The Metabolism of Labelled Hexadecyl Sulphate Salts in the Rat, Dog and Human

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(Received 4 November 1974)

The metabolic fate of [1-¹⁴C]hexadecyl sulphate and hexadecyl [³⁵S]sulphate, administered intravenously as the sodium and trimethylammonium salt to dogs and orally as the erythromycin salt to dogs, rats and humans, was studied. Studies with rats indicated that the compounds were well absorbed and rapidly excreted in the urine. However, after oral administration of the ¹⁴C- and ³⁵S-labelled hexadecyl sulphate erythromycin salt to dogs, considerable amounts of radioactivity were excreted in the faeces as unmetabolized hexadecyl sulphate. Studies with two humans showed that orally administered erythromycin salt of [1-¹⁴C]hexadecyl sulphate was well absorbed in one person but poorly absorbed in the other. Radioactive metabolites in urine were separated by t.l.c. in two solvent systems. The main metabolite of hexadecyl sulphate in the dog, rat and human was identified as the sulphate ester of 4-hydroxybutyric acid. In addition, γ -[¹⁴C]butyrolactone as a minor metabolic product of [1-¹⁴C]hexadecyl sulphate was also isolated from the urine of rat, dog and man. However, there was still another metabolite in dog urine, which comprised about 20% of the total urinary radioactivity and carried both ¹⁴C and ³⁵S labels. This metabolite was absent from rat urine. The metabolite in dog urine was isolated and subsequently identified by t.l.c. and g.l.c. and by isotope-dilution experiments as the sulphate ester of glycollic acid. Small amounts (about 5% of the total recovered radioactivity in excreta) of labelled glycollic acid sulphate were also found in human urine after ingestion of erythromycin [1-¹⁴C]hexadecyl sulphate.

The surface-active properties of hexadecyl sulphate have been known since the early days of organic chemistry after hexadecyl hydrogen sulphate and its potassium salt were first prepared by Dumas & Peligot (1836). Despite the wide use of hexadecyl sulphate as a detergent and foaming agent in industrial and domestic preparations, no metabolic studies in mammals with identification of the products of degradation have been reported. However, Denner *et al.* (1969) have published results on the metabolism of potassium dodecyl [³²S]sulphate in the rat. They identified the main metabolite of dodecyl sulphate in rat urine as the sulphate ester of 4-hydroxybutyric acid. Since dodecyl sulphate is a homologue of hexadecyl sulphate possessing four less carbon atoms, I thought that the metabolic degradation of these two compounds should proceed along similar patterns.

The present investigation is about the metabolism of [1-¹⁴C]hexadecyl sulphate and hexadecyl [³⁵S]sulphate administered intravenously to dogs and orally to rats, dogs and humans. The ester has been found to be metabolized to butyric acid 4-sulphate as the main product and γ -butyrolactone as a minor product in the rat, dog and man. However, in the dog and man, but not in the rat, some of it is broken down further to glycollic acid sulphate.

Materials and Methods

Labelled drugs

Trimethylammonium salt of [1-¹⁴C]hexadecyl sulphate. It was prepared by Mr. Arthur Alter (Abbott Laboratories, North Chicago, Ill., U.S.A.), from labelled palmitic acid (New England Nuclear Corp., Boston, Mass., U.S.A.) by the reduction to hexadecan-1-ol and reaction with SO₃-trimethylamine complex (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.). It was found both chemically and radiochemically to be at least 95% pure and had a specific radioactivity of 61.5 μ Ci/mg.

Sodium salt of hexadecyl [³⁵S]sulphate. It was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.), and had an initial specific radioactivity of 18.75 μ Ci/mg. The salt was used within 1 month of delivery.

Erythromycin salt of [1-¹⁴C]hexadecyl sulphate. The salt was synthesized by Mr. Alter by the reaction of trimethylammonium salt of [1-¹⁴C]hexadecyl sulphate with erythromycin A base (Abbott Laboratories). Its purity (>99.5%) was checked by t.l.c. in three different solvent systems, by i.r. and n.m.r. spectrometry, and by an isotopic-dilution technique. Its specific radioactivity was 16.7 μ Ci/mg.

Erythromycin salt of hexadecyl [^{35}S]sulphate. It was synthesized from the sodium salt of hexadecyl [^{35}S]sulphate and erythromycin A base by Dr. G. J. Ikeda at Abbott Laboratories. It was found to be at least 93% radiochemically pure by t.l.c. in three solvent systems and chemically pure by i.r. and n.m.r. spectrometry. It had an initial specific radioactivity of 4.74 $\mu\text{Ci}/\text{mg}$ and was used within 1 month after its synthesis.

Chemicals

Silica-gel Q1F Quanta/Gram plates (5 cm $\times$ 20 cm) and preparative plates (20 cm $\times$ 20 cm) were purchased from Quantum Industries (Fairfield, N.J., U.S.A.). [$1\text{-}^{14}\text{C}$]Glycollic acid (sodium salt) was purchased from Amersham/Searle Corp. (Arlington Heights, Ill., U.S.A.). Methanol-toluene-based scintillation solution was obtained from Burdick and Jackson (Muskegon, Mich., U.S.A.). Aquasol scintillation solution and $\text{H}_2^{35}\text{SO}_4$ were purchased from New England Nuclear Corp. Glycollic acid, γ -butyrolactone, *p*-nitrobenzyl bromide, *p*-bromophenacyl bromide and *p*-phenylphenacyl bromide were the products of Aldrich Chemical Co. *NO*-Bis(trimethylsilyl)acetamide was purchased from Pierce Chemical Co. (Rockford, Ill., U.S.A.). Dimethylformamide was obtained from Mallinckrodt Chemical Works (St. Louis, Mo., U.S.A.). Potassium butyric acid 4-sulphate was synthesized by the procedure of Denner *et al.* (1969). Its structure was verified by t.l.c. and n.m.r. spectrometry.

