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

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Intraperitoneal administration of sodium oestrone [^{35}S]sulphate to male and female free-ranging guinea pigs is followed by excretion of most of the radioactivity mainly as inorganic [^{35}S]sulphate in the urine within 72 h. The remainder of the radioactivity in the urine was found in oestrone [^{35}S]sulphate, two unidentified metabolites (A and B) and traces of oestradiol-17 β 3-[^{35}S]sulphate. When injected intraperitoneally into animals with bile-duct and bladder cannulae, most of the dose was excreted in the bile. Unchanged oestrone [^{35}S]sulphate was the main biliary component excreted in males and females, but the latter also excreted appreciable amounts of oestradiol-17 β 3-[^{35}S]sulphate and metabolites A and B. The urine from these animals also contained these metabolites, inorganic [^{35}S]sulphate and also oestrone [^{35}S]sulphate, but in small amounts. Metabolite A was present only in samples from males. Whole body radioautography pinpointed the liver and kidney as the possible sites of metabolism of the ester. The ester underwent little desulphation in the isolated perfused female guinea-pig liver and in animals in which kidney function had been eliminated, and was excreted unchanged in the bile. These results and the observed low oestrogen sulphotase and arylsulphotase C activities found in guinea-pig liver and kidney support the view that the two enzymes are identical.

Previous studies on the metabolism of oestrone [^{35}S]sulphate in the rat have shown that the ester is rapidly hydrolysed *in vivo*, leading to the excretion of inorganic [^{35}S]sulphate in the urine (Dolly *et al.*, 1971). This hydrolysis principally occurred in the liver. It is also known that, in the rat, liver is by far the richest source of oestrogen sulphotase, an enzyme that is probably identical with the so-called arylsulphotase C (Dolly *et al.*, 1972). It therefore seemed likely that this enzyme was responsible for the desulphation of oestrone [^{35}S]sulphate by the rat. To test this possibility further, the metabolism of the ester has been studied in the guinea pig, an animal with very low hepatic arylsulphotase C activity (Roy, 1958; Milsom, 1970).

Materials and Methods

Chemicals

Unlabelled and ^{35}S -labelled phenolic sulphate esters of oestrone and oestradiol-17 β were prepared (as sodium salts) as described by Dolly *et al.* (1971). The specific radioactivity of the labelled compounds was approx. 1.9 mCi/mmol. Aluminium oxide ("Camag" M.F.C., about 100–240 mesh, neutral Brockmann activity of 1) and Celite 545 were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Experimental animals

Adult male and female guinea pigs (Hartley strain, 600–700 g body wt., obtained from Goodchild Bros., Black Corner, Pound Hill, near Crawley, Sussex, U.K.) were used unless otherwise stated. Oestrone [^{35}S]sulphate was administered intraperitoneally and the animals were placed in metabolism cages that permitted separate collection of urine and faeces. During the experiments animals were allowed food and water without restriction. Urine samples were collected after 6, 12, 24, 48 and 72 h and faeces were collected after 72 h.

In experiments on guinea pigs with cannulae in bile duct and bladder, animals were lightly anaesthetized with ether and the trachea and jugular vein were cannulated (see Flynn *et al.*, 1967). The animals were allowed to regain partial consciousness before administration of phenobarbitone (Nembutal, veterinary grade) via the jugular cannula. It was essential to inject the phenobarbitone (approx. 4.5 mg/kg body wt.) very slowly otherwise the animals died. The gall bladder was tied off and the bile duct and bladder were cannulated to permit the collection of bile and urine samples at suitable time-intervals after administration (via the jugular cannula) of the labelled ester. It was necessary to inject 5 ml of 5% (w/v) mannitol as a diuretic at the start of each experiment to ensure a good production of urine.

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

At the end of each experiment, animals were killed and their carcasses taken for determination of residual ^{35}S .

Methods

Whole-body radioautography. Young female guinea pigs (100–120 g body wt.) each received an intraperitoneal injection of sodium oestrone [^{35}S]sulphate (3.3 mg dissolved in 1 ml of 0.95% NaCl). The animals 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 *et al.* (1967).

Liver perfusion. The apparatus was essentially the same as that used for perfusing isolated rat liver (Curtis *et al.*, 1971) except that a larger reservoir was employed. Isolated guinea-pig liver was perfused with homologous blood containing oestrone [^{35}S]sulphate by the procedure of Flynn *et al.* (1967).

