Biochemical Pharmacology, Vol. 18, pp. 181–195. Pergamon Press 1969. Printed in Great Britain

THE INFLUENCE OF SOME PHYSICO-CHEMICAL FACTORS ON THE BILIARY EXCRETION OF A SERIES OF STRUCTURALLY RELATED ARYL SULPHATE ESTERS*

D. J. HEARSE,† GILLIAN M. POWELL, A. H. OLAVESEN and K. S. DODGSON

Department of Biochemistry, University College, Cardiff

(*Received* 7 *June* 1968; *accepted* 15 *August* 1968)

Abstract—The metabolic fates and modes of excretion of biphenylyl 4-^{35}S-sulphate, cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate were investigated in the rat. In experiments with free-ranging animals the bulk of the administered radioactivity was recovered in the urine. The proportion of urinary radioactivity appearing as inorganic ^{35}S-sulphate was different for each ester (biphenylyl 4-^{35}S-sulphate, 36 per cent in the male and 19 per cent in the female; cyclohexylphenyl 4-^{35}S-sulphate, 33 per cent in the male and 19 per cent in the female; cyclohexylphenyl 2-^{35}S-sulphate, less than 2 per cent in each sex). In experiments in which the esters were administered to rats with bile fistulae significant biliary elimination was recorded (biphenylyl 4-^{35}S-sulphate, up to 18 per cent; cyclohexylphenyl 4-^{35}S-sulphate, up to 81 per cent; cyclohexylphenyl 2-^{35}S-sulphate, up to 70 per cent). In each case a single biliary metabolite was detected and this was identified as a glucuronic acid conjugate of the administered ester. Factors which might govern the respective levels of biliary excretion are discussed.

THE EXCRETION of simple phenolic substances is frequently preceded by sulphate conjugation with the formation of ester sulphates. The strongly polar nature of these conjugates facilitates their rapid urinary elimination and sulphate esters are common constituents of mammalian urines. The appearance of such compounds in urines has resulted in a widely held view that aryl sulphate esters represent the end products of metabolism only and are essentially inert *in vivo*. This view is supported by studies[1] in which administered phenyl sulphate was found to be eliminated unchanged via the urine. More recently, however, it has become apparent that aryl sulphate esters are not necessarily inert *in vivo*. Thus both *p*-nitrophenyl sulphate[2] and 2-hydroxy-5-nitrophenyl-sulphate[3] undergo considerable metabolism when injected into rats. Furthermore, in studies with 2-hydroxy-5-nitrophenyl-sulphate some biliary elimination was noted.

In the present investigation the metabolism and mode of excretion of aryl sulphate esters has been further explored with particular reference to more complex molecules containing more than one carbon ring system. Thus, the behaviour *in vivo* of potassium

* D.J.H. is grateful to the Medical Research Council for a Research Studentship. The work was supported by U.S. Public Health Service Research Grant No. AMO1982 from the Arthritis and Metabolic Diseases Division.

† Present Address: Department of Pharmacology, New York University Medical Center, 550, First Avenue, New York, N.Y. 10016, U.S.A.

biphenylyl 4-^{35}S-sulphate, potassium cyclohexylphenyl 4-^{35}S-sulphate and potassium cyclohexylphenyl 2-^{35}S-sulphate was investigated. In contrast to simple ester sulphates such as phenyl sulphate, these three esters exhibit significant lipophilic properties, so rendering them less suitable for urinary elimination. It was thus of considerable interest to determine whether this lipophilic component of the molecule would facilitate biliary elimination.

MATERIALS AND METHODS

Aryl sulphate esters

The ^{35}S-labelled sulphate esters of 4-hydroxybiphenyl, 4-cyclohexylphenol and 2-cyclohexylphenol were prepared as described by Hearse, Olavesen and Powell.[4] The specific radioactivities of the preparations were 26 μc/mg, 36 μc/mg and 37 μc/mg respectively.

Thin-layer chromatography (TLC)

TLC was performed on silica gel (Kieselgel G, Merck Ltd., Darmstadt, W. Germany) using plates (20 × 5 cm) with a gel thickness of 0·25 mm (1·5 mm for preparative work). All plates were activated at 110° for 45 min before use. The plates were developed by ascending chromatography over a distance of 10 cm in a pre-equilibrated tank. Solvent systems used were:—A, 2-propanol:chloroform:methanol:water (10:10:5:2 by volume); B, 1-butanol:acetic acid:water (60:20:20 by volume).

Detection of radioactivity on TLC's

Radioactive areas were located either by autoradiography, when plates were in contact with X-ray film (Ilford Industrial B) for periods of up to 7 days, or by scanning (Packard Model 7200 Radiochromatogram Scanner). The relative amount of radioactivity associated with each spot was estimated from the record of the scanner as described by Jones and Dodgson.[5]

Measurement of radioactivity in urine, bile and blood

Urine, bile and blood samples were made up to a known volume with water and aliquots were removed for estimation of inorganic ^{35}S-sulphate (as $Ba^{35}SO_4$) and total ^{35}S-sulphate (as $Ba^{35}SO_4$ after acid hydrolysis) according to the method of Lloyd.[6] The precipitated $Ba^{35}SO_4$ was plated at infinite thickness in plastic planchets, giving a surface area of 1 cm^2. Measurement of radioactivity was made using IDL Type 6050 automatic scaling equipment. Corrections for background and coincidence were made and sufficient counts were recorded to give a S.E. of less than $\pm$ 2 per cent.