Animals and drug administration

Male albino Long-Evans rats (Blue Spruce Co., Altamont, N.Y., U.S.A.) and pure-bred female beagle dogs were starved for 18 h before experimentation, but were allowed water *ad libitum*. The animals were placed in metabolism cages that permitted separate collection of faeces and urine. Dogs were dosed intravenously (saphenous vein) with a mixture of hexadecyl [^{35}S]sulphate or [$1\text{-}^{14}\text{C}$]hexadecyl sulphate and non-labelled drug. The intravenous dose was administered as a freshly prepared solution of hexadecyl sulphate in aq. 10% (v/v) ethanol at a dosage of 4.4 mg/kg for the ^{14}C -labelled drug and 2.9 mg/kg for the ^{35}S -labelled drug. Unanaesthetized rats and dogs were dosed orally by gavage with a mixture of [$1\text{-}^{14}\text{C}$]hexadecyl sulphate and non-labelled drug or hexadecyl [^{35}S]sulphate and non-labelled drug in the form of a freshly prepared 0.3% tragacanth suspension at a dosage of 14.4 mg/kg. The animals were allowed food (Purina Lab Chow or Gaines Dog Food) without restriction 6 h after dosage. Three rats were kept in controlled-environment metabolism cages (Delmar-Roth, model no. DS-

7005) and the expired CO_2 was trapped in ethanol-amine-ethanol (1:1, v/v).

Sample collection. Urine samples were filtered through a glass-wool plug and, whenever possible, were assayed immediately by t.l.c.; otherwise they were stored frozen at -10°C and assayed not more than 1 week later. Blood samples were withdrawn at designated times from the rat (tail vein) and dog (jugular vein) into heparinized vials. Plasma was separated from cells by centrifugation at 1000g for 10 min.

Human studies

Two adult healthy male human volunteers were in this experiment; subject N.J.V. weighed 79.5 kg and subject D.J.D. weighed 92.7 kg. Each subject ingested a capsule containing 360 mg of erythromycin hexadecyl sulphate (equivalent to 250 mg of erythromycin base) with 48.4 μCi of ^{14}C -labelled hexadecyl sulphate. Both had given informed consent before the study. The study proposal had been reviewed and approved by the isotope and human research committees at the hospital.

Analytical procedures

Measurement of radioactivity. The ^{14}C or ^{35}S content of samples was determined in a refrigerated Tri-Carb liquid-scintillation spectrometer (Packard, model 3380). The settings were as follows: gain 17%, discriminators 50–1000. Sufficient counts were recorded to give a standard error of less than 2%. Corrections were made for background (usually about 20 c.p.m.), decay (for ^{35}S), and quenching (by the use of an internal [^{14}C]toluene standard). When the sample was in the form of an aqueous solution, 2.5–4 ml portions were mixed with 10 ml of Aquasol scintillation solution and the radioactivity of the resulting gel was assayed. Counting efficiencies for the gels ranged from 78 to 86%. For non-aqueous samples in the methanol-toluene-based scintillation solution in the presence of the suspended solid particles of silica, the counting efficiency exceeded 90%.

Determination of [^{35}S]sulphate. Inorganic [^{35}S]sulphate (as $\text{Ba}^{35}\text{SO}_4$) and total [^{35}S]sulphate (as $\text{Ba}^{35}\text{SO}_4$ after acid hydrolysis) in urine was determined by the method of Lloyd (1961) with slight modifications. The inorganic [^{35}S]sulphate fraction of urine was precipitated as follows. To 1.5 ml of the sample in a Pyrex test tube (13 mm $\times$ 100 mm) was added 0.5 ml of 4M-HCl and 0.9 ml of 0.15M- H_2SO_4 , followed by 1.2 ml of aq. 10% (w/v) BaCl_2 . After mixing, the tube was kept at 4°C for 4 h. The precipitate was centrifuged down (15 min at 2000g) and washed with 3×8 ml of warm (about 50°C) water, followed with 8 ml lots of acetone and ethanol, with

intermediate centrifuging. Finally the precipitate was suspended in 2.5 ml of water and mixed with 10 ml of Aquasol scintillation solution. The resulting gel was assayed for radioactivity. Counting efficiency of the precipitate ranged between 55 and 83%.

For the determination of the combined ester [^{35}S]-sulphate and inorganic [^{35}S]-sulphate, the sample was hydrolysed by refluxing in 1 M-HCl for 22 h as suggested by Denner *et al.* (1969). After cooling, $\text{Ba}^{35}\text{SO}_4$ was precipitated as described above.