Thin-layer chromatography. Samples (1–2 μl) were applied to silica gel G plates and developed in benzene–methanol–ammonia (16:8:1, by vol.).

Paper electrophoresis. Samples (1–5 μl) were subjected to electrophoresis on Whatman no. 1 paper with 0.1M-sodium acetate–acetic acid buffer, pH 4.5, for 2 h at a potential gradient of 9 V/cm.

Detection of radioactive areas on chromatograms and electrophoretograms. This was done by the methods of Dolly *et al.* (1971). The relative amounts of each metabolite in the samples were calculated from the areas of the peaks on the appropriate chromatogram and electrophoretogram scans as described by Jones & Dodgson (1965).

Measurement of radioactivity in biological samples. The samples were prepared for scintillation counting and their total radioactive contents were measured by the procedures outlined by Dolly *et al.* (1971).

Whenever possible, urine, bile and blood samples were assayed for radioactivity immediately; otherwise they were stored at -15°C until required.

Purification of oestrone [^{35}S]sulphate from bile. The procedure of Hobkirk *et al.* (1969) was employed for the purification of oestrone [^{35}S]sulphate from the bile produced during the perfusion of isolated guinea-pig liver with oestrone [^{35}S]sulphate. The bile samples were combined (20 ml) and extracted three times with ether at pH 6.8. The aqueous phase was then extracted five times with ethyl acetate at pH 7.0 after addition of 4 g of NaCl. The ethyl acetate fraction was adjusted to pH 9.6 with a few drops of NH_3 (sp.gr. 0.88), evaporated to dryness at 40°C and the residue was partitioned three times between butan-1-ol and 0.1M-NaOH. The butan-1-ol extracts (140 ml) were washed with a small volume of saturated NaCl solution (approx. 25 ml) and evaporated to dryness

at 40°C . The residue was dissolved in 15 ml of methanol (containing 0.31 g of NaOH/500 ml) and chromatographed on an aluminium oxide column (29 cm $\times$ 1.5 cm) in the same methanol–NaOH solvent. Radioactive samples having the same R_F as oestrone [^{35}S]sulphate on t.l.c. (fractions 3–6, each 10 ml) were pooled, evaporated to dryness at 40°C and the residue was dissolved in 6 ml of toluene–butan-1-ol–0.1M- NH_3 (6:4:5, by vol.); the insoluble material was filtered off. The solution was then chromatographed on a column of Celite (30 cm $\times$ 1.2 cm) by the procedure of Siiteri (1963) and the radioactive samples having the same mobility as oestrone [^{35}S]sulphate on t.l.c. were evaporated to dryness. The identity of the material was confirmed by reverse isotope dilution. Unlabelled sodium oestrone sulphate (160 mg) was added and the whole was recrystallized six times from methanol–ether (approx. 1:1, by vol.). A sample of each crop of crystals (approx. 5 mg) was removed, dried *in vacuo* over P_2O_5 (to constant wt.) and its radioactive content measured.

Assay of oestrogen sulphatase and arylsulphatase C activities. Enzyme activities were measured by the methods described in the accompanying papers (Dolly *et al.*, 1972; Milsom *et al.*, 1972). Protein was determined by the procedure of Gornall *et al.* (1949).

Experimental and Results

Experiments on free-ranging animals

Free-ranging guinea pigs (male and female) were injected intraperitoneally with sodium oestrone [^{35}S]sulphate [1 mg (5 μCi)/200 g body wt., dissolved in 0.3 ml of 0.95% NaCl]. In both male and female animals most of the radioactivity of the dose appeared in the urine within 72 h, with smaller amounts in the faeces and carcass (Table 1). Quantitative t.l.c. of the 6 h urine samples showed the presence of inorganic [^{35}S]sulphate, oestrone [^{35}S]sulphate and at least two unidentified metabolites. Later samples mainly contained inorganic [^{35}S]sulphate. One of the unidentified metabolites (metabolite A) had a slightly lower R_F value (0.39) than oestradiol-17 β 3-[^{35}S]sulphate (0.41), whereas the other (metabolite B) had a much lower R_F (0.18). No attempts were made to identify the metabolites, but it was noteworthy that female guinea pigs excreted 14.9% of the dose as metabolite A whereas male guinea pigs excreted traces only. Only traces of oestradiol-17 β 3-[^{35}S]sulphate could be detected in urine from animals of either sex.

