 ml) were removed hourly and assayed for inorganic [³⁵S]-sulphate and total [³⁵S]sulphate. Hydrolysis of the ester was not detected over the 6 h period.

Diethylstilboestrol di[³⁵S]sulphate (10 mg) was added to the perfusate (100 ml) and rat livers (male and female) were perfused for 6 h. Samples of blood (0.5 ml) and bile were collected at 30 min and 60 min after the introduction of the ester into the liver perfusion system, and thereafter at hourly intervals. The blood and bile samples were assayed for inorganic [³⁵S]sulphate and total [³⁵S]sulphate.

After introduction of the liver, there was an initial rapid hydrolysis of the ester during the first 30 min, when the amount of inorganic [³⁵S]sulphate reached 62% (expressed in terms of the total radioactivity of the blood sample). The relative amount of inorganic [³⁵S]sulphate in the blood increased over 3 h, at which time all the circulating radioactivity was present in this form and ester [³⁵S]sulphate could not be detected (see Fig. 1).

Analysis of bile samples showed that the amount of ³⁵S excreted in the bile (expressed as a percentage of ³⁵S initially added to the blood) was 11%; this ³⁵S in bile was present entirely in the form of ester sulphate. Chromatographic analysis of bile by t.l.c. in solvents A and B revealed the presence of a major radioactive component chromatographically identical with metabolite A.

Experiments with diethylstilboestrol mono[³⁵S]sulphate

Whole-body radioautography. Male and female rats (30–50 g body wt.) received intraperitoneal injections of potassium diethylstilboestrol mono[³⁵S]sulphate (1 mg/animal) and were killed at intervals ranging from 5 min to 6 h. Whole-body radioautograms demonstrated the rapid accumulation of radioactivity in the liver. Considerable amounts of radioactivity were associated with the gastrointestinal tract, indicating substantial elimination of radioactivity in bile. There was some accumulation of radioactivity in the cartilage of the long bones and intercostal cartilages, suggesting that some desulphation of the ester had occurred (see Plate 1).

Intravenous administration. Rats (three male and three female) with bile-duct and ureter cannulae received intravenous injections of potassium diethylstilboestrol mono[³⁵S]sulphate (1 mg/200 g body wt.).

Urine and bile were collected at hourly intervals over 6 h and assayed for total and inorganic [³⁵S]-sulphate. The results are shown in Table 2. The most striking feature of these results is the extent of elimination of ³⁵S in bile in comparison with that in the urine. Thus the amount of radioactivity appearing in the bile in 6 h was approx. 46.9% of the injected dose in male rats and 49.4% in female rats. The corresponding values for urine were 25.1% and 32.5%. Although the ³⁵S in bile was present largely as ester [³⁵S]sulphate, the radioactivity present in the urine was almost entirely inorganic [³⁵S]sulphate.

A further series of experiments was carried out in which male rats with bile-duct and ureter cannulae received intravenous injections of the ester at two further dosages (5 and 10 mg/200 g body wt.). Substantially the same percentage of injected radioactivity appeared in the bile as previously recorded at the 1 mg/200 g body wt. dose. All the radioactivity in urine was present as inorganic [³⁵S]sulphate.

Qualitative analysis of bile and urine samples. Urine and bile samples were collected over 3 h from male and female rats receiving diethylstilboestrol mono[³⁵S]sulphate and subjected to t.l.c. in solvents A and B and electrophoresis. Radioactive areas were located by scanning and radioautography. The major radioactive component in urine was chromatographically and electrophoretically identical with inorganic [³⁵S]sulphate. Bile samples contained one major radioactive component (95% of biliary radioactivity), which was chromatographically identical with metabolite A. On some chromatograms, trace amounts (less than 1%) of diethylstilboestrol mono[³⁵S]sulphate were detected in bile samples collected up to 1 h after its administration. The major metabolite in bile was identified as diethylstilboestrol mono[³⁵S]sulphate monoglucuronide as described above.

Isolated-liver-perfusion studies. Preliminary experiments showed that hydrolysis of diethylstilboestrol mono[³⁵S]sulphate did not occur in the perfusate in the absence of the liver, under the conditions of the experiment. Potassium diethylstilboestrol mono[³⁵S]sulphate was added to whole rat blood (1 mg/10 ml), and rat livers (three male and one female) were perfused for 6 h. Samples of blood (0.5 ml) and bile were collected at 30 min and 60 min after the introduction of the ester into the liver-perfusion system, and thereafter at hourly intervals. The blood samples were subjected to electrophoresis; electrophoretograms were scanned and the inorganic [³⁵S]sulphate and ester [³⁵S]sulphate contents determined. Bile samples were assayed for total [³⁵S]sulphate and inorganic [³⁵S]sulphate contents.

