The Structure of the Glucuronide of Sulphadimethoxine Formed in Man

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1. The major metabolite of 2,4-dimethoxy-6-sulphanilamidopyrimidine (sulphadimethoxine) in urine in man is a non-reducing glucuronide, which has been isolated and characterized as its *S*-benzylthiuronium salt. 2. The same compound was made synthetically by standard methods from sodium sulphadimethoxine and methyl 2,3,4-tri-*O*-acetyl-1-bromoglucuronate. 3. On hydrolysis with acid, the glucuronide yielded sulphanilic acid, glucuronic acid and barbituric acid, and with β -glucuronidase it slowly yielded sulphadimethoxine and glucuronic acid. 4. Evidence based on infrared spectra and other data showed that the urinary and synthetic glucuronide was 1-deoxy-1-[*N*^{1'}-(2'',4''-dimethoxypyrimidin-6''-yl)sulphanilamido- β -D-glucosid]uronic acid or sulphadimethoxine *N*¹-glucuronide. 5. *N*¹-Methyl- and *N*^{ring}-methyl derivatives of sulphadimethoxine and 4-methoxy-6-sulphanilamidopyrimidine were prepared and their infrared and ultraviolet spectra determined for comparison.

Sulphadimethoxine (2,4-dimethoxy-6-sulphanilamidopyrimidine; Madribon) was synthesized by Bretschneider & Kloetzer (1955) and its antibacterial properties were reported by Semenitz (1954) and Schnitzer, DeLorenzo & Grunberg (1959). It is regarded as a long-acting sulphonamide of low toxicity (Randall, Bagdon & Engelberg, 1959), valuable in the treatment of respiratory and urinary infections. It has the advantage that its major metabolite in man is a relatively water-soluble glucuronide, whereas many other sulphonamides tend to form relatively insoluble acetyl derivatives that could produce renal damage. Previous attempts to isolate and characterize this glucuronide have been made (e.g. Koechlin, Kern & Engelberg, 1959), but only a crude product was obtained which contained sulphonamide and glucuronic acid in equimolecular amounts. This product was not hydrolysed by β -glucuronidase but with acid it gave sulphanilic acid. In a preliminary account of this glucuronide (Bridges, Kibby & Williams, 1964) it was suggested that glucuronic acid was attached to the *N*¹-position of sulphadimethoxine. The evidence for this is given in the present paper.

EXPERIMENTAL

Materials and synthesis of reference compounds

6-Amino-2,4-dimethoxypyrimidine, m.p. 150–152°, sulphadimethoxine, m.p. 200–201°, 2-methoxy-4-sulphanilamidopyrimidine, m.p. 186–187°, 5-methoxy-4-sulphanil-

amidopyrimidine, m.p. 200–201°, 2,5-dimethoxy-4-sulphanilamidopyrimidine, m.p. 190–193°, 4-methoxy-2-methyl-6-sulphanilamidopyrimidine (sulphamethomidine), m.p. 172°, 2,4-dimethyl-6-sulphanilamidopyrimidine (sulphasomidine), m.p. 240–241°, and 4,5-dimethoxy-6-sulphanilamidopyrimidine (sulphorthodimethoxine), m.p. 194–197°, were given by Roche Products Ltd., Welwyn Garden City, Herts.; 4-methoxy-6-sulphanilamidopyrimidine, m.p. 201–203°, was given by Imperial Chemical Industries (Pharmaceutical Division), Alderley Park, Cheshire; *N*⁴-acetylsulphanilyl chloride m.p. 149° was given by May and Baker Ltd., Dagenham, Essex; 4,6-dihydroxypyrimidine, m.p. 230°, was purchased from Koch-Light Ltd., Colnbrook, Bucks.