Studies on cannulated guinea pigs

The relatively large amounts of inorganic [^{35}S]sulphate found in the urine samples could arise in part from enterohepatic circulation and subsequent

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

T, Total ^{35}S radioactivity; I, inorganic [^{35}S]sulphate; O₁S, oestrone [^{35}S]sulphate; A and B, unidentified metabolites. For experimental details see the text. Typical results for one animal of each sex are given.

Sample	Time (h)	Percentage of injected radioactivity recovered									
		Male					Female				
		T	I	O ₁ S	A	B	T	I	O ₁ S	A	B
Urine	0-6	30.5	16.2	11.1	Trace	3.2	54.3	16.0	12.9	14.9	10.5
	6-12	35.4	31.9	2.1	Trace	1.4	11.1	10.9	0	0	0.2
	12-24	8.3	8.3	0	0	0	3.3	3.1	0	0	0.2
	24-48	3.2	3.2	0	0	0	1.7	1.7	0	0	0
	48-72	1.2	1.2	0	0	0	1.0	1.0	0	0	0
Total		78.6	60.8	13.2		4.6	71.4	32.7	12.9	14.9	10.9
Faeces	0-72	13.0					5.6				
Carcass		7.5					18.0				
Total recovery		99.1					95.0				

desulphation of the ester in the gut. Further experiments were therefore carried out on guinea pigs with bile-duct and bladder cannulae.

Table 2 shows the excretion of ^{35}S in urine and bile after intravenous injection of sodium oestrone [^{35}S]sulphate (1 mg/200g body wt., dissolved in 0.3 ml of 0.95% NaCl) into anaesthetized guinea pigs (male and female) with bile-duct and bladder cannulae. In contrast with the results obtained with cannulated rats (Dolly *et al.*, 1971), most of the radioactivity (57.7-75.8% of the dose) was excreted in the bile within 4-6 h. Most of the dose was eliminated in the bile of male and female animals within the first 30 min after the injection of oestrone [^{35}S]sulphate, and this was all as ester sulphate. Quantitative t.l.c. of bile samples established that oestrone [^{35}S]sulphate was the major labelled component in samples from male and female guinea pigs, but appreciable amounts of oestradiol-17 β 3- ^{35}S]sulphate and metabolites A and B were also present in the bile samples from females. In contrast with the results obtained with free-ranging animals (Table 1) relatively small quantities of radioactivity (14.8-35.4% of the dose) appeared in the urine samples, and examination by t.l.c. of samples from female animals showed the presence of oestrone [^{35}S]sulphate, oestradiol-17 β 3- ^{35}S]sulphate, inorganic [^{35}S]sulphate and metabolites A and B. Metabolite A could not be detected in samples from males, but otherwise the excretion pattern was similar. One possible explanation for the discrepancies between the recoveries of radioactivity in the urine of cannulated and free-ranging animals (Table 1) is that any radioactivity eliminated in the bile of free-ranging guinea pigs was reabsorbed and subsequently excreted in the urine. Relatively larger

amounts of inorganic [^{35}S]sulphate were present in the urine of free-ranging animals than in the urine samples from cannulated guinea pigs. It thus seemed likely that some hydrolysis of ester sulphate occurred in the gut.

Collectively the findings indicated that practically no hydrolysis of oestrone [^{35}S]sulphate occurred in guinea pigs when enterohepatic circulation was prevented. The results also indicate that guinea pigs, unlike rats, can rapidly eliminate oestrone [^{35}S]sulphate and its sulphate metabolites into the bile.

Whole-body radioautography

The most striking feature of the whole-body radioautograms was the cellular accumulation of radioactivity in the liver and kidney. Thus radioautograms of animals killed 5 min after injection showed significant amounts of radioactivity within the liver and kidney. The concentration of radioactivity in these organs increased up to 30 min and then gradually declined, and only trace amounts remained after 3 h. Some radioactivity was also present in the bladder 10 min after injection. After 1 h practically all of the radioactivity had accumulated in the gut, presumably as a result of the excretion of ^{35}S -labelled ester in the bile. In all cases only traces of radioactivity were present in the blood.