T.l.c. Separation of the radioactive metabolites of hexadecyl sulphate, administered in the form of its sodium, trimethylammonium or erythromycin salt, was performed on silica-gel plates in two solvent systems. Solvent mixture A, consisting of acetic acid, water and butan-1-ol (1:1:4, by vol.) has been described by Denner *et al.* (1969). Solvent mixture B contained acetic acid, methanol and chloroform (1:3:16, by vol.). After the plates had been developed (which usually took 4 h in solvent A and 1 h in solvent B), 0.5 cm sections were sequentially scraped by an automatic scraper (Analabs Co., North Haven, Conn., U.S.A.) into counting vials containing 10 ml of methanol-toluene scintillation solution and were assayed for radioactivity. No corrections for quenching were made.

Urine samples could be spotted directly on t.l.c. plates. In some experiments, when the drug was labelled with ^{35}S , it was desirable to exclude possible inorganic sulphate. In this case, urine samples (3 ml) were freeze-dried and the residue was extracted with 3 × 3 ml of boiling methanol. The combined methanol extracts were centrifuged at 1000g for 15 min and evaporated under N_2 to about 1 ml before application to silica-gel plates. Faecal samples were homogenized in aq. 80% (v/v) ethanol and the centrifuged extracts were spotted on silica-gel t.l.c. plates. For the analyses of plasma samples, 2 ml portions were freeze-dried and the residues were extracted with 3 × 3 ml of boiling methanol. The combined methanol solutions were centrifuged and evaporated under N_2 to about 0.5–1 ml for t.l.c. The methanol-extraction residue was suspended in 2 ml of water and 10 ml of Aquasol and the radioactivity of the resulting gel was assayed.

Gas-liquid chromatography. G.l.c. was carried out with a Varian Aerograph series 1400 instrument equipped with a flame ionization detector. Two stainless-steel columns (180 cm long, 3.2 mm inner diam.) were used: one was packed with 10% (w/w) diethylene glycol adipate on 80–100-mesh Chromosorb Q and operated at 110°C; the other column was packed with 40–60-mesh Chromosorb W, coated with 20% (w/w) neopentyl glycol succinate and operated at 100°C or 130°C. The injection-chamber temperature was 220°C and the detector temperature was 230°C. The N_2 carrier gas-flow rate was 20 ml/min. The columns were connected with a splitter which directed 90% of the gas flow through a stainless-steel tube (50 cm long,

2 mm diam.). This tubing was encased in 6 mm-diam. copper tubing except for 35 mm which was protruding, uncovered, from the end of the copper tubing. Heating tape (1.27 cm × 122 cm; Clas-Col Apparatus Co., Terre Haute, Ind., U.S.A.), was wrapped around the copper tubing and the temperature was kept at 180–200°C. Exciting gases were trapped as they bubbled through 10 ml of methanol-toluene-based scintillation liquid contained in counting vials. These vials were changed manually every minute, immediately capped and assayed for radioactivity.

Experimental and Results

Recoveries of radioactivities, due to the administered labelled hexadecyl sulphate doses, in 0–48 h excreta exceeded 91% in rats; in dogs they ranged from 79 to 92% and in humans from 86 to 88%. Only 3–7% of the oral erythromycin [$1\text{-}^{14}\text{C}$]hexadecyl sulphate dose was expired as $^{14}\text{CO}_2$ in rats. The complete information of the recoveries of excreted radioactivity is shown in Table 1.

In the ^{35}S - and ^{14}C -labelled erythromycin hexadecyl sulphate molecules both labels are located in the hexadecyl sulphate portion. Therefore assuming complete dissociation of the erythromycin salt in water solution, the radioactivity in the excreta would represent hexadecyl sulphate and/or its metabolites rather than the intact salt, erythromycin hexadecyl sulphate.

Metabolic patterns

Plasma. The rapid appearance of radioactivity in plasma of dogs and humans after oral administration of erythromycin [$1\text{-}^{14}\text{C}$]hexadecyl sulphate indicated rapid absorption of the drug. Fig. 1 depicts dog plasma concentrations of [$1\text{-}^{14}\text{C}$]hexadecyl sulphate and its main metabolite, [$4\text{-}^{14}\text{C}$]butyric acid 4-sulphate, from 15 min to 6 h after an oral dose of [$1\text{-}^{14}\text{C}$]hexadecyl sulphate erythromycin salt. The compounds were separated by t.l.c. The top plasma concentrations of hexadecyl sulphate were reached 30–60 min after an oral dose of 14.4 mg/kg. Then 6 h later the plasma hexadecyl sulphate concentrations had decreased to about one-tenth of the peak values, which reflects relatively rapid elimination of the drug. Comparison of hexadecyl sulphate concentrations in plasma after administration of its sodium or erythromycin salts by different routes is shown in Table 2. Fig. 2 shows the concentrations of [$1\text{-}^{14}\text{C}$]hexadecyl sulphate and its main metabolite, [$4\text{-}^{14}\text{C}$]butyric acid 4-sulphate, in plasma of human subjects. It can be seen that there is a marked difference in the two individuals, apparently due to poor absorption of the drug in subject D.J.D. After ingestion of [$1\text{-}^{14}\text{C}$]hexadecyl sulphate erythromycin salt, human plasma radioactivity is almost entirely due to the presence of labelled hexadecyl sulphate.

Table 1. Recoveries of excreted radioactivities after administration of labelled hexadecyl sulphate salts to rats, dogs and humans
Details are given in the text.