Continuous monitoring of radioactivity appearing in bile and urine

The levels of radioactivity in bile and urine issuing from cannulated bile ducts and ureters were measured continuously. The cannulae were arranged so that bile and urine flowed on to a moving ($\frac{3}{4}$ in./min) strip of chromatography paper (Whatman No. 1, 1·5 in. wide). The strip was dried and the levels of radioactivity on the strip determined by scanning. In experiments made to determine the rapidity of elimination of radioactivity via urine and bile, suitable corrections were made for the dead volumes of the cannulae.

Measurement of ^{35}S-content of faeces and carcasses

The total ^{35}S-contents of faeces and of carcasses were precipitated and counted as $Ba^{35}SO_4$ after oxidation with fuming nitric acid according to the method of Young, Edson and McCarter.[7]

Isotope dilution analysis

The procedure of Dodgson, Powell, Rose and Tudball[8] was adopted. Isolated radioactive material was mixed with a solution of 50 mg of unlabelled authentic material and the authentic material recrystallized, the crystals isolated and dried and a sample (3 mg) was retained for determination of radioactivity. The solubilization, recrystallization and isolation procedure was repeated a further five times; at each stage a sample (3 mg) of dried crystals was retained. The specific activity (in counts/mg/min) of each sample was determined.

Determination of protein binding

The extent to which each of the esters was bound to plasma protein was determined by ultrafiltration. Blood (6 ml) containing the ^{35}S-labelled ester was centrifuged (2000 *g* for 30 min). The plasma was subjected to ultrafiltration through a Sartorius cellulose nitrate membrane filter (LSG 60; average pore size 5μ) under a pressure of 100 lb/in.^{2} of nitrogen.

Samples (250 μl) of ultrafiltrate and untreated plasma were assayed for total ^{35}S-sulphate. The difference between the ^{35}S-sulphate content of untreated plasma and the ultrafiltrate represented the amount bound to protein.

Experimental animals

Male and female MRC hooded rats (approximately 200 g body wt.), maintained on a standard diet, were used throughout. In experiments with free ranging animals, intraperitoneal injections were carried out while the animals were under light ether anaesthesia. Animals were subsequently placed in metabolism cages designed to permit separate collection of urine and faeces, and allowed water without restriction. Urine samples were collected at suitable time intervals, funnel washings being added to the appropriate sample. Samples were either assayed immediately or stored at 4° until required.

In experiments on rats with bile duct and ureter cannulae, animals were anaesthetized with ether and the trachea exposed and cannulated. The right jugular vein was then cannulated towards the heart and phenobarbitone (Nembutal veterinary grade) was administered (3 mg/200 g body wt. dissolved in 0·1 ml of water) via the jugular vein cannula. Further similar doses of phenobarbitone were administered as necessary to maintain anaesthesia throughout the experiments. The jugular vein cannula was also used for intravenous administration of compounds and for withdrawing blood samples. Ureters and bile duct were cannulated through a midline abdominal incision and urine and bile samples were collected at suitable time intervals.

For experiments involving functional renalectomy, animals were anaesthetized (phenobarbitone) and following a mid-line abdominal incision both renal pedicles were firmly ligatured.

Isolated liver perfusion

The perfusion apparatus was essentially that described by Miller, Bly, Watson and Bale.[9] Isolated rat livers were perfused with homologous blood collected from anaesthetized (ether) rats by cannulation of the abdominal aorta. Blood (approximately 60 ml) was collected into a vessel containing 25 ml of heparinized saline (5000 units of heparin/25 ml of saline) and the whole filtered through nylon mesh before being introduced into the perfusion apparatus. Rats (approximately 300 g body wt.) fasted for 24 hr, served as liver donors and livers were removed by the procedure described by Miller *et al.*[9]

RESULTS

Free-range studies

In preliminary experiments, rats received intraperitoneal injections of aqueous solutions (1 mg/100 g body wt.) of the appropriate esters. Six animals (three male and three female) were used for experiments with any one ester. Each animal was placed in a metabolism cage designed to permit the separate collection of urine and faeces. Urines were collected at intervals of 12 hr, 24 hr and 48 hr after injection and assayed for total and inorganic ^{35}S-sulphate. Faeces were collected after 48 hr and at this time animals were killed by a blow on the back of the head. Faeces and carcasses were taken for the determination of ^{35}S-content. The results are shown in Table 1.

In all experiments between 76 and 98 per cent of the injected radioactivity appeared in the urine over 48 hr, the major proportion appearing during the first 12 hr. Only trace amounts of radioactivity were associated with carcasses and in experiments with cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate only small amounts of radioactivity were detected in the faeces. In animals receiving biphenylyl 4-^{35}S-sulphate up to 20 per cent of the injected radioactivity appeared in the faeces.

Further analysis of urine samples revealed a wide variation in the amounts of radioactivity appearing as inorganic ^{35}S-sulphate. Thus, in urines obtained from animals receiving biphenylyl 4-^{35}S-sulphate, between 18 and 38 per cent of the injected radioactivity appeared as inorganic ^{35}S-sulphate. However, closer examination of the results revealed a significant sex difference in the level of inorganic ^{35}S-sulphate with an average value of 36 per cent for male urine and a corresponding figure of 19 per cent for female urine. In contrast, the wide variation in inorganic ^{35}S-sulphate content of urines in experiments with cyclohexylphenyl 4-^{35}S-sulphate (28–60 per cent) and cyclohexylphenyl 2-^{35}S-sulphate (7–33 per cent) could not be explained simply on the basis of a sex difference. It seemed possible that variations in the amounts of inorganic ^{35}S-sulphate found in urines could result from biliary circulation of the esters and subsequent variable desulphation by the intestinal flora.

