Metabolism of Sodium Oestrone [^{35}S]Sulphate in the Rat

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Intraperitoneal, intravenous or oral administration of sodium oestrone [^{35}S]sulphate to male and female Medical Research Council hooded rats is followed by the rapid excretion of the bulk of the radioactivity in urine in the form of inorganic [^{35}S]sulphate. Pre-treatment of rats with an antibiotic regimen does not affect the results except in the case of oral administration, when relatively large amounts of the dose are recovered as ester [^{35}S]sulphate in faeces. Intravenous administration of the labelled ester to male and female rats with cannulae in bile duct and ureter gave results similar to those obtained with free-range animals. Only small amounts of radioactivity appeared in bile and this was mainly in the form of ester sulphate, including both oestrone [^{35}S]sulphate and oestradiol-17 β 3 [^{35}S]sulphate. Whole-body radioautography pinpointed the liver as the probable site of the desulphation of the sulphate ester and this was confirmed by liver and kidney perfusion experiments and by studies with rats in which kidney function had been eliminated by ligation of the renal pedicles.

Oestrone sulphate is a naturally occurring sulphate ester that was first isolated by Schachter & Marrian (1938) from urine of a pregnant mare and was subsequently identified as a component of human pregnancy urine (Butenandt & Hofstetter, 1939; McKenna, Menini & Norymberski, 1961). The ester has also been noted in the urines of males and non-pregnant females after the administration of labelled oestrogen precursors (Hobkirk, Nilsen & Purre, 1966; Hobkirk, Nilsen & Blakey, 1969) whereas its presence in human plasma (Purdy, Engel & Oncley, 1961) and (in high concentration) in stallion testes (Raeside, 1969) has also been demonstrated.

Studies on the metabolic fate of injected oestrone [^{35}S]sulphate in the rat (Hanahan, Everett & Davis, 1949; Hanahan & Everett, 1950; Davis *et al.* 1950) and humans (Lewison, Levi, Jones, Jones & Silberstein, 1951) have established that the ester undergoes appreciable desulphation with the consequent rapid excretion of inorganic [^{35}S]sulphate in the urine. There have been several reports in the literature that rat and human tissues are able to desulphate oestrone sulphate (see Pulkkinen, 1961; Pulkkinen & Paunio, 1963; Zuckerman & Hagerman, 1969; French & Warren, 1966; Sandberg & Jenkins, 1966), the responsible enzyme being localized in the microsomal fraction of the cell. However, the site of the desulphation of the ester after its injection into rats has never been positively established. The present investigation pinpoints the liver as the principal site of this process at the

dosages employed. A preliminary account of some of this work has already been made (Dolly, Curtis, Dodgson & Rose, 1970).

MATERIALS AND METHODS

Preparation of sulphate esters. Unlabelled and ^{35}S -labelled phenolic sulphate esters of oestrone and oestradiol were prepared (as sodium salts) by the methods of Levitz (1963) and Kirdani (1965) respectively. The products (sp. radioactivity of labelled materials was approx. 6 mCi/mmol) behaved as homogeneous compounds when subjected to t.l.c. and paper electrophoresis. Analysis of the esters gave values agreeing with theory and u.v. and i.r. spectra were identical with those quoted in the literature (Kirdani, 1965; Fex, Lundvall & Olsson, 1968; Neudert & Ropke, 1965). The esters were stored *in vacuo* over P_2O_5 at -15°C for at least 3 months without apparent deterioration.

Other materials. Oestrone, oestradiol and a reference sample of oestrone sulphate were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and their purity was checked by t.l.c. (Bishara & Jakovljevic, 1969; Flickinger & Touchstone, 1968; Wusteman, Dodgson, Lloyd, Rose & Tudball, 1964) and i.r. spectroscopy. Triton X-100 (scintillation grade) was purchased from Nuclear Enterprises Ltd., Edinburgh, U.K., and 2,5-diphenyloxazole was supplied by Beckman Instrument Inc., Calif., U.S.A.

Experimental animals. Adult male and female Medical Research Council hooded rats (200–250 g body wt.) were employed except where otherwise stated. Some animals received prior treatment with antibiotics (Dodgson, Powell, Rose & Tudball, 1961). Oestrone [^{35}S]sulphate was administered by intraperitoneal, intravenous and oral

routes to animals under light ether anaesthesia. Animals were placed in metabolism cages that permitted separate collection of urine and faeces and were allowed water and food without restriction. Urine samples were collected after 6, 12, 24 and 48 h and faeces after 48 h.