Species	Group of	Route of administration	Dose	Sample collection (h)	% of administered dose recovered		
					Urine	Faeces	CO ₂
Rat*	Group of 6	Oral	Hexadecyl [³⁵ S]sulphate erythromycin salt, 14.4 mg/kg	0-96	94	5	—
	Group of 5	Oral	[1- ¹⁴ C]Hexadecyl sulphate erythromycin salt, 14.4 mg/kg	0-72	87	4	5
Dog	no. 1	Intravenous	Hexadecyl [³⁵ S]sulphate	0-72	83	3	—
	no. 2	Intravenous	sodium salt, 2.9 mg/kg	0-72	92	—	—
	no. 4	Intravenous	[1- ¹⁴ C]Hexadecyl sulphate trimethylammonium salt, 4.4 mg/kg	0-48	81	4	—
	no. 6	Oral	Hexadecyl [³⁵ S]sulphate	0-72	50	41	—
	no. 7	Oral	erythromycin salt, 14.4 mg/kg	0-72	54	33	—
	no. 8	Oral	[1- ¹⁴ C]Hexadecyl sulphate	0-72	50	40	—
	no. 9	Oral	erythromycin salt, 14.4 mg/kg	0-72	79	12	—
Human	N.J.V.	Oral	[1- ¹⁴ C]Hexadecyl sulphate	0-72	80	7	—
	D.J.D.	Oral	erythromycin salt, 250 mg	0-118	20	73	—

* Mean values reported.

Fig. 1. Plasma concentration of labelled hexadecyl sulphate and its main metabolite, butyric acid 4-sulphate, after oral administration of [1-¹⁴C]hexadecyl sulphate or hexadecyl [³⁵S]sulphate erythromycin salts to dogs

The dose was 14.4 mg/kg. These compounds were separated and quantified by silica-gel t.l.c. in solvent A. ●, ○, Concn. of hexadecyl sulphate (µmol/l); ▲, △, concn. of butyric acid 4-sulphate (µmol/l). ○, △, dog 1; ●, ▲, dog 2.

Faeces. Although the faecal excretion of orally administered labelled dose was negligible in the rat, dogs excreted considerable amounts of radioactivity in the faeces. Further, the elimination of the labelled dose in faeces was rather slow in the dog. The 24-48 h faeces contained about 30 % of the orally administered ³⁵S dose (dog 6) and 39 % of the ¹⁴C dose (dog 8). The urinary elimination of radioactive products from 24 to 48 h was relatively small, thus assuring less contamination of faeces. The metabolite patterns of these two faecal samples were determined by t.l.c. The results revealed that in the dog all of the faecal radioactivity was due to unmetabolized labelled hexadecyl sulphate. Absorption of orally administered erythromycin salt of [1-¹⁴C]hexadecyl sulphate proceeded differently in the two human subjects used in the experiment. During the 0-48 h time-interval, the drug had been relatively well absorbed in subject N.J.V. but poorly absorbed in subject D.J.D. Analysis of the faecal excreta by t.l.c. in two solvent systems revealed that all the faecal radioactivity was due to unmetabolized [1-¹⁴C]hexadecyl sulphate.

Urine. The urinary excretion of the labelled dose occurred primarily during the first 24 h in rat, dog and man. Therefore 0-24 h urine samples were used for the determination of metabolic patterns of the drug. These patterns are presented for the rat and dog in Fig. 3.

Identification of labelled metabolites in urine

Identification of inorganic sulphate. The work on the metabolism of orally administered hexadecyl [³⁵S]-sulphate salts showed that the ester undergoes some desulphation. Freeze-dried urine residues from both

Table 2. Hexadecyl sulphate concentrations ($\mu\text{g/ml}$) in dog plasma after administration of labelled hexadecyl sulphate salts to the animals by different routes

Details are given in the text.

Dose ...	Hexadecyl [^{35}S]sulphate sodium salt, 2.9 mg/kg intravenously		Hexadecyl [^{35}S]sulphate erythromycin salt, 14.4 mg/kg orally		[1- ^{14}C]Hexadecyl sulphate erythromycin salt, 14.4 mg/kg orally	
Time after dose	Dog 1	Dog 2	Dog 6	Dog 7	Dog 8	Dog 9
1 min	34.5	35.3	—	—	—	—
15 min	9.4	9.0	0.30	0.12	0.06	0.02
30 min	4.3	4.8	1.22	2.45	3.81	3.13
1 h	1.4	1.5	1.29	2.00	4.50	6.40
2 h	0.23	0.31	0.72	1.06	1.91	2.32
4 h	0.09	0.03	0.36	0.56	0.70	0.53
6 h	0.02	0.06	0.19	0.33	0.25	0.29

Fig. 2. Plasma concentration of [1- ^{14}C]hexadecyl sulphate and its main metabolite, [4- ^{14}C]butyric acid 4-sulphate, after oral administration of 250 mg of [1- ^{14}C]hexadecyl sulphate erythromycin salt to two humans

These compounds were separated and quantified by silica-gel t.l.c. in solvent A. (a) Subject N.J.V.; (b) subject D.J.D. ●, Concn. of [1- ^{14}C]hexadecyl sulphate ($\mu\text{mol/l}$); ○, concn. of [4- ^{14}C]butyric acid 4-sulphate ($\mu\text{mol/l}$).

the rat and dog contained 16–24% of the total ^{35}S -labelled dose which was not extractable with boiling methanol. From a corresponding ^{14}C -labelled dose, only 2–3.5% was insoluble in methanol. It was concluded that the difference in the amounts of the two labels in the urine residues was due to the presence of inorganic sulphate in rat urine. Inorganic [^{35}S]sulphate content in the rat and dog urine was determined directly by precipitation (as $\text{Ba}^{35}\text{SO}_4$) with BaCl_2 solution. Values ranging from 14.2 to 22.0% were obtained with both dog and rat urine. These values compare favourably with the 17% inorganic [^{35}S]sulphate content in rat urine resulting from the metabolism of dodecyl [^{35}S]sulphate as reported by Denner *et al.* (1969).