In order to check this possibility these preliminary experiments were repeated using animals which had been treated with sulphasuxidine (total dose of 1·5 g/rat) and terramycin (total dose of 0·3 g/rat) *per os* over a period of three days before injection (Dodgson *et al.*[8]).

It is evident from the results (see Table 2) that in animals receiving biphenylyl 4-^{35}S-sulphate the gut flora did not make any contribution to the desulphation of the ester. In contrast, desulphation of cyclohexylphenyl 2-^{35}S-sulphate can be almost completely attributed to gut flora since only trace amounts of inorganic ^{35}S-sulphate were detected in the urines of both male and female rats. The elimination of the gut

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN URINE, FAECES AND CARCASSES OF RATS FOLLOWING THE I.P. INJECTION (1 mg/100 g BODY WT.) OF ^{35}S-LABELLED ARYL SULPHATE ESTERS

T = Total ^{35}S-sulphate; I = Inorganic ^{35}S-sulphate; E = Ester ^{35}S-sulphate; M = Male rat; F = Female rat.

Compound	Animal	% Injected radioactivity recovered in:														
		Urine												Faeces	Carcass	Total
		12 hr			24 hr			48 hr			Total					
		T	I	E	T	I	E	T	I	E	T	I	E	T	T	T
Biphenylyl 4-^{35}S-sulphate	M_1	53	18	35	22	12	10	8	3	5	83	33	50	18	0	101
	M_2	49	24	25	23	11	12	8	3	5	80	38	42	20	0	100
	M_3	61	17	44	18	16	2	4	4	0	83	37	46	19	0	102
	F_1	70	12	58	12	4	8	4	3	1	86	19	67	13	0	99
	F_2	66	9	57	11	6	5	5	3	2	82	18	64	14	1	97
	F_3	75	13	62	10	4	6	4	3	1	89	20	69	11	0	100
Cyclohexylphenyl 4-^{35}S-sulphate	M_1	58	38	20	14	11	3	10	8	2	82	57	25	4	6	92
	M_2	62	42	20	16	12	4	8	6	2	86	60	26	5	4	95
	M_3	80	26	54	6	4	2	4	3	1	90	33	57	3	2	95
	F_1	86	21	65	8	5	3	2	2	0	96	28	68	2	2	100
	F_2	61	36	25	10	9	1	5	4	1	76	49	27	11	4	91
	F_3	75	19	56	9	5	4	3	2	1	87	26	61	5	2	94
Cyclohexylphenyl 2-^{35}S-sulphate	M_1	87	5	82	6	1	5	5	1	4	98	7	91	1	1	100
	M_2	90	30	60	5	1	4	2	2	0	97	33	64	2	1	101
	M_3	73	18	55	15	6	9	4	2	2	92	26	66	4	3	99
	F_1	86	18	68	5	2	3	2	1	1	93	21	72	4	1	98
	F_2	90	7	83	4	3	1	3	1	2	97	11	86	3	2	102
	F_3	82	11	71	9	5	4	4	2	2	95	18	77	4	2	101

flora in animals receiving cyclohexylphenyl 4-^{35}S-sulphate led to a significant reduction in the amount of urinary inorganic ^{35}S-sulphate and revealed a sex difference in the ability of rats to desulphate cyclohexylphenyl 4-^{35}S-sulphate similar to that observed with biphenylyl 4-^{35}S-sulphate.

TABLE 2. INORGANIC ^{35}S-SULPHATE APPEARING IN THE URINE OF NORMAL AND ANTIBIOTIC TREATED RATS FOLLOWING INTRAPERITONEAL ADMINISTRATION (1 mg/100 g BODY WT.) OF ARYL SULPHATE ESTERS

Each result is expressed as a percentage of the total urinary radioactivity. M = Male rat; F = Female rat.

Compound	Animal	% Urinary radioactivity as inorganic ^{35}S-sulphate in: Urine from Normal rats	Urine from antibiotic treatedrats
		%	%
Biphenylyl 4-^{35}S-sulphate	M_1	33	36
	M_2	38	38
	M_3	37	35
	F_1	20	21
	F_2	18	18
	F_3	19	17
Cyclohexylphenyl 4-^{35}S-sulphate	M_1	57	35
	M_2	33	30
	M_3	60	—
	F_1	28	18
	F_2	49	17
	F_3	26	22
Cyclohexylphenyl 2-^{35}S-sulphate	M_1	26	2
	M_2	7	1
	M_3	33	1
	F_1	11	1
	F_2	18	2
	F_3	21	1

Cannulation studies

In subsequent experiments each of the ^{35}S-labelled esters was administered (1 mg/100 g body wt.) i.v. to rats (three male and three female) with bile duct and ureter cannulae. Bile and urine were collected in hourly samples over 6 hr and assayed for total and inorganic ^{35}S-sulphate (see Table 3).

In experiments with biphenylyl 4-^{35}S-sulphate, between 50 and 77 per cent of the injected radioactivity was recovered in urine and bile over the 6-hr period; of the injected radioactivity between 17 and 18 per cent appeared in the bile of male rats and between 7 and 12 per cent in the bile of female rats. Inorganic ^{35}S-sulphate was not present in the bile of either sex. Analysis revealed that in male rats approximately 47 per cent of the urinary radioactivity appeared as inorganic ^{35}S-sulphate with a corresponding figure of 24 per cent for female rats.

In studies with cyclohexylphenyl 4-^{35}S-sulphate the major proportion (88–99 per cent) of the radioactive dose was excreted during the 6-hr period, between 70 and 81 per cent of the dose appearing in the bile of both male and female rats as ester sulphate.

The percentage of urinary radioactivity appearing as inorganic ^{35}S-sulphate was approximately 48 per cent in the male and 29 per cent in the female.