In experiments on rats with cannulae in bile duct and ureter, animals were anaesthetized with ether, the jugular vein was cannulated and phenobarbitone (Nembutal, veterinary grade) was administered (10–15 mg/kg body wt.) via the cannula. The bile duct and ureters were cannulated and bile and urine samples collected at suitable time-intervals after administration (via the jugular cannula) of the labelled ester.

For experiments involving functional renalectomy animals were anaesthetized (phenobarbitone) and both renal pedicles were ligatured.

Whole-body radioautography. Young male and female rats (30–50 g body wt.) under light ether anaesthesia each received an intraperitoneal injection of oestrone [^{35}S]-sulphate (1 mg dissolved in 0.3 ml of 0.95% NaCl). Rats were killed at 5, 10 and 30 min and 1, 3, 6, 12 and 24 h and whole-body radioautograms were prepared by the method of Powell, Curtis & Dodgson (1967).

Liver and kidney perfusion. The perfusion apparatus was that described by Curtis, Powell & Stone (1971). Isolated rat livers from male and female rats were perfused with homologous blood containing oestrone [^{35}S]-sulphate by the method of Flynn, Dodgson, Powell & Rose (1967) and kidney was perfused according to the procedure of Curtis, Powell & Dodgson (1969).

Thin-layer chromatography. Samples were applied to silica gel G plates and developed in either benzene-methanol-ammonia (16:8:1, by vol.) or benzene-methylethylketone-ethanol-water (3:3:3:1, by vol.). Unlabelled oestrogens and oestrogen sulphates were detected by spraying the dried plates with aq. 50% H_2SO_4 and heating at 140°C (Wusteman *et al.* 1964).

Paper electrophoresis. Urine, bile and plasma samples were subjected to electrophoresis on Whatman no. 1 paper with 0.1 M-sodium acetate-acetic acid buffer, pH 4.5, for 2 h at a potential gradient of 9 V/cm. Electrophoresis of some samples was also carried out in 0.05 M-barium acetate-HCl buffer, pH 4.5, for 2.5 h with a potential gradient of 6 V/cm. In some experiments suitably diluted supernatant preparations of aqueous homogenates of liver, kidney, intestine and faeces were similarly examined. Unlabelled oestrogens and oestrogen sulphate esters were detected on electrophoretograms with *m*-dinitrobenzene reagent (Smith, 1960).

Detection of radioactive areas on chromatograms and electrophoretograms. This was achieved by radioautography on X-ray film (Ilford Industrial B) for periods of up to 4 weeks and also by scanning with a Packard Radiochromatogram Scanner (model 7200). The relative amount of radioactivity associated with each spot was measured from the record of the scanner as described by Jones & Dodgson (1965).

Preparation of samples for determination of radioactivity. Whenever possible urine, bile and blood samples were immediately assayed for radioactivity; otherwise they were stored at -15°C until required. Urine and bile samples were made up to a known volume with water and their total ^{35}S and inorganic [^{35}S]sulphate contents were determined as follows. For total ^{35}S

content, 75 μl of 0.15 M- K_2SO_4 , 120 μl of 4 M-HCl and 100 μl of water were mixed with 150 μl of sample. After keeping at 2°C for 2 min the mixture was centrifuged and portions of the supernatant were taken for scintillation counting. In parallel experiments the inorganic [^{35}S]-sulphate was first removed by substituting 100 μl of a 10% solution of BaCl_2 for water in the above procedure. The inorganic [^{35}S]sulphate contents of samples could then be calculated by difference. Previous work (Dodgson & Tudball, 1960) has established that the amount of labelled organic forms of sulphur (e.g. mercapturic acids) present in rat urine in this type of experiment is very small and, for all practical purposes, may be neglected. The remainder of the radioactivity present in the samples after removal of inorganic [^{35}S]sulphate can thus be assumed to be ester [^{35}S]sulphate and this was confirmed in the present work by determining the latter in a few samples according to the procedure of Lloyd (1961). Preliminary experiments also showed that oestrone [^{35}S]sulphate was not hydrolysed under the assay conditions employed and that inorganic [^{35}S]sulphate could be recovered quantitatively from rat urine or bile in the presence of the labelled ester.

The relative amounts of inorganic and ester [^{35}S]-sulphate in plasma and in faecal and tissue homogenates were calculated from the areas of the peaks on the appropriate electrophoretogram scans. For estimation of total ^{35}S content of whole blood and plasma, samples (175 μl) were mixed with 0.2 M-NaOH (1.2 ml) and left overnight. They were then diluted to 10 ml with water and a portion of each was taken for scintillation counting. Total ^{35}S contents of faeces, tissue homogenates and carcasses were determined by scintillation counting of portions of the digests (diluted 15-fold) resulting from the oxidation of the material with fuming nitric acid according to the method of Young, Edson & McCarter (1949). As a check on the method the total ^{35}S radioactivity in the acid digests was also determined (as $\text{Ba}^{35}\text{SO}_4$) by the method of Lloyd (1961).