Identification of butyric acid 4-sulphate. In the rat and dog the main metabolite (R_f 0.44–0.53 in solvent

system A) contained both ^{14}C and ^{35}S labels. This compares favourably with the R_f (0.45) of 4-hydroxybutyric acid sulphate ester, which is the main metabolite of dodecyl sulphate in the rat according to Denner *et al.* (1969). They established the identity of butyric acid 4-sulphate, among other ways, by refluxing it in HCl , which converted it into γ -butyrolactone. This, in turn, was identified by g.l.c.

We have established the identity of the main metabolite of hexadecyl sulphate in dog and rat urine in a similar way. The metabolites in the urine samples were separated by preparative t.l.c. on silica gel in solvent system A. The plates were then sequentially scraped in 0.5 cm sections into vials. Each section was extracted with 20 ml of methanol, portions of which were assayed for radioactivity. The fractions comprising the peak of the main metabolite were then

Fig. 3. T.l.c. of labelled hexadecyl sulphate metabolites after oral administration of $[1-^{14}\text{C}]$ hexadecyl sulphate or hexadecyl $[^{35}\text{S}]$ sulphate erythromycin salts to (a) rats and (b) dogs

The dose was 14.4 mg/kg. Metabolites were separated on silica-gel plates in solvent A as described in the text. ●, Metabolic patterns of $[1-^{14}\text{C}]$ hexadecyl sulphate; ○, metabolic patterns of hexadecyl $[^{35}\text{S}]$ sulphate. I, Inorganic sulphate; II, glycollic acid sulphate; III, unknown; IV, butyric acid 4-sulphate; V, hexadecyl sulphate and γ -butyrolactone.

Fig. 4. G.l.c. identification of $[1-^{14}\text{C}]$ hexadecyl sulphate metabolites in dog urine

(a) γ - $[^{14}\text{C}]$ Butyrolactone derived from $[4-^{14}\text{C}]$ butyric acid 4-sulphate on a 10% diethylene glycol adipate column; (b), silylated $[2-^{14}\text{C}]$ glycollic acid derived from $[2-^{14}\text{C}]$ glycollic acid sulphate on a 20% neopentyl glycol succinate column. Radioactive fractions are shown by shaded areas. Experimental details are given in the text.

pooled and evaporated *in vacuo*. To the residue was added 1.6mg of unlabelled potassium butyrate 4-sulphate as a marker and carrier, and the mixture was refluxed for 20h in 15ml of 1M-HCl. The solution was then shaken with 4 × 20ml of benzene, the pooled benzene solutions were dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to about 0.1–0.2ml. A solution of about 2mg of authentic γ -butyrolactone in benzene was treated the same way.

Fig. 4(a) demonstrates a g.l.c. tracing for γ -butyrolactone derived from the potassium butyrate 4-sulphate marker and the main metabolite in dog urine. The γ -butyrolactone marker and radioactivity peak emerged at the same time, after 12min, from the 10% diethylene glycol adipate column at 110°C. The authentic γ -butyrolactone (Aldrich Chemical Co.) had a retention time of 12½min. Similar results were obtained with rat urine on a diethylene glycol adipate column. On a 20% neopentyl glycol succinate column at 130°C the retention time of γ -butyrolactone was 12½min and its peak coincided again with the radioactivity peaks derived from dog, rat and human urine. The recoveries of injected radioactivities on both columns were 100%. Thus it can be concluded that the main metabolite of hexadecyl sulphate in the urine of rat, dog and man was butyric acid 4-sulphate.

Identification of γ -butyrolactone. Fig. 3 shows that the tracings of ¹⁴C and ³⁵S labels closely followed each other except in two zones. The first zone, with R_F 0.05, had a ³⁵S peak representing inorganic sulphate and naturally no ¹⁴C radioactivity was expected here. The ³⁵S-labelled peak was absent when a methanol extract of freeze-dried urine was chromatographed. The second zone, with R_F in the range 0.7–0.8, contained a relatively small ³⁵S peak which was overshadowed by a bigger ¹⁴C peak. Hexadecyl sulphate and γ -butyrolactone chromatographed in this zone. Since separation of these two substances in solvent system A was not obtained, solvent system B was used for quantifications of these two compounds. The presence of ¹⁴C-labelled γ -butyrolactone in rat and dog urine, after oral administration of [1-¹⁴C]-hexadecyl sulphate, was verified by the combination of preparative t.l.c. and g.l.c. techniques. The corresponding zones on silica-gel plates with R_F values of 0.65–0.85 in solvent system A were extracted with methanol as described above. The methanol was evaporated *in vacuo* and the residue was dissolved in 0.1ml of benzene containing 1mg of γ -butyrolactone as a carrier. Then 10 μ l of the benzene solution was injected into the gas chromatograph. The radioactivity peak and the tracing of γ -butyrolactone peak emerged at the same time, both having a retention time of 12½min on a 10% diethylene glycol adipate column at 110°C.