When animals received cyclohexylphenyl 2-^{35}S-sulphate, between 80 and 100 per cent of the radioactivity was excreted in 6 hr. However, there was a relative difference in the extent of biliary and urinary elimination of radioactivity in males and females. Thus a greater proportion of the dose appeared in male bile (between 62 and 70 per cent) than in female bile (between 24 and 50 per cent). Only trace amounts of inorganic ^{35}S-sulphate could be detected in bile and urine samples of either sex.

In all experiments there was a rapid elimination of radioactivity following the administration of the esters. This is illustrated in Fig. 1 which shows the urinary and biliary excretion of radioactivity with time in the male rat following the administration of cyclohexylphenyl 2-^{35}S-sulphate, the bulk of the radioactivity appearing during the first hour. This observation was also made in experiments with biphenylyl 4-^{35}S-sulphate and cyclohexylphenyl 4-^{35}S-sulphate.

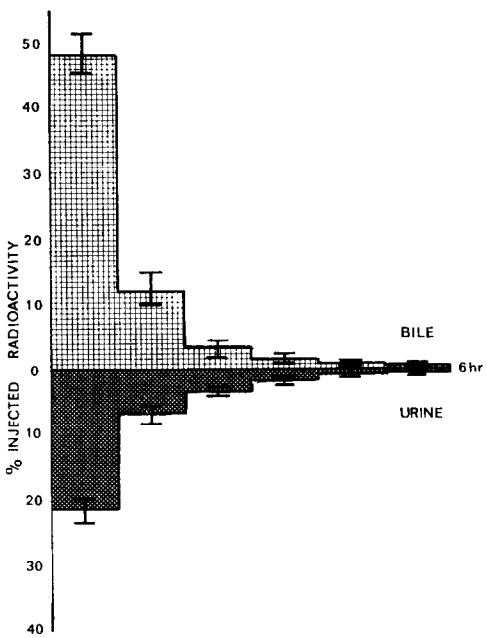

FIG. 1. Distribution of radioactivity in bile and urine in the male rat over the 6-hr period following the intravenous administration (1 mg/100 g body wt.) of cyclohexylphenyl 2-^{35}S-sulphate. Each percentage represents the mean of results obtained from three animals. The limits of variation are included.

The rapidity of the biliary elimination of radioactivity was further investigated by continuous analysis of bile. The results obtained in experiments with cyclohexylphenyl 4-^{35}S-sulphate (Fig. 2) are typical of the three esters studied; radioactivity was detected in bile approximately 3 min after injection of the ester; the level of activity then rose to a maximum after 20 min and subsequently declined steadily. Similar results were obtained in experiments to determine the speed of urinary elimination.

TABLE 3. THE DISTRIBUTION OF RADIOACTIVITY IN THE BILE AND URINE OF RATS FOR THE 6-hr PERIOD FOLLOWING THE I.V. ADMINISTRATION OF ^{35}S-LABELLED ARYL SULPHATE ESTERS

T = Total ^{35}S-sulphate; I = Inorganic ^{35}S-sulphate; E = Ester ^{35}S-sulphate; M = Male rat; F = Female rat.

Compound	Animal	% Radioactive dose recovered in: Bile T	Bile I	Urine T	Urine I	Urine I/T × 100	Total T
Biphenylyl 4-^{35}S-sulphate	M_1	18	1	43	21	49	61
	M_2	18	0	44	23	52	62
	M_3	17	1	33	15	45	50
	F_1	12	1	65	22	34	77
	F_2	7	0	49	8	17	56
	F_3	12	1	45	9	20	57
Cyclohexylphenyl 4-^{35}S-sulphate	M_1	75	1	20	8·0	40	95
	M_2	77	0	11	5·8	52	89
	M_3	81	1	18	9·2	51	99
	F_1	77	0	17	5·0	29	94
	F_2	73	0	17	5·1	30	90
	F_3	70	0	18	5·2	29	88
Cyclohexylphenyl 2-^{35}S-sulphate	M_1	62	0	37	1·3	4	99
	M_2	67	0	30	1·3	4	97
	M_3	70	0	31	0·9	3	100
	F_1	43	0	37	0·7	2	80
	F_2	50	0	55	1·5	3	105
	F_3	24	0	70	0·7	1	94

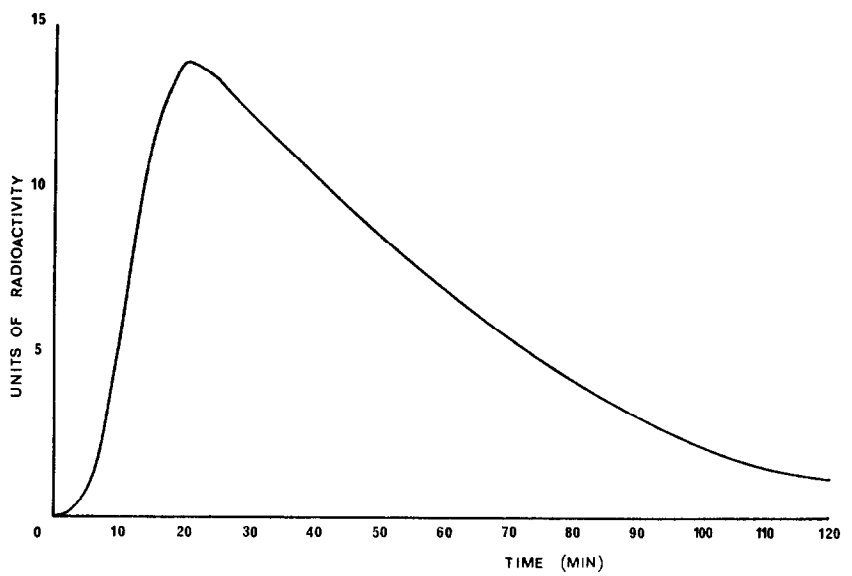

FIG. 2. The appearance of radioactivity in bile with time following the i.v. administration (1 mg/100 g body wt.) of cyclohexylphenyl 4-^{35}S-sulphate to the male rat.