There was an initial rapid rise in the concentration of inorganic [³⁵S]sulphate over the first 30 min, at which time 76% of the circulating radioactivity was present as inorganic [³⁵S]sulphate. After 3 h

all the circulating radioactivity was in this form. Unchanged diethylstilboestrol mono[^{35}S]sulphate was detected in the first (30 min) sample only (see Fig. 1).

The amount of radioactivity in bile (expressed as a percentage of ^{35}S initially added to the perfusate) varied considerably from one experiment to another: values recorded were 25.7, 48.9, 60.5 and 27.1%. The major component of all bile samples was chromatographically identical with diethylstilboestrol mono[^{35}S]sulphate monoglucuronide and traces only of other radioactive components could be detected.

Hydrolytic activity of subcellular fractions towards the [^{35}S]sulphate esters of diethylstilboestrol

Preliminary experiments with each subcellular fraction showed that the distribution of hydrolytic activity with respect to diethylstilboestrol di[^{35}S]sulphate was substantially the same as that for the microsomal marker enzyme arylsulphatase C (Dolly *et al.*, 1972). Since the highest relative specific activity was associated with the microsomal fraction, all subsequent experiments were carried out with this fraction only.

The rate of hydrolysis of the microsomal fraction towards diethylstilboestrol di[^{35}S]sulphate measured at 5 min intervals over 20 min was constant. Reaction mixtures were subjected to electrophoresis, and electrophoretograms were subsequently scanned. All samples contained three radioactive components, unchanged diethylstilboestrol di[^{35}S]sulphate, diethylstilboestrol mono[^{35}S]sulphate and inorganic [^{35}S]sulphate. Further, since the amount of radioactivity associated with inorganic [^{35}S]sulphate in each reaction mixture was always greater than the amount of radioactivity associated with diethylstilboestrol mono[^{35}S]sulphate, the monosulphate derivative is also a substrate for the microsomal sulphohydrolase. This was confirmed in experiments in which diethylstilboestrol mono[^{35}S]sulphate was incubated with the microsomal fraction.

The effect of substrate concentration on the microsomal hydrolytic activity was measured over the range 20–200 μM -diethylstilboestrol di[^{35}S]sulphate with an incubation period of 15 min. The value for K_m was 76 μM .

A value for K_m of 52 μM -diethylstilboestrol mono[^{35}S]sulphate was obtained in experiments in which microsomal sulphohydrolase activity towards this substrate was measured over the concentration range 20–250 μM .

Discussion

Studies with diethylstilboestrol mono[^{35}S]sulphate and diethylstilboestrol di[^{35}S]sulphate have shown that both compounds undergo desulphation in the rat, with the liberation of inorganic [^{35}S]sulphate

in the urine. Proportions of both compounds are eliminated via the biliary route as a glucuronic acid conjugate, diethylstilboestrol mono[^{35}S]sulphate monoglucuronide. However, the extent of biliary elimination was significantly greater after administration of the monosulphate ester.

Whole-body radioautograms were prepared from animals receiving either diethylstilboestrol mono[^{35}S]sulphate or diethylstilboestrol di[^{35}S]sulphate. The most striking difference between the two sets of radioautograms was the appearance of much larger amounts of radioactivity in the gastrointestinal tract of animals receiving the monosulphate than in those receiving the disulphate, reflecting the different extents of biliary elimination. The concentration of radioactivity in the livers of animals receiving either diethylstilboestrol mono[^{35}S]sulphate or diethylstilboestrol di[^{35}S]sulphate implicated this organ as a major site of metabolism of both esters. This was confirmed by using isolated perfused rat livers. These experiments showed that rat liver possesses the two facilities necessary for the metabolism of diethylstilboestrol monosulphate and diethylstilboestrol disulphate, namely desulphation and the formation of diethylstilboestrol monosulphate monoglucuronide.

At relatively high dosages there was no significant biliary elimination of either mono- or di-sulphate esters of diethylstilboestrol, although diethylstilboestrol disulphate does fulfil the requirements of molecular weight, anionic character and lipophilic nature suggested (Hirom *et al.*, 1972) as necessary for biliary elimination. Either there is no mechanism for the biliary elimination of these esters or the metabolism is so rapid that no biliary elimination of the compound is observed.