In a separate experiment hexadecyl [³⁵S]sulphate metabolites were isolated from dog urine by preparative t.l.c. on silica gel with solvent A. Fractions with

R_F values 0.45–0.55 were extracted with methanol as described above. The methanol extract did not contain any inorganic [³⁵S]sulphate as determined by precipitation with BaCl₂. However, after acid hydrolysis, all the radioactivity in the sample could be precipitated as Ba³⁵SO₄, indicating that the main metabolite appeared as the ester sulphate in dog urine.

Identification of glycollic acid sulphate. Fig. 3 shows that there was a difference between the metabolic patterns of hexadecyl sulphate in urine of rat and dog. Silica-gel thin-layer plates, developed in solvent system A, separated a metabolite (R_F 0.20) from dog urine which contained about 20% of the total dog urinary radioactivity. This metabolite was totally absent from rat urine. The R_F 0.20 metabolite in dog urine contained both the ¹⁴C and ³⁵S labels, which suggested the presence of an intact sulphate ester linkage. It was decided to hydrolyse this suspected sulphate ester linkage and then prepare derivatives of the liberated alcohol, suspected to be glycollic acid. For that purpose a silica-gel preparative thin-layer plate was spotted with 0–24h urine from a dog, which had been given orally [1-¹⁴C]hexadecyl sulphate erythromycin salt, and developed in solvent system A. Fractions with R_F values between 0.20 and 0.25 were extracted with methanol as described above, pooled and the methanol was evaporated *in vacuo* at 25°C. The residue was dissolved in 10ml of 1M-HCl, refluxed for 20h and the solution was freeze-dried. The residue after freeze-drying was dissolved in 5ml of water, of which 1.8ml was taken for g.l.c. determination, 3ml was used for isotope-dilution experiments and 0.2ml was used for rechromatography on silica-gel plates.

(a) T.l.c. When the hydrolysed material was rechromatographed on silica-gel plates, the radioactive zones (R_F 0.43 in solvent A and R_F 0.10 in solvent B) coincided with the mobilities of authentic [1-¹⁴C]-glycollic acid reference standard in these solvent systems.

(b) Isotope-dilution experiments. To the 3ml of water solution containing the suspected [2-¹⁴C]-glycollic acid was added 600mg of unlabelled glycollic acid. The solution was neutralized with 1M-KOH to pH5 and divided into three equal parts of about 4ml each. To one was added 0.2g of *p*-nitrobenzyl bromide, to another 0.2g of *p*-bromophenacyl bromide and to the third 0.2g of *p*-phenylphenacyl bromide (McElvain, 1949). The mixtures were refluxed and enough ethanol was added to ensure a homogeneous boiling solution. After boiling for 1h, the solutions were refrigerated at 4°C overnight. The separated esters were recrystallized from aq. 70% (v/v) ethanol and dried *in vacuo* over CaCl₂. About 2–11 mg was accurately weighed out into 10ml of scintillation solution for radioactivity assay. The rest was recrystallized twice more from aq. 95% (v/v) ethanol and dried *in vacuo* over CaCl₂; each time an accurately weighed

Table 3. *Isotope-dilution assay of [2-¹⁴C]glycollic acid esters*

Details are given in the text.

Ester	First recrystallization from 70% (v/v) ethanol		Second recrystallization from 95% (v/v) ethanol		Third recrystallization from 95% (v/v) ethanol	
	M.p. (°C)	Sp. radioactivity (c.p.m./mmol)	M.p. (°C)	Sp. radioactivity (c.p.m./mmol)	M.p. (°C)	Sp. radioactivity (c.p.m./mmol)
<i>p</i> -Nitrobenzyl	108	9220	108	8710	108	7920
<i>p</i> -Bromophenacyl	128–135	7480	138	7700	138	7590
<i>p</i> -Phenylphenacyl	117–120	7290	123	7740	123	8050

portion was kept for radioactivity assay and also some material for melting-point determination. The results, expressed in c.p.m./mmol, are shown in Table 3. Within the limits of experimental error, esters of constant molecular radioactivity were obtained.

(c) G.l.c. To the 1.8 ml of water solution containing suspected ¹⁴C-labelled glycollic acid was added 1 mg of unlabelled glycollic acid and the solution was freeze-dried and dried *in vacuo* over P₂O₅. The residue was dissolved in 100 μl of dimethylformamide, and 50 μl of bis-(trimethylsilyl)acetamide, a highly reactive trimethylsilyl donor (Klebe *et al.*, 1966), was added. The solution was kept for 1–3 min at 50°C and 2 μl portions of it were injected into the chromatographic column containing 20% neopentyl glycol succinate on Chromosorb W at 100°C. Fig. 4 shows that the trimethylsilylated glycollic acid marker peak had a retention time of 9½ min and the fraction with the highest radioactivity emerged between 9 and 10 min. Similarly on a diethylene glycol adipate column at 110°C the trimethylsilylated glycollic acid marker peak coincided with the peak of radioactivity. In both cases the radioactive peak contained more than 80% of the injected radioactivity.