Further experiments were made in which female rats received intravenous injections of the sulphate esters at two other dose levels. Thus rats received biphenylyl 4-^{35}S-sulphate, cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate at a dose level of 0·1 mg/100 g body wt. (one tenth the dose level used in previous experiments). With biphenylyl 4-^{35}S-sulphate and cyclohexylphenyl 4-^{35}S-sulphate the relative distributions of radioactivity in urine and bile were the same as those previously recorded. In contrast, with cyclohexylphenyl 2-^{35}S-sulphate the entire radioactive dose was eliminated via the urine. In other experiments animals received 10 mg/100 g body wt. of either cyclohexylphenyl 4-^{35}S-sulphate or cyclohexylphenyl 2-^{35}S-sulphate. The relative distribution of radioactivity in urine and bile was the same as that recorded at a dose level of 1 mg/100 g body wt. but in the case of cyclohexylphenyl 2-^{35}S-sulphate the bulk of the radioactivity was excreted over a 3-hr period in contrast to the 1-hr period previously recorded. Due to the limited solubility of biphenylyl 4-^{35}S-sulphate (approximately 3 mg/ml) it was not possible to investigate excretion patterns at a dose level of 10 mg/100 g body wt., the ester was therefore injected at a dose of 2 mg/100 g body wt. There was no deviation from the previously recorded excretion pattern.

Chromatographic analysis of urine and bile

Urine and bile samples were collected for 2 hr from animals (male and female) receiving i.v. injections of the ^{35}S-labelled esters (1 mg/100 g body wt.) and subjected to TLC in the presence of appropriate marker compounds (sufficient urine or bile was applied to thin-layer plates in order to give approximately 2000 cpm measured with a Panax monitor). Radioactive areas were located by scanning and autoradiography.

Chromatography of urines from rats receiving biphenylyl 4-^{35}S-sulphate revealed the presence of three major radioactive components viz: unchanged biphenylyl 4-^{35}S-sulphate (identified by isotope dilution analysis), inorganic ^{35}S-sulphate and an unidentified metabolite (metabolite A). Several other minor radioactive components representing less than 1 per cent of the dose were detected.

In contrast, in urines from animals receiving cyclohexylphenyl 4-^{35}S-sulphate only trace amounts of parent ester could be detected. However, urine contained two major radioactive components viz: inorganic ^{35}S-sulphate and a metabolite (metabolite B). Traces of other radioactive components were also detected.

Urines from rats receiving cyclohexylphenyl 2-^{35}S-sulphate contained two major radioactive components in approximately equivalent amounts; unchanged parent ester (identified by isotope dilution analysis) and a metabolite (metabolite C). Only trace amounts of inorganic ^{35}S-sulphate were detected.

Table 4 shows the relative concentrations of the urinary components and their respective R_f values.

Analysis of bile from animals receiving biphenylyl 4-^{35}S-sulphate, cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate revealed the presence of only one radioactive component in each case.

Identification of biliary metabolites

Bile samples obtained following the injection of the ^{35}S-labelled esters were subjected to thin-layer chromatography in two solvent systems (A and B) as previously described. The major radioactive spot was located by autoradiography following

which the thin-layer plates were sprayed with a mixture of phosphoric acid: 0·2 per cent alcoholic naphthoresorcinol (1:22 by volume) and heated at 105° for 10 min (Randerath[10]). Each bile sample contained a component which gave the characteristic blue colour typical for glucuronic acid conjugates and furthermore the coloured areas on the plates coincided with the major radioactive spots. It therefore seemed probable that in each bile sample the major biliary metabolite could be identified as a glucuronic acid conjugate. This view was substantiated by experiments in which bile samples were subjected to treatment with β-glucuronidase.

TABLE 4. THE RELATIVE CONCENTRATIONS AND R_f VALUES OF RADIOACTIVE URINARY COMPONENTS OBTAINED FOLLOWING THE I.V. ADMINISTRATION (1 mg/100 g BODY WT.) OF ^{35}S-LABELLED ARYL SULPHATE ESTERS TO RATS

(R_f values were obtained using solvent A) I = Inorganic ^{35}S-sulphate; E = Ester ^{35}S-sulphate.

Compound	R_f Value				Urinary composition		
	I	E	Metabolite A/B/C	Bile metabolite	I %	E %	Metabolite A/B/C %
Biphenylyl 4-sulphate	Baseline	0·66	0·60	0·06	25	65	10
Cyclohexylphenyl 4-sulphate	Baseline	0·63	0·52	0·06	40	0	60
Cyclohexylphenyl 2-sulphate	Baseline	0·61	0·43	0·06	0	50	50

Portions (0·2 ml) of each bile sample were each mixed with an equal volume of a solution of β-glucuronidase (Sigma bacterial preparation type I, 1000 Fishman units/ml in 0·1 M phosphate buffer at pH 6·5) and incubated at 37° for 3 hr. Portions (20 μl) of the incubation mixtures together with untreated bile and appropriate markers were subjected to TLC in solvent systems A and B. Autoradiograms showed a marked reduction in the concentration of the biliary metabolites with the concomitant appearance of other ^{35}S-labelled spots which in each case exhibited chromatographic properties identical with the respective major urinary metabolites (metabolites A, B and C).