Synthesis of sulphadimethoxine N¹-glucuronide. The method of Okumura (1962) was modified. Sodium sulphadimethoxine (6.0 g.) and methyl 2,3,4-tri-*O*-acetyl-1-bromoglucuronate (7.7 g.; m.p. 106–109°; Bollenback, Long, Benjamin & Lindquist, 1955) were dissolved in a mixture of water (12 ml.) and acetone (50 ml.) and the solution was kept at 37° for 4 hr. The dark-red solution was kept at 0° overnight and then treated with propan-2-ol (25 ml.) followed by water (200 ml.), whereby the triacetyl methyl ester of sulphadimethoxine *N*¹-glucuronide was precipitated and then filtered and dried at the pump to give a buff powder (6.5 g.), m.p. 110–120°. The crude ester in acetone (5 ml.) was passed through a column (2.5 cm. $\times$ 25 cm.) of activated alumina (type H; 100–200 mesh) (P. Spence Ltd., Widnes, Lancs.) and eluted with acetone (300 ml.). The first fraction (100 ml.) was collected and evaporated to dryness. The product was recrystallized from propan-2-ol to give white platelets of the pure methyl 2,3,4-tri-*O*-acetyl-1-deoxy-1-[*N*^{1'}-(2'',4''-dimethoxypyrimidin-6''-yl)sulphanilamidoglucosid]uronate, m.p. 154–155° and $[\alpha]_D^{20} + 87^\circ$ (c 1 in methanol) (Found: C, 47.4; H, 5.2; N, 8.6. C₂₅H₃₀N₄O₁₃S requires C, 47.9; H, 4.8; N, 8.9%).

The above ester (1.84 g.) in methanol (50 ml.) was treated with 0.5N-barium methoxide in methanol (5 ml.). After the mixture had been kept for 3 days at 0°, 2N-H₂SO₄ (1.25 ml.) and water (25 ml.) were added. The BaSO₄ that separated was removed and the solution passed through a column (2.5 cm. × 10 cm.) of Amberlite CG-120 (H⁺ form; 100–200 mesh) (British Drug Houses Ltd., Poole, Dorset). The column was washed with water (10 ml.) and eluted with 2N-NH₃ solution (150 ml.) followed by water (100 ml.). The combined eluates were evaporated to dryness *in vacuo* at 45° to give a solid (1.1 g.), which was then dissolved in methanol (10 ml.). The addition of propan-2-ol to the solution precipitated the ammonium salt of the glucuronide, which was recrystallized from methanol. Ammonium 1-deoxy-1-[N¹-(2',4'-dimethoxypyrimidin-6"-yl)sulphanilamidoglucosid]uronate (0.7 g.) was obtained as clusters of small prisms, m.p. 150–160° (decomp.) and $[\alpha]_D^{25} + 4.5^\circ$ (c 5 in water) (Found: C, 40.4; H, 5.2; N, 12.9; S, 5.8; loss at 2 mm. Hg at room temp., 4.4. C₁₈H₂₅N₅O₁₀S·1.5H₂O requires C, 40.7; H, 5.3; N, 13.2; S, 6.0; H₂O, 5.1%). This compound did not reduce Benedict's solution or Fehling's solution.

The salt (90 mg.) was dissolved with warming in a saturated aqueous solution of *S*-benzylthiuronium chloride (5 ml.). The solution was filtered (charcoal) and allowed to crystallize. *S*-Benzylthiuronium 1-deoxy-1-[N¹-(2',4'-dimethoxypyrimidin-6"-yl)sulphanilamidoglucosid]uronate (42 mg.) had m.p. 178–179° and $[\alpha]_D^{25} - 13^\circ$ (c 1 in water) (Found: C, 46.5; H, 4.8; N, 12.3; S, 10.3; loss at 110°, 2.8%. C₂₆H₃₂N₆O₁₀S₂·H₂O requires C, 46.6; H, 5.1; N, 12.5; S, 9.6; H₂O, 2.7%). This salt was also non-reducing.