In a separate experiment hexadecyl [³⁵S]sulphate metabolites were isolated from dog urine by preparative t.l.c. on silica gel with solvent A. Fractions with *R_F* values 0.18–0.25 were extracted with methanol as described above. The content of inorganic [³⁵S]-sulphate in this extract was negligible as determined by precipitation with BaCl₂. However, after acid hydrolysis, all the radioactivity in the sample could be precipitated as Ba³⁵SO₄, indicating that the compound appeared as the ester sulphate in dog urine.

Composition of metabolites of hexadecyl sulphate in urine of rat, dog and man

Table 4 summarizes the *R_F* values of t.l.c. on silica gel for the identified hexadecyl sulphate metabolites and for some related compounds in two solvent systems. These *R_F* values were used for the identification of radioactive peaks on silica-gel chromatograms obtained with rat, dog and human urines. Table 5

Table 4. *R_F values of some hexadecyl sulphate metabolites and related compounds on silica-gel t.l.c. in two solvent systems*

Compound	Solvent A	Solvent B
Inorganic sulphate	0.0–0.05	0.0
Glycollic acid sulphate	0.20–0.26	0.10
Glycollic acid	0.40–0.45	0.10–0.15
Butyric acid 4-sulphate	0.44–0.53	0.10
γ-Butyrolactone	0.75–0.85	0.90–0.96
Hexadecyl sulphate	0.75–0.85	0.44–0.50
Hexadecan-1-ol	0.93–0.96	0.93–0.96

gives the numerical values for hexadecyl sulphate urinary metabolites in rat after oral administration of erythromycin [1-¹⁴C]hexadecyl sulphate and erythromycin hexadecyl [³⁵S]sulphate. Table 5 also compares in the dog the metabolism of orally administered erythromycin salt and intravenously administered trimethylammonium salt of [1-¹⁴C]hexadecyl sulphate with the metabolism of erythromycin hexadecyl [³⁵S]sulphate (given orally) and sodium hexadecyl [³⁵S]sulphate (given intravenously). The metabolic patterns of all three salts of hexadecyl sulphate were quite similar and were not affected by the route of administration. Composition of orally administered erythromycin [1-¹⁴C]hexadecyl sulphate metabolites in human excreta is also given in Table 5.

Discussion

For the separation of metabolites silica-gel t.l.c. was chosen, since silica gel has high capacity for inorganic salts (Donike *et al.*, 1973). Thus urine could be applied to silica-gel plates without the previous removal of salts. Labelling the hexadecyl sulphate molecule either with ¹⁴C or ³⁵S enabled us to follow the fate of the alcohol part or the sulphuric acid part of the ester molecule. When hexadecyl [³⁵S]sulphate was administered to rats and dogs, most of the radioactivity was excreted in urine within 24 h, approx. 20% being eliminated as inorganic [³⁵S]sulphate. After administration of [1-¹⁴C]hexadecyl sulphate,

Table 5. *Urinary metabolic patterns of labelled hexadecyl sulphate in dog, rat and human*

Metabolites were separated by t.l.c. on silica-gel plates developed in two solvent systems. Details of the solvent systems are given in the text. Results are given as mean values $\pm$ s.e.m. for dog and rat. Numbers in parentheses refer to numbers of determinations in each group. Human excretion data include faeces.

Compound	Dog				Rat		Human			
	% of ^{14}C chromatographed		% of ^{35}S chromatographed		[1- ^{14}C]Hexadecyl sulphate, 14.4 mg/kg orally (5) chromatographed (% of ^{14}C)	Hexadecyl [^{35}S]sulphate, 14.4 mg/kg orally (5) (% of ^{35}S chromatographed)	[1- ^{14}C]Hexadecyl sulphate erythromycin salt, 250mg orally (% of ^{14}C recovered)		Subject N.J.V.	
	...		...				Subject D.J.D.	Subject N.J.V.		
	Dose	[1- ^{14}C]Hexadecyl sulphate erythromycin salt, 14.4 mg/kg orally (6)	[1- ^{14}C]Hexadecyl sulphate tri-methylammonium salt, 4.4 mg/kg intravenously (3)	Hexadecyl [^{35}S]sulphate erythromycin salt, 14.4 mg/kg orally (3)	Hexadecyl [^{35}S]sulphate sodium salt, 2.9 mg/kg intravenously (4)			Urine	Faeces	Urine
Inorganic sulphate	0	0	22.5 $\pm$ 1.7	24.1 $\pm$ 0.7	0	19.7 $\pm$ 1.1	0	0	0	0
Glycollic acid sulphate	21.0 $\pm$ 1.0	17.8 $\pm$ 1.3	23.6 $\pm$ 0.6	18.1 $\pm$ 1.2	0	0	4.6	0	5.0	0
Unknown	6.6 $\pm$ 0.5	8.4 $\pm$ 0.6	7.2 $\pm$ 0.4	5.1 $\pm$ 0.5	3.6 $\pm$ 0.4	4.9 $\pm$ 0.7	0	0	4.1	0
Butyric acid 4-sulphate	54.3 $\pm$ 0.7	58.7 $\pm$ 0.7	43.2 $\pm$ 1.0	47.8 $\pm$ 1.2	85.7 $\pm$ 0.9	73.8 $\pm$ 1.3	16.0	0	78.2	0
Hexadecyl sulphate*	6.9 $\pm$ 0.9	6.9 $\pm$ 0.2	3.4 $\pm$ 0.9	5.0 $\pm$ 0.6	6.1 $\pm$ 0.2	1.6 $\pm$ 0.2	0.3	78.2	0	7.5
γ -Butyrolactone*	11.1 $\pm$ 0.4	9.9 $\pm$ 2.0	0	0	3.2 $\pm$ 0.9	0	0.9	0	5.2	0

* Separated in solvent B.