These studies with β-glucuronidase, coupled with the specific colour reaction obtained with naphthoresorcinol provide evidence of the identity of the biliary metabolites as glucuronic acid conjugates. Further, the chromatographic similarities of the urinary metabolites (A, B and C) with the β-glucuronidase degradation products of bile samples strongly indicates that the urinary metabolites A, B and C can be identified as hydroxylated derivatives of biphenylyl 4-^{35}S-sulphate, cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate respectively. Urinary excretion of these hydroxylated derivatives may be accounted for by cleavage of glucuronic acid from the biliary metabolite in the intestinal tract followed by reabsorption and urinary excretion of the aglycone.

Intravenous administration of isolated biliary metabolites of cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate

Following the administration of cyclohexylphenyl 4-^{35}S-sulphate or cyclohexylphenyl 2-^{35}S-sulphate, a large proportion of the radioactive dose appeared in the

bile as the respective glucuronic acid conjugate. It was of considerable interest to investigate the route of excretion of these double conjugates following intravenous administration.

Male rats (four) were injected i.v. with cyclohexylphenyl 4-^{35}S-sulphate (1 mg/100 g body wt.). Bile samples were collected for 2 hr, pooled and applied as streaks to a preparative thin-layer chromatography plate which was developed in solvent B. The position of the major biliary metabolite was located by autoradiography, the appropriate area of silica gel was removed from the plate and the metabolite eluted with water (total volume, approximately 5 ml). The eluate was concentrated *in vacuo* at room temperature to a volume of approximately 0·5 ml. The preparation contained only one radioactive component which was identical with the biliary metabolite as shown by TLC. When the isolated double conjugate (0·4 ml; the radioactive content of the injected dose was comparable with that associated with 2 mg of the ^{35}S-labelled parent ester) was administered intravenously to a male rat with bile duct and ureter cannulae, it was eliminated unchanged within 4 hr, 90 per cent appearing in the bile and 10 per cent in the urine.

An identical experiment was performed in which the biliary metabolite of cyclohexylphenyl 2-^{35}S-sulphate was isolated, purified and injected into a male rat. The double conjugate of this ester was also eliminated unchanged within 4 hr, 60 per cent appearing in the bile and 40 per cent in the urine.

Further investigations with biphenylyl 4-^{35}S-sulphate

The chemical structure of biphenylyl 4-sulphate is in many ways similar to the chemical structures of cyclohexylphenyl 4-sulphate and cyclohexylphenyl 2-sulphate. However, in contrast to the latter two compounds only relatively small amounts of radioactivity (up to 20 per cent of the dose) are eliminated via the bile following the administration of biphenylyl 4-^{35}S-sulphate. It seemed possible that this low level of biliary elimination of the glucuronic acid conjugate might be explained by the rapid clearance of the injected radioactivity by the kidneys resulting in minimal exposure of the ester to the liver enzymes (cf. Flynn *et al.*[3]).

It was also possible that the results could be attributed to the inability of the ester to penetrate the liver cells. These possibilities were investigated in the first instance by studying the metabolic fate of the ester in an isolated perfused liver system.

Biphenylyl 4-^{35}S-sulphate (40 mg) was added to the perfusate (80 ml) and an aliquot (5 ml; control blood sample) of this mixture was incubated at 37° for 6 hr. Rat livers (male and female) were perfused for 6 hr during which time blood samples (2 ml; test blood samples) were removed from the apparatus at intervals of 1 hr. Bile was collected for 6 hr. Blood samples (tests and control) and bile were assayed for total and inorganic ^{35}S-sulphate. Up to 7 per cent of the radioactivity added to the perfusate appeared in the bile largely as ester ^{35}S-sulphate. This level of biliary elimination of radioactivity is comparable with that already recorded in cannulation experiments with whole animals and it is thus clear that in these experiments the low level of biliary elimination cannot be accounted for by rapid clearance of the injected radioactivity by the kidneys.

The results of analyses of test blood samples are presented in Fig. 3 which shows the progressive appearance of inorganic ^{35}S-sulphate in the perfusate with time. Thus after 6 hr, between 25 and 29 per cent of the radioactivity added to the perfusate

appeared as inorganic ^{35}S-sulphate and it is thus clear that the liver is capable of contributing significantly to the hydrolysis of the ester *in vivo*. Analysis of control blood showed that less than 2 per cent hydrolysis had occurred over 6 hr.

The results of the liver perfusion experiments demonstrated that appreciable desulphation of biphenylyl 4-^{35}S-sulphate occurred under the "closed system" conditions but there was no significant increase in the level of biliary elimination when

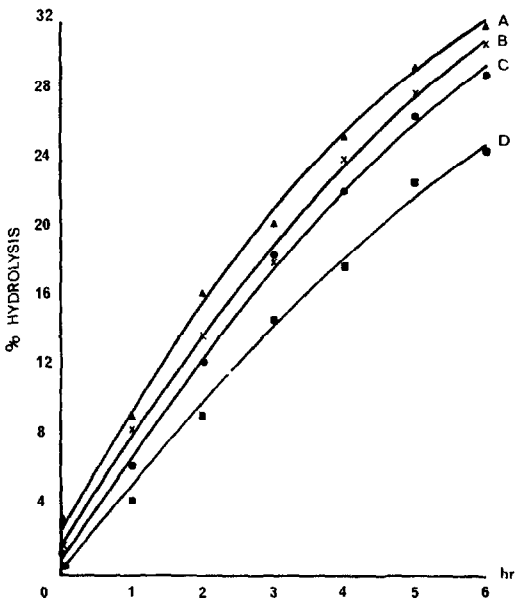

FIG. 3. Time hydrolysis curves for biphenylyl 4-^{35}S-sulphate as recorded in: A, renalectomized male rat; B, renalectomized female rat; C, isolated perfused female liver; D, isolated perfused male liver. For details see text.