These observations demonstrated that after intraperitoneal administration of sodium oestrone [^{35}S]sulphate, radioactivity rapidly accumulated in liver and kidney and was later excreted into the gut. These results, taken in conjunction with the results of experiments on cannulated animals, demonstrate that guinea-pig liver has an efficient mechanism for the

Table 2. *Distribution of ^{35}S in the urine, bile and carcass after intravenous injection of sodium oestrone [^{35}S]sulphate (1 mg/200g body wt.) into anaesthetized guinea pigs (male and female) with bile-duct and bladder cannulae*

The abbreviations are as in Table 1. Oestradiol-17 β 3-[^{35}S]sulphate (O₂S) and metabolite A were not sufficiently well separated on the thin-layer chromatograms to allow each of them to be measured separately. The figures given below under the heading 'O₂S+A' represent the additive figures for the two metabolites. For experimental details see the text. Typical results for one animal of each sex are given.

Sample	Time (min)	Percentage of injected radioactivity recovered								
		Male				Female				
		T	I	O ₂ S	O ₂ S+A	T	I	O ₂ S	O ₂ S+A	B
Urine	15	18.7	Trace	14.0	4.7	1.3	0	1.0	0.3	0
	30	10.9	Trace	8.2	2.7	3.9	0.2	1.8	1.8	0.1
	45	2.6	Trace	1.6	1.0					
	60	1.0	0.1	0.7	0.2	4.4	0.3	1.2	2.3	0.6
	90	1.2	0.1	1.0	0.1	2.6	1.0	0.4	0.8	0.4
	120	0.2	0.1	0.1	0	1.3	0.1	0.3	0.4	0.5
	180	0.7	0.1	0.4	0.2					
	240	0.1	0	0.1	0	1.0	0.1	0.2	0.3	0.4
	360	0	0	0	0	0.3	0	0	0.1	0.2
Total		35.4	0.4	26.1	8.9	14.8	1.7	4.9	6.0	2.2
Bile	15	47.4	0	47.4	0	29.4	0	26.4	1.5	1.5
	30	6.2	0	6.2	0	29.8	0	22.5	4.4	2.9
	45	1.9	0	1.9	0					
	60	0.9	0	0.9	0	6.6	0	4.2	0.9	1.5
	90	0.9	0	0.9	0	2.9	0	1.7	0.5	0.7
	120	0.2	0	0.2	0	2.0	0	0.8	0.3	0.9
	180	0.1	0	0.1	0					
	240	0.1	0	0.1	0	3.2	0	1.5	0.5	1.2
	360	0	0	0	0	1.9	0	0.7	0.2	1.0
Total		57.7	0	57.7	0	75.8	0	57.8	8.3	9.7
Carcass		7.3				14.7				
Total recovery		100.4				105.3				

biliary excretion of oestrone [^{35}S]sulphate and its sulphated metabolites.

Liver perfusion

Isolated female guinea-pig liver (28 g) was perfused for 6 h at the rate of 55–66 ml/min with blood (130 ml) containing sodium oestrone [^{35}S]sulphate (25 mg). Control experiments showed that no hydrolysis of the ester could be detected in the absence of the liver. Blood and bile samples were collected at zero time and at subsequent time-intervals and their radioactive contents were analysed quantitatively and qualitatively. Electrophoresis of plasma samples (no significant amounts of ^{35}S -labelled material sedimented with the cells) revealed that samples taken at 0, 15 and 30 min contained one radioactive component and its electrophoretic mobility was identical with that of oestrone [^{35}S]sulphate. Later

samples (1, 1.5, 2 and 3 h) contained appreciable amounts of inorganic [^{35}S]sulphate, and the 6 h sample contained only inorganic [^{35}S]sulphate. However, the total radioactivity in the blood at the end of the experiment accounted for only 8% of the total amount added initially. Most of the radioactivity (84.7% of the dose) was excreted in the bile within 6 h. Radioautograms of thin-layer chromatograms of sequential samples of bile showed that oestrone [^{35}S]sulphate was the major component and only minute traces of oestradiol-17 β 3-[^{35}S]sulphate and metabolites A and B were found in any of the samples. Oestrone [^{35}S]sulphate was purified as described previously and was found to be homogeneous when subjected to t.l.c. Radiochemical purity of the ester was confirmed by reverse isotope dilution. Within the limits of experimental error, material of constant specific radioactivity was obtained after the first recrystallization.

These results clearly showed that relatively very

little hydrolysis of oestrone [^{35}S]sulphate occurred in guinea-pig liver.