Studies *in vitro* showed that both diethylstilboestrol monosulphate and diethylstilboestrol disulphate are desulphated by a rat liver microsomal enzyme, arylsulphatase C, which has been shown to be identical with oestrogen sulphatase (Dolly *et al.*, 1972). Presumably desulphation of diethylstilboestrol disulphate occurs in the liver to yield the monosulphate derivative, which has a free hydroxyl group available for conjugation with glucuronic acid. However, quantitatively the fate of diethylstilboestrol monosulphate formed *in vivo* from desulphation of the disulphate is different from that of injected diethylstilboestrol monosulphate. In the latter case, most of the dose appeared in bile as the glucuronic acid conjugate. In contrast, after administration of diethylstilboestrol disulphate, relatively small amounts appear in bile as the diconjugate. These differences cannot be explained on the basis of liver arylsulphatase C activity, since the K_m values for both sulphate esters are of the same order and at these concentrations the rates of hydrolysis are similar. Moreover, the liver-perfusion studies gave no indication of any major

difference in the rates of uptake by the liver. The quantitative difference in metabolism of the esters may reflect some differences in the transport and location of the two esters within the liver cell and/or the affinity of a glucuronyltransferase for diethylstilboestrol monosulphate. It is pertinent that, when the metabolite diethylstilboestrol monosulphate monoglucuronide, isolated from bile, was reinjected into rats, it was rapidly eliminated in the bile unchanged. Therefore either the metabolite is not a substrate for the sulphatase or its rapid elimination does not allow desulphation to occur.

These studies with diethylstilboestrol monosulphate and diethylstilboestrol disulphate have shown that both compounds, in company with others previously studied (see, e.g., Gatehouse *et al.*, 1972; Curtis *et al.*, 1969; Hearse *et al.*, 1969), represent sulphate esters that are not metabolically inert *in vivo*.

Financial support by the Wellcome Trust is gratefully acknowledged. P. A. G. is grateful to the Medical Research Council for a Studentship.

References

- Abou-El-Makarem, M. M., Millburn, P., Smith, R. L. & Williams, R. T. (1967) *Biochem. J.* **105**, 1289–1293
- Bishop, P. M. F., Richards, N. A. & Perry, W. L. M. (1951) *Lancet* **260**, 818–820
- Curtis, C. G., Powell, G. M. & Dodgson, K. S. (1969) *Biochem. Pharmacol.* **18**, 2551–2558
- Curtis, C. G., Powell, G. M. & Stone, S. L. (1971) *J. Physiol. (London)* **213**, 14P–15P
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Dolly, J. O., Dodgson, K. S. & Rose, F. A. (1972) *Biochem. J.* **128**, 337–345
- Flynn, T. G., Dodgson, K. S., Powell, G. M. & Rose, F. A. (1967) *Biochem. J.* **105**, 1003–1010
- Gatehouse, P. W., Roy, A. B., Dodgson, K. S., Powell, G. M., Lloyd, A. G. & Olavesen, A. H. (1972) *Biochem. J.* **127**, 661–668
- Hearse, D. J., Powell, G. M., Olavesen, A. H. & Dodgson, K. S. (1969) *Biochem. Pharmacol.* **18**, 181–195
- Herbst, A. L., Kurman, R. L. & Scully, R. E. (1972) *Obstet. Gynecol.* **40**, 287–298
- Hirrom, P. C., Millburn, P., Smith, R. L. & Williams, R. T. (1972) *Biochem. J.* **129**, 1071–1077
- Jones, J. G. & Dodgson, K. S. (1965) *Biochem. J.* **94**, 331–336
- Klaasen, C. D. (1973) *Toxicol. Appl. Pharmacol.* **24**, 142–149
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Milsom, D. W., Rose, F. A. & Dodgson, K. S. (1972) *Biochem. J.* **128**, 331–336
- Powell, G. M., Curtis, C. G. & Dodgson, K. S. (1967) *Biochem. Pharmacol.* **16**, 1997–2001
- Randerath, K. (1963) *Thin-Layer Chromatography*, p. 200, Academic Press, New York and London
- Talalay, P., Fishman, W. H. & Huggins, C. (1946) *J. Biol. Chem.* **166**, 757
- Umberger, E. J. (1975) *Toxicology* **3**, 3–21
- Young, L., Edson, M. & McCarter, J. A. (1949) *Biochem. J.* **44**, 179–185