compared with previous results with cannulated animals. In order to simulate these conditions in the living animal, biphenylyl 4-^{35}S-sulphate (1 mg/100 g body wt.) was injected into rats (male and female) in which kidney function had been eliminated by ligation of the renal pedicles. Blood samples (0·2 ml) were withdrawn at hourly intervals over a period of 6 hr and assayed for total and inorganic ^{35}S-sulphate. Bile collected over the 6-hr period was similarly assayed for radioactivity and was found to contain approximately 13 per cent of the injected radioactivity, largely as ester ^{35}S-sulphate. This percentage is comparable with that observed in previous cannulation experiments. Analysis of blood samples showed that considerable hydrolysis of the ester occurred over 6 hr (see Fig. 3) the results being in close agreement with those obtained in isolated liver perfusion studies.

Protein binding studies

It is quite probable that the biliary elimination of compounds may be in part determined by the extent to which these compounds may be bound to plasma proteins. For this reason the ability of plasma proteins to bind biphenylyl 4-^{35}S-sulphate, cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate was investigated.

Each ester was administered to a rat at a dose level of 1 mg/100 g body wt. and after 20 min the blood was collected from the abdominal aorta and the degree of protein binding determined for each ester. With biphenylyl 4-^{35}S-sulphate approximately 90 per cent of the ester was bound to protein and the corresponding figures for cyclohexylphenyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate were approximately 70 and 95 per cent respectively.

DISCUSSION

The investigations reported here on the metabolic fates and modes of excretion of biphenylyl 4-sulphate, cyclohexylphenyl 4-sulphate and cyclohexylphenyl 2-sulphate provide further support for the view that aryl sulphate esters are not necessarily inert *in vivo*. All three of the aryl sulphate esters investigated were metabolized by the rat but, in spite of certain common structural similarities, the compounds were metabolized to varying extents.

In experiments in which free-ranging rats received intraperitoneal injections of the ^{35}S-labelled esters, the bulk of the radioactivity was recovered in the urine. In all cases the greater proportion of the urinary radioactivity was in the form of ester sulphate and, following either biphenylyl 4-^{35}S-sulphate or cyclohexylphenyl 2-^{35}S-sulphate administration, significant amounts of the ester sulphate fraction could be accounted for by the presence of unchanged parent ester. However, in the case of cyclohexylphenyl 4-^{35}S-sulphate, only trace amounts of unchanged parent ester were detected. The proportion of urinary radioactivity appearing as inorganic ^{35}S-sulphate varied considerably in experiments with the three esters. Thus, urines obtained following administration of either biphenylyl 4-^{35}S-sulphate or cyclohexylphenyl 4-^{35}S-sulphate contained considerable amounts of the radioactive dose in the form of inorganic ^{35}S-sulphate and in both instances the amount of inorganic ^{35}S-sulphate in male urine was approximately twice as much as that in female urine. In contrast only traces of inorganic ^{35}S-sulphate were detected in urines of animals receiving cyclohexylphenyl 2-^{35}S-sulphate.

Subsequent experiments with rats with bile duct fistulae revealed that the biliary excretion of the administered radioactivity varied markedly with each compound. Thus, following the administration of biphenylyl 4-^{35}S-sulphate, up to 18 per cent of the radioactivity appeared in the bile, with corresponding figures of up to 81 per cent with cyclohexylphenyl 4-^{35}S-sulphate and up to 70 per cent with cyclohexylphenyl 2-^{35}S-sulphate. In studies with both biphenylyl 4-^{35}S-sulphate and cyclohexylphenyl 2-^{35}S-sulphate there was a sex difference in the amount of radioactivity eliminated via the bile: this was small in the case of biphenylyl 4-^{35}S-sulphate but with cyclohexylphenyl 2-^{35}S-sulphate the difference was very marked; in the male between 62 and 70 per cent of the administered dose appeared in the bile with a corresponding range of 24–50 per cent in the female. Further analysis revealed that in experiments with each ester only one major radioactive component was present in the bile and in each case this was in the form of ester sulphate, subsequently identified as a glucuronic acid conjugate.

The investigations have further revealed that following the biliary elimination of the administered esters as glucuronic acid conjugates, the latter undergo degradation in the gastrointestinal tract. This results in the loss of the glucuronic acid residue, reabsorption of the aglycone and its subsequent urinary excretion.

At the present time the factors which determine the extent of urinary or biliary excretion of compounds are not completely understood. However, a number of workers have investigated various parameters relating to problems of routes of elimination and have suggested a number of criteria which may assume importance in determining the route of excretion. Sperber[11] put forward the view that relatively small ions (with a molecular weight of less than 400), not strongly protein bound, are efficiently excreted by the renal tubules while the liver preferentially excretes somewhat larger ions even if they exhibit strong protein binding. Smith and Williams[12] and Millburn, Smith and Williams[13] suggest that a possible prerequisite for biliary excretion is a certain balance of hydrophilic and lipophilic properties of the molecule in question. They further suggest that the extent of biliary elimination of foreign compounds by rats depends on their molecular weight and their possessing a strongly polar anionic group. In addition, they propose a minimum value (325 ± 50) for molecular weight below which little biliary excretion occurs.