Attempts to prepare sulphadimethoxine N¹-glucuronide by fusing sodium sulphadimethoxine with glucuronic acid or by heating concentrated aqueous solutions of glucuronic acid and sulphadimethoxine with H₂SO₄ according to the method of Tsukamoto, Yoshimura & Tatsumi (1963a) for the preparation of meprobamate *N*-glucuronide, were unsuccessful.

Methylation of sulphadimethoxine. The drug (2.5 g.) was mixed with ether (2 ml.), and an excess of diazomethane in ether was distilled into the stirred mixture [cf. Shepherd, Bratton & Blanchard (1942) on the methylation of sulphapyridine]. After being kept at room temperature overnight, the mixture was warmed to remove excess of diazomethane and filtered. The solid residue was now extracted with ether containing 3% (v/v) of ethanol (2 × 100 ml.) and the extracts were evaporated to dryness *in vacuo*. The white crystalline residue was dissolved in methanol and, on keeping, N¹-methylsulphadimethoxine (0.95 g.) crystallized (m.p. 150–152°). The residue from the extraction was shaken with 10% (w/v) NaOH to remove unchanged drug and the residue from this was heated with 6N-acetic acid (50 ml.) for 5 min., filtered hot and the residue retained. The filtrate, on cooling, yielded a mixture of methylated sulphadimethoxines from which N¹-methylsulphadimethoxine was removed by extraction with hot methanol m.p. 148–151° (yield, 0.25 g.). The two crops of N¹-methylsulphadimethoxine were combined and recrystallized from methanol. 2,4-Dimethoxy-6-(N¹-methylsulphanilamido)pyrimidine formed white needles, m.p. 151–152° (Found: C, 48.5; H, 5.0; N, 17.3; S, 10.3. C₁₂H₁₄N₄O₄S requires C, 48.1; H, 4.9; N, 17.3; S, 9.9%).

The residue from the acetic acid extraction and that from the second crop of N¹-methylsulphadimethoxine above were combined and washed with hot methanol to remove any

N¹-methyl isomer still present. This residue (0.5 g.) was now recrystallized from 6N-acetic acid and there was obtained 0.46 g. of small white plates, m.p. 169–173° (Found: C, 47.8; H, 4.8; N, 17.1; S, 9.9. C₁₃H₁₆N₄O₄S requires C, 48.1; H, 4.9; N, 17.3; S, 9.9%), which appears, from infrared spectra (see below), to be a mixture of the two N¹-methylsulphadimethoxines, i.e. 2,4-dimethoxy-1- and 2,4-dimethoxy-3-methyl-6-sulphanilamidopyrimidine. Attempts to separate these were not successful.

N⁴-Acetyl-N¹-methylsulphadimethoxine. (1) N¹-Methylsulphadimethoxine (0.5 g.) was dissolved with warming in acetic anhydride-pyridine (1:1, v/v) mixture. The solution was kept at room temperature for 4 hr. and then diluted with aq. 50% (v/v) ethanol (50 ml.) with warming to dissolve any separated material. On keeping, 6-(N⁴-acetyl-N¹-methylsulphanilamido)-2,4-dimethoxypyrimidine separated and was recrystallized from methanol. It formed small white plates (0.28 g.), m.p. 142–144° (Found: C, 49.1; H, 4.9; N, 15.2; S, 8.8. C₁₅H₁₈N₄O₅S requires C, 49.2; H, 4.9; N, 15.3; S, 8.7%).