Determination of radioactivity. The radioactivity of samples (50–100 μl) was counted with the Packard Tri-Carb (model 3375) liquid-scintillation spectrometer in a toluene medium (1 litre of toluene, 5 g of 2,5-diphenyloxazole and 500 ml of Triton X-100). Quench correction was made by the external standard procedure. For solid counting, $\text{Ba}^{35}\text{SO}_4$ was plated at 'infinite thickness' in plastic planchets giving a surface area of 1 cm^2 and the radioactivity was counted with a thin-end-window Geiger-Müller tube (IDL type 6051 automatic scaling equipment). In all cases sufficient counts were recorded to ensure counting errors of less than $\pm 2\%$.

RESULTS

Studies on free-ranging animals. In preliminary experiments free-ranging rats (male and female) received sodium oestrone [^{35}S]sulphate [1 mg (16 μCi)/200 g body wt., dissolved in 0.3 ml of 0.95% NaCl] via intraperitoneal, intravenous and oral routes. In all cases the bulk of the radioactivity of the dose appeared in the urine within 48 h, mainly as inorganic [^{35}S]sulphate (Table 1). Excretion patterns after intravenous or intraperitoneal injection were very similar but radioactivity was

Table 1. *Distribution of ^{35}S in the urine and faeces of rats receiving sodium oestrone [^{35}S]sulphate (1 mg/200 g body wt.)*

T, Total ^{35}S radioactivity; I, inorganic [^{35}S]sulphate; E, ester [^{35}S]sulphate. Each result is a mean value for two animals. For experimental details see the text.

Percentage of injected radioactivity recovered												
Urine											Faeces T	Total recovery T
Intraperitoneal administration	6h		12h		24h		48h		Total			
	I	E	I	E	I	E	I	E	I	E		
Male	45.5	4.5	17.1	2.8	4.9	1.0	3.1	0.6	70.6	8.9	15.2	94.7
Female	65.2	6.6	8.0	1.2	3.5	0.6	2.3	0.4	79.0	8.8	8.1	95.9
Male*	32.4	3.4	18.8	3.1	4.1	0.8	1.8	0.4	57.1	7.7	17.9	82.7
Female*	42.4	2.6	8.9	1.0	2.9	0.4	1.9	0.4	56.1	4.4	16.7	77.2
Oral administration												
Male	7.8	0.7	23.0	2.9	15.5	2.7	7.7	1.0	54.0	7.3	21.5	82.8
Female	15.4	1.5	21.9	2.3	10.3	1.4	12.1	1.6	59.7	6.8	18.8	85.3
Female*	2.3	0.3	6.4	1.0	5.9	0.8	6.9	0.9	22.5	3.0	41.5	67.0

* Animals pretreated with antibiotics.

lower in urine and higher in faeces after oral administration. Control experiments showed that oestrone [^{35}S]sulphate was not hydrolysed to any significant extent in the stomach.

The relatively large amounts of inorganic [^{35}S]sulphate found in urine could arise in part from biliary circulation and subsequent desulphation of the ester by the intestinal flora. Further experiments were therefore carried out with rats that had been pretreated with antibiotics. The results (see Table 1) indicated that the intestinal flora does not play a significant role in the metabolism of intraperitoneally or intravenously administered oestrone [^{35}S]sulphate. However, when given orally to antibiotic-treated rats relatively little of the radioactivity of the dose appeared in urine, over 40% being recovered in faeces in ester form.

The action of the intestinal flora of normal rats was studied further by incubating crude homogenates (40% in 0.95% NaCl) of the contents of the small and large intestine with oestrone [^{35}S]sulphate (1.3 mM final concn.) at 37°C and pH 7.0 for 48h. Paper electrophoresis of supernatants of incubation mixtures showed that some hydrolysis of the ester occurred (approx. 10 and 1% respectively for small and large intestine). These findings posed the problem of whether oestrone [^{35}S]sulphate was partially hydrolysed *in vivo* before its absorption from the gut. The labelled ester (1 mg dissolved in 0.3 ml of 0.95% NaCl) was injected into the duodenum of an anaesthetized rat with cannulae in the bile duct and hepatic portal vein. Analysis of hepatic portal blood samples showed that in the first 25 min after injection only oestrone [^{35}S]sulphate could be detected and this was

followed by the appearance of inorganic [^{35}S]sulphate in progressively increasing amounts. At the end of the experiment (5.5h after injection) considerable amounts of oestrone [^{35}S]sulphate (approx. 50% of the dose) still remained in the intestine.