The results of the present investigation were examined in the light of these criteria. In general the results were found to be compatible with these ideas but several interesting points emerged. Firstly, the extent to which these particular esters are bound to plasma proteins bears little relation to their respective levels of biliary excretion. Although all three esters are bound to similar extents, the degree of biliary elimination varies considerably. With reference to the balance of hydrophilic and lipophilic properties of the molecule it is of interest to compare the metabolic fate and mode of excretion of biphenylyl 4-sulphate with that of cyclohexylphenyl 4-sulphate. Cyclohexylphenyl 4-sulphate undergoes extensive metabolism and considerable biliary excretion, while biphenylyl 4-sulphate is metabolized to a lesser extent and shows a much lower level of biliary elimination. The striking difference in the metabolic behaviour of these two structurally very similar compounds is explicable only on the basis of the loss of aromatic character of the ring remote from the sulphate moiety. It seemed unlikely that replacement of one aromatic ring of biphenyl 4-sulphate with the cyclohexyl ring of cyclohexylphenyl 4-sulphate would lead to any great change in the lipophilic nature of the molecule and this view was supported by the observation that both compounds exhibit very similar, low, water solubilities (3–4 mg/ml).

In biphenylyl 4-sulphate and cyclohexylphenyl 4-sulphate, the sulphate moiety is in the para position with respect to the lipophilic part of the molecule. In contrast, in cyclohexylphenyl 2-sulphate, the sulphate moiety lies in the ortho position with respect to the cyclohexyl group. This close association of the highly charged sulphate group with the lipophilic cyclohexyl group would be expected to effectively decrease the overall lipophilic nature of the molecule. This expectation was realized since cyclohexylphenyl 2-sulphate exhibited an extremely high water solubility and was able to form micelles in dilute aqueous solution.

These modifications to the physico-chemical properties of cyclohexylphenyl sulphate resulting from translocation of the sulphate moiety from the 4- to the 2-position led to some reduction in the level of biliary elimination. However, it should be noted that the biliary excretion is still considerably greater than that recorded with biphenylyl 4-sulphate whose lipophilic character is comparable with that of cyclohexylphenyl 4-sulphate. These collective findings strongly suggest that for the three compounds studied in this investigation, the balance of lipophilic and hydrophilic

properties of the respective molecules is not an over-riding factor in determining the route of elimination.

The molecular weight of each of the sulphate esters studied is less than 300 and in view of the principles put forward by Millburn *et al.*[13] major biliary elimination would not be expected. The appearance of only trace amounts of unchanged parent ester in bile was in accordance with these principles. However, varying amounts of parent ester in conjugation with glucuronic acid were eliminated in the bile and it is noteworthy that, assuming monosubstitution with glucuronic acid, molecular weights in excess of 440 would be achieved. In experiments with cyclohexylphenyl 4-sulphate the expected high level of biliary elimination was realized but with cyclohexylphenyl 2-sulphate the extent of biliary elimination was considerably lower than this while with biphenyl 4-sulphate values of 18 per cent and below were recorded.

The collective findings with the three aryl sulphate esters suggest that a possible controlling factor in biliary elimination is the ability of the administered compound to form a glucuronic acid conjugate at appropriate sites within the liver. This may in turn be governed by the reactivity of the compound and its ability to reach the site of conjugating enzymes. The route of elimination of glucuronic acid conjugates might then be determined by such factors as polarity, molecular weight and lipophilic character.

Some insight into the influence of a sulphate moiety on the extent of biliary excretion of a compound can be gained by comparing the results of Millburn *et al.*[13] with the present findings. Thus, following the administration of biphenyl to the rat up to 17 per cent of the dose appeared in the bile as a glucuronic acid conjugate. This value is virtually identical with that obtained when biphenylyl 4-^{35}S-sulphate was administered to rats. This suggests that the presence of the sulphate moiety does not influence the extent of biliary excretion and this in turn may be explained by the absence of a specific sulphate transporting mechanism. Further, the biliary elimination of aryl sulphate esters may thus depend, in the first instance, upon the formation of glucuronic acid conjugates and, secondly, on the presence of a suitable transporting mechanism for these conjugates.

REFERENCES

1. J. B. Hawkins and L. Young, *Biochem. J.* **56**, 166 (1954).
2. K. S. Dodgson and N. Tudball, *Biochem. J.* **74**, 154 (1960).
3. T. G. Flynn, K. S. Dodgson, G. M. Powell and F. A. Rose, *Biochem. J.* **105**, 1003 (1967).
4. D. J. Hearse, A. H. Olavesen and G. M. Powell, *Biochem. Pharmac.* **18**, 173 (1969).
5. J. G. Jones and K. S. Dodgson, *Biochem. J.* **94**, 331 (1965).
6. A. G. Lloyd, *Biochem. J.* **80**, 572 (1961).
7. L. Young, M. Edson and J. A. McCarter, *Biochem. J.* **44**, 179 (1949).
8. K. S. Dodgson, G. M. Powell, F. A. Rose and N. Tudball, *Biochem. J.* **79**, 209 (1961).
9. L. L. Miller, C. G. Bly, M. L. Watson and W. F. Bale, *J. exp. Med.* **94**, 431 (1951).
10. K. Randerath, *Thin-Layer Chromatography*. Academic Press, New York and London (1963).
11. I. Sperber, *The Biliary System* (Ed. W. Taylor). Blackwell, Oxford (1965).
12. R. L. Smith and R. T. Williams, *Glucuronic Acid Free and Combined.* (Ed. G. J. Dutton), Academic Press, New York and London (1966).
13. P. Millburn, R. L. Smith and R. T. Williams, *Biochem. J.* **105**, 1275 (1967).