(2) 2,4-Dichloro-6-methylaminopyrimidine, m.p. 184–186°, was synthesized from barbituric acid via 2,4,6-trichloropyrimidine according to the methods of Winkelman (1927) and Baddiley & Topham (1944). It (0.5 g.) was dissolved in hot methanol (10 ml.) and added to sodium methoxide in methanol (0.24 g. of Na in 5 ml. of methanol). The mixture was heated in a sealed tube for 5 hr. at 120°. The methanol was evaporated and the residue recrystallized from water to give 2,4-dimethoxy-6-methylaminopyrimidine (0.22 g.), m.p. 134–136° (Found: C, 49.5; H, 6.1; N, 25.1. C₇H₁₁N₃O₂ requires C, 49.7; H, 6.5; N, 24.9%). Brederick, Kupsch & Wieland (1959) prepared it by another route and gave m.p. 135–136°. 2,4-Dimethoxy-6-methylaminopyrimidine (0.1 g.) and *N*-acetylsulphanil chloride (0.2 g.) were dissolved in pyridine (0.5 ml.) and the solution was kept at room temperature for 24 hr. Water (5 ml.) was added and the solution warmed to dissolve the precipitate that formed. On standing, N⁴-acetyl-N¹-methylsulphadimethoxine separated and was recrystallized from methanol, m.p. 142–144° (yield, 47 mg.) (Found: C, 49.1; H, 5.0; N, 15.2; S, 8.9%). This sample was identical in all respects (mixed m.p. and infrared spectrum) with the sample obtained by acetylation of N¹-methylsulphadimethoxine.

Methylation of 4-methoxy-6-sulphanilamidopyrimidine. This compound was methylated as described above for sulphadimethoxine. 4-Methoxy-6-(N¹-methylsulphanilamido)pyrimidine gave white needles from methanol, m.p. 152° (Found: C, 48.9; H, 4.9; N, 19.0; S, 11.0. C₁₂H₁₄N₄O₃S requires C, 49.0; H, 4.8; N, 19.0; S, 10.9%). 4-Methoxy-N¹-methyl-6-sulphanilamidopyrimidine was recrystallized from 6N-acetic acid to give white platelets, m.p. 179–180°, of 4-methoxy-1- or 4-methoxy-3-methyl-6-sulphanilamidopyrimidine (Found: C, 49.0; H, 5.0; N, 18.9; S, 11.0. C₁₂H₁₄N₄O₃S requires C, 49.0; H, 4.8; N, 19.0; S, 10.9%). The material from its infrared spectrum and m.p. appeared to contain only one isomer, but the position of the methyl group was not determined.

6-(N⁴-Acetyl-N¹-methylsulphanilamido)-4-methoxy-pyrimidine. (1) 4-Methoxy-6-(N¹-methylsulphanilamido)pyrimidine (1.0 g.) was dissolved in acetic anhydride-pyridine (1:1, v/v) mixture and the solution kept at room temperature overnight. Treatment with ice-water gave crystals that were recrystallized from aq. 40% (v/v)

methanol to give white needles (0.68 g.) of 6-(*N*⁴-acetyl-*N*¹'-methylsulphanilamido)-4-methoxy-*N*-methylpyrimidine, m.p. 178–179° (Found: C, 49.7; H, 4.8; N, 16.5; S, 9.6. C₁₄H₁₆N₄O₄S requires C, 50.0; H, 4.8; N, 16.7; S, 9.5%).

(2) 4-Methoxy-6-methylaminopyrimidine, m.p. 82–83°, was synthesized from 4,6-dihydroxypyrimidine via 4,6-dichloropyrimidine (m.p. 64–65°) and 4-chloro-6-methylaminopyrimidine (m.p. 137–138°) according to the methods of Hull (1951) and Brown (1955). It (1.5 g.) was dissolved in pyridine (6 ml.) together with *N*-acetylsulphanilyl chloride (3 g.) and kept overnight at room temperature. On pouring into ice-water, crystals formed that on recrystallization from aq. 40% (v/v) methanol gave white needles, m.p. 178–179° (yield 1.2 g.) (Found: C, 50.0; H, 5.1; N, 16.5; S, 9.7%), identical in all respects with the sample obtained by acetylation of 4-methoxy-6-(*N*¹'-methylsulphanilamido)-pyrimidine. 6-(*N*⁴-Acetylsulphanilamido)-4-methoxy-*N*¹'-methylpyrimidine was obtained by treatment of 4-methoxy-6-(*N*¹'-methylsulphanilamido)-pyrimidine (0.2 g.) with acetic anhydride-pyridine (1:1, v/v) mixture. The compound was recrystallized from 6*N*-acetic acid to give 6-(*N*⁴-acetylsulphanilamido)-4-methoxy-1- or 6-(*N*⁴-acetylsulphanilamido)-4-methoxy-3-methylpyrimidine, m.p. 235–236° (Found: C, 49.8; H, 4.9; N, 16.5; S, 9.7. C₁₄H₁₆N₄O₄S requires C, 50.0; H, 4.8; N, 16.7; S, 9.5%). This compound