Kidney ligation experiment

Bile samples collected from the female guinea pig with bile-duct and bladder cannulae contained appreciable quantities of oestradiol-17 β 3- ^{35}S]sulphate and metabolites A and B (Table 2). In contrast, the bile samples produced by the isolated perfused female guinea-pig liver contained only traces of these compounds. These results suggested that the formation of oestradiol-17 β 3- ^{35}S]sulphate and metabolites A and B in the cannulated animals also occurred in some organ other than the liver. To investigate if the kidneys were responsible, sodium oestrone [^{35}S]sulphate (1 mg/200 g body wt., dissolved in 0.3 ml of 0.95% NaCl) was injected intravenously into a female guinea pig in which kidney function was eliminated by ligation of the renal pedicles. Bile samples were collected at time-intervals and examined quantitatively and qualitatively for radioactive components. Most of the radioactivity (74% of the dose) was excreted in the bile within 2.5 h. As with bile samples from perfused liver only traces of oestradiol 17 β 3- ^{35}S]sulphate and metabolites A and B could be detected. Thus it seemed possible that in the cannulated guinea pigs the kidneys may have been involved in the conversion of oestrone [^{35}S]sulphate into other sulphated metabolites. The accumulation of radioactivity in the kidney cells after the injection of oestrone [^{35}S]sulphate into guinea pigs, as seen in the whole-body radioautograms, might also implicate the

kidney as a site of metabolism of the ^{35}S -labelled ester.

Oestrogen sulphatase and arylsulphatase C activities in various tissues of the guinea pig

Table 3 shows the oestrogen sulphatase and arylsulphatase activities in female guinea-pig tissues. These activities were measured by using the optimum conditions for the rat liver enzymes (Dolly *et al.*, 1972; Milsom *et al.*, 1972). However, it is clear that both enzyme activities were very much lower in guinea-pig liver and kidney than in the corresponding rat tissues (Dolly *et al.*, 1972). The oestrogen sulphatase activity in all the other guinea-pig tissues was fairly similar to the activities of the enzyme in the rat tissues.

Discussion

The work of Dolly *et al.* (1971, 1972) has established that oestrone [^{35}S]sulphate is readily hydrolysed when injected into rats, the principal site of the process being the liver. Further, it was suggested that an insoluble microsomal enzyme was responsible for the desulphation and that this enzyme, oestrogen sulphatase, is identical with the well-known arylsulphatase C. The results of the present investigation have established that the very low activities of oestrogen sulphatase found in guinea-pig liver and kidney are paralleled by low activities of arylsulphatase C, as would be expected if the two enzymes were identical. However, activities of the two enzymes in other tissues, e.g. adrenals, ovaries, intestine and uterus, differ widely. The reason for this is not understood but it may be a reflection of differences in the properties or membrane localization of the enzymes in these tissues.

It might also be expected that oestrone [^{35}S]sulphate would not undergo appreciable desulphation in guinea-pig liver, and this has been clearly established with liver-perfusion experiments and experiments with appropriately cannulated animals. Only in free-ranging guinea pigs did appreciable amounts of inorganic [^{35}S]sulphate appear in urine after intraperitoneal injection of the ^{35}S -labelled ester. However, it seems likely that this may have been a consequence of the excretion of the ester into the bile and its subsequent desulphation in the gut. Re-absorption of the liberated inorganic [^{35}S]sulphate, followed by excretion in the urine, might then be expected to occur.

A further finding has been that guinea pigs appear to have an efficient mechanism for the biliary excretion of oestrone [^{35}S]sulphate and oestradiol-17 β 3- ^{35}S]sulphate.

Finally, the results suggest that oestrogen sulphatase (arylsulphatase C) is a physiologically active

Table 3. *Oestrogen sulphatase and arylsulphatase C activities of female guinea-pig tissues*

Enzyme activities are expressed as nmol of substrate hydrolysed/h per mg of protein at 37°C. Typical results for one animal are given; for experimental details see the Materials and Methods section.

Tissue	Oestrogen sulphatase	Arylsulphatase
Adrenals	34.6	12.1
Ovaries	19.3	12.9
Lung	18.4	19.2
Spleen	17.0	10.9
Small intestine	12.4	2.2
Brain	11.4	6.9
Large intestine	10.7	4.3
Uterus	10.4	3.5
Heart	7.8	3.3
Kidney	3.7	3.1
Liver	2.9	1.5
Pancreas	0	0

enzyme although its precise physiological role is still largely obscure.

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