These results suggest that oestrone [^{35}S]sulphate was absorbed into the circulation before undergoing hydrolysis, although the possibility remains that some hydrolysis of the ester might occur in the intestinal wall where oestrone sulphohydrolase activity has been detected (unpublished results). The collective observations also suggest that the differences between the excretion patterns after oral administration of the ester to normal and antibiotic-treated rats might be explained in terms of impeded absorption of the ester in the treated rats.

Experiments with cannulated rats. After intravenous injection of oestrone [^{35}S]sulphate into rats with cannulae in bile duct and ureter, 53.4% of the dose was recovered in urine within 12.5h, 95% of that amount being excreted as inorganic [^{35}S]sulphate (Fig. 1). These values generally agree with those obtained with free-range rats. Relatively small amounts (5.6%) of the dose appeared in bile and this was mainly ester [^{35}S]sulphate. Similar results were obtained with both male and female rats and the collective results indicate that relatively little biliary circulation of ^{35}S occurred.

Qualitative examination of urine and bile of rats receiving oestrone [^{35}S]sulphate. Urine samples from both free-range rats and those with cannulae in bile duct and ureter were subjected to paper electrophoresis and t.l.c. Radioautograms of electrophoresis strips showed the presence of a major

Fig. 1. Excretion of ^{35}S radioactivity in (a) urine and (b) bile of a male rat receiving sodium oestrone [^{35}S]-sulphate (1 mg/200 g body wt.) by intravenous injection. Amounts recorded over the total period of the experiment are shown on the right-hand side of the diagram where ■ represents inorganic [^{35}S]-sulphate and ■ represents ester [^{35}S]-sulphate.

radioactive component together with a number of minor metabolites. The mobility of the major component was identical with that of inorganic [^{35}S]-sulphate in either the sodium acetate or barium acetate buffer systems. Radioautograms of thin-layer chromatograms of sequential samples of urines from cannulated female and male rats confirmed that inorganic [^{35}S]-sulphate was the major metabolite in urine, although traces of oestrone [^{35}S]-sulphate were always detected in the early samples (15 and 30 min) from female rats. Inorganic [^{35}S]-sulphate was also the major radioactive component of urine samples from free-ranging animals (normal and antibiotic-treated) receiving oral, intravenous and intraperitoneal doses of the labelled ester.

Chromatographic analysis of bile samples showed the presence of at least six radioactive components including inorganic [^{35}S]-sulphate, oestrone [^{35}S]-sulphate and oestradiol-17 β 3[^{35}S]-sulphate.

Whole-body radioautography. The most striking feature of whole-body radioautograms was the cellular accumulation of radioactivity in the liver. In female rats 10 min after injection the bulk of the

radioactivity was localized in the liver with smaller amounts in the cortex of the kidney (Plate 1). After 1 h all the radioactivity had left the liver and was accumulating in bladder and gut whereas the 3 h, 6 h, 12 h and 24 h radioautograms were typical of those obtained after the administration of inorganic [^{35}S]-sulphate. In all radioautograms only traces of radioactivity were associated with circulating blood. Male rats gave similar results except that no ^{35}S accumulated in kidney cortex.

The collective results were compatible with the view that the liver was the major site of desulphation of oestrone [^{35}S]-sulphate.

Liver perfusion experiments. Isolated rat livers (male and female) were perfused with blood (90 ml per liver) containing sodium oestrone [^{35}S]-sulphate (18–28 mg per liver). Control experiments established that relatively little hydrolysis of the ester occurred (approx. 1% in 8 h) in the absence of the liver. Blood and bile samples were collected at zero time and at subsequent time-intervals and portions were taken for measurement of ^{35}S contents. The remainder of each blood sample was centrifuged to remove erythrocytes (no significant amounts of

EXPLANATION OF PLATE I

Radioautograms showing the distribution of radioactivity in young female rats at time-intervals after the intraperitoneal injection of 1 mg of sodium oestrone [^{35}S]sulphate. Time after injection: (a) 10 min; (b) 1 h; (c) 3 h.

Fig. 2. Time-course of the desulphation of sodium oestrone [^{35}S]sulphate (18mg) by the isolated perfused male rat liver. O, Disappearance of sodium oestrone [^{35}S]sulphate; ●, appearance of inorganic [^{35}S]sulphate.