* Separated in solvent B.

the excretion of inorganic sulphate could naturally not be demonstrated, but the elimination of 3–7% of the ^{14}C dose in the form of $^{14}\text{CO}_2$ in rats could be measured. Likewise the urinary excretion of γ -butyrolactone, which comprised 3% of the total urinary radioactivity in rat and up to 10% in dog, could be measured with ^{14}C label; with the ^{35}S label it would have been completely missed.

The metabolic fate of hexadecyl sulphate was independent of the cation part of the salt, since sodium, trimethylammonium and erythromycin salts produce similar urinary metabolic patterns. The metabolic fate of hexadecyl sulphate was not affected by the route of administration.

The classical experiments by Verkade (Verkade *et al.*, 1933; Verkade & van der Lee, 1934a,b; Verkade *et al.*, 1935) suggested ω -oxidation as an alternative pathway to the direct β -oxidation of fatty acids in mammalian tissues. The importance of this pathway is apparent for compounds in which β -oxidation is blocked by substituents at the α or β carbon: the molecule could be ω -oxidized and then β -oxidized from the unsubstituted end (Flaschen-träger *et al.*, 1934; Bergström *et al.*, 1954). Enzyme systems involved in the ω -oxidation of fatty acids and their derivatives have been found in the liver microsomal fraction of guinea pigs (Wakabayashi & Shimazono, 1963) and of rats (Preiss & Bloch, 1964). The metabolism of potassium dodecyl [^{35}S]sulphate in rat seems to go along an analogous pathway according to Denner *et al.* (1969). They suggested that the metabolism is initiated by ω -oxidation of the terminal methyl group. The resulting substituted fatty acid could then undergo oxidation, analogous to normal β -oxidation, with the successive elimination of C_2 fragments. They found that the final degradation product of dodecyl sulphate in rat was butyric acid 4-sulphate and that for some reason further β -oxidation of this ester to glycollic acid sulphate did not occur. The results of the present investigation of the metabolism of hexadecyl sulphate confirm the conclusion of Denner *et al.* (1969) that sulphuric acid esters of long-chain fatty alcohols are metabolized in rat to the sulphate ester of 4-hydroxybutyric acid. In the present study the major metabolite of hexadecyl sulphate in dog and man has also been found to be the sulphate ester of 4-hydroxybutyric acid. However, I found that although further β -oxidation of butyric acid 4-sulphate in the rat did not occur, some of the 4-hydroxybutyric acid sulphate ester had obviously been further metabolized in the dog and human, so that of the total recovered radioactivity about 20% was associated with glycollic acid sulphate in dog urine and 5% in human urine. Why the process, by which hexadecyl sulphate is shortened, should stop or slow down at the stage of butyric acid 4-sulphate is not readily apparent.

The following colleagues at Abbott Laboratories have made valuable contributions to this investigation, which I here gratefully acknowledge. Labelled erythromycin salts of hexadecyl sulphate were synthesized by Dr. G. J. Ikeda and Mr. A. Alter. Mr. C. B. Estep, Mr. W. W. Hunter and Mr. C. J. Contreras dosed the animals and collected the samples. Dr. R. J. Powers directed the clinical investigation.

References

- Bergström, S., Borgström, B., Tryding, N. & Westöö, G. (1954) *Biochem. J.* **58**, 604–608
- Denner, W. H. B., Olavesen, A. H., Powell, G. M. & Dodgson, K. S. (1969) *Biochem. J.* **111**, 43–51
- Donike, M., Suberg, H. & Jaenicke, L. (1973) *J. Chromatogr.* **85**, 9–17
- Dumas & Peligot (1836) *Ann. Pharmacol.* **19**, 290–296
- Flaschenträger, B., Bernhard, K., Lövenberg, C. & Schläpfer, M. (1934) *Hoppe-Seyler's Z. Physiol. Chem.* **225**, 157–167
- Klebe, J. F., Finkbeiner, H. & White, D. M. (1966) *J. Amer. Chem. Soc.* **88**, 3390–3395
- Lloyd, A. G. (1961) *Biochem. J.* **80**, 572–578
- McElvain, S. M. (1949) *The Characterization of Organic Compounds* pp. 185, 187, The Macmillan Co., New York
- Preiss, B. & Bloch, K. (1964) *J. Biol. Chem.* **239**, 85–88
- Verkade, P. E. & van der Lee, J. (1934a) *Biochem. J.* **28**, 31–40
- Verkade, P. E. & van der Lee, J. (1934b) *Hoppe-Seyler's Z. Physiol. Chem.* **225**, 230–244
- Verkade, P. E., Elzas, M., van der Lee, J., de Wolff, H. H., Verkade-Sandbergen, A. & van der Sande, D. (1933) *Hoppe-Seyler's Z. Physiol. Chem.* **215**, 225–227
- Verkade, P. E., van der Lee, J. & Holwerda, K. (1935) *Hoppe-Seyler's Z. Physiol. Chem.* **234**, 21–26
- Wakabayashi, K. & Shimazono, N. (1963) *Biochim. Biophys. Acta* **70**, 132–142