^{35}S -labelled material sedimented with the cells) and portions of the supernatants (1–5 μl) were subjected to electrophoresis. Blood taken at zero time contained one radioactive component only and its electrophoretic mobility was identical with that of oestrone [^{35}S]sulphate. After 15 min large amounts of inorganic [^{35}S]sulphate could be detected in blood and after 2 h practically all the circulating radioactivity was in this form (Fig. 2). Total radioactivity in blood at the end of the experiment (8 h) accounted for 70% of the amount added initially. A further 11% was excreted in the bile (several components were present including inorganic [^{35}S]sulphate, oestrone [^{35}S]sulphate and oestradiol-17 β 3 [^{35}S]sulphate) and 9% was still present in the liver (as inorganic [^{35}S]sulphate). Clearly, the liver is an effective organ for the desulphation of oestrone sulphate.

Kidney ligation and perfusion experiments. Sodium oestrone [^{35}S]sulphate (3 mg/200 g body wt. dissolved in 0.3 ml of 0.95% NaCl) was injected intravenously into male and female rats in which kidney function was eliminated by ligation of the renal pedicles. Blood samples (0.2 ml) were withdrawn at time-intervals and examined qualitatively and quantitatively for radioactive components. Oestrone [^{35}S]sulphate rapidly disappeared from the circulating blood and only inorganic [^{35}S]sulphate could be detected after the first 10 min. The latter accounted for about 7% of the injected dose at the end of the experiment (3 h). At that time, radioactivity in the bile accounted for 8% of the dose and reflected the presence of a number of labelled compounds, including inorganic [^{35}S]sulphate, oestrone [^{35}S]sulphate, oestradiol-17 β 3 [^{35}S]sulphate. Liver

and intestine contained 6 and 20% respectively, all as inorganic [^{35}S]sulphate, whereas the remaining radioactivity could be accounted for in the rest of the carcass. The relatively high recovery of inorganic [^{35}S]sulphate from the intestine compared with bile suggests that sulphate can be excreted into the intestine by a route other than that of the biliary system.

In further experiments rat (female) kidney was perfused with blood (180 ml) containing 12.5 mg of oestrone [^{35}S]sulphate. Urine and venous blood samples were collected over a 2 h perfusion period and examined by electrophoresis. Less than 1% hydrolysis of the ester occurred during the whole period.

Apparently the kidneys are not significantly involved in the desulphation of oestrone [^{35}S]sulphate.

DISCUSSION

The present work clearly establishes that relatively large doses of sodium oestrone [^{35}S]sulphate are readily desulphated by the rat and pinpoints the liver as the principal organ where this process occurs. Unpublished results from this laboratory have established that in the Medical Research Council hooded rat the liver is by far the richest source of oestrone sulphohydrolase, an insoluble microsomal enzyme having properties similar to those of arylsulphohydrolase C. It is therefore tempting to conclude that this sulphohydrolase is responsible for the desulphation of oestrone [^{35}S]sulphate *in vivo* demonstrated in the present study. In this connexion it is noteworthy that the whole-body radioautography experiments indicated that the kidney cortex was a secondary site of accumulation of radioactivity in female rats only. Further, in the cannulation experiments oestrone [^{35}S]sulphate could be detected in early samples of urine from female rats but not from male rats. The reason for these sex differences is obscure but it is interesting that the oestrone sulphohydrolase activity in female rat liver is only about 60% of that of the male (cf. arylsulphohydrolase C, Dodgson, Spencer & Thomas, 1953). Possibly the secondary accumulation of radioactivity in female kidney reflects small amounts of oestrone [^{35}S]sulphate that have escaped hydrolysis by the liver enzyme in the initial phases of the experiments at this dosage.

Meanwhile, it is still difficult to assess the biological role (if any) of oestrone sulphate. Certainly its appearance in the urine of some species in relatively large amounts (e.g. human, Butenandt & Hofstetter, 1939; the mare, Schachter & Marrian, 1938) suggests that it may represent an end product of metabolism in these species. However, Brooks, Leithauser, de Loecker & de Wever (1969) have claimed that sulphation of oestrone is a prerequisite

of the hormone's stimulatory effect on protein synthesis in rat and rabbit uterine preparations.

The physiological function of the sulphohydrolase is, as yet, equally obscure although Alonso & Troen (1966) have suggested that human oestrone sulphohydrolase plays a significant role in maintaining the balance between conjugated and free forms of oestrogens in the placental foetal unit.

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