was shown to be different from the *N*⁴-acetyl-*N*¹-methyl isomer by infrared spectra, mixed m.p. and chromatography.

Sulphadimethoxine N⁴-glucuronide. Sulphadimethoxine (6.3 g.) dissolved in dimethylformamide (20 ml.) was mixed with a solution of sodium glucuronate (2.4 g.) in ethylene glycol (30 ml.). Acetic acid (0.5 ml.) was added to bring the pH to 3–4 and the solution was kept at 70° for 10 min. On keeping overnight at 0°, crystals (3.9 g.) separated, and these were filtered and washed with dimethylformamide, then acetone and finally ether. Attempts to purify the compound were made by dissolving it in 0.02*N*-NH₃ (10 ml.) and adding acetone (25 ml.). On keeping at 0° overnight, crystals separated and these were filtered and washed with acetone (yield, 2.1 g.), m.p. 149° (decomp.). The compound, which was expected to be sodium 1-deoxy-1-[*p*-(2',4'-dimethoxy-*pyrimidin*-6'-ylsulphamoyl)anilinoglucosid]uronate (sulphadimethoxine *N*⁴-glucuronide sodium salt), was very labile and was never obtained free of uncombined sulphadimethoxine. However, it was used as such for chromatographic purposes (see Table 1) and on paper it could easily be distinguished from sulphadimethoxine *N*¹-glucuronide. It reduced Benedict's solution and Fehling's solution readily on warming, and the ratio of sulphadimethoxine to glucuronic acid in the compound was 1.03.

Table 1. *Chromatography and colour reactions of sulphadimethoxine and related compounds*

Descending chromatography on Whatman no. 4 paper was used. The solvent systems employed were: *A*, butan-1-ol-aq. NH₃ (sp.gr.0.88)-water (10:1:1, by vol.); *B*, aq. 80% (v/v) ethanol; *C*, butan-1-ol-acetic acid-water (4:1:2, by vol.). Chromatograms were run for 6 hr. in solvents *A* and *C* and for 4 hr. in solvent *B*. Abbreviations: DSA, diazotized sulphanilic acid; DMAC, *p*-dimethylaminocinnamaldehyde; NR, naphtharesorcinol.

Compound	<i>R_F</i> values			Fluorescence in u.v. light (254 mμ)	Colour reactions		
	Solvent <i>A</i>	Solvent <i>B</i>	Solvent <i>C</i>		With DSA	With DMAC	With NR
Sulphadimethoxine	0.40	0.95	0.95	q*	None	Purple	None
<i>N</i> ⁴ -Acetylsulphadimethoxine	0.65	0.95	0.95	q*	None	None (purple overnight)†	None
Sulphadimethoxine <i>N</i> ⁴ -glucuronide sodium salt	0.00	0.25	—‡	q*	None	Purple (slowly)	Blue
Sulphadimethoxine <i>N</i> ¹ -glucuronide ammonium salt (synthetic)	0.15	0.55	0.80	q*	None	Purple	Blue-brown
Sulphadimethoxine glucuronide ammonium salt (from urine)	0.15	0.55	0.80	q*	None	Purple	Blue-brown
Glucuronic acid	0.00	0.37	0.23	q*	None	None	Blue
Glucuronolactone	0.03	0.65	0.39	q*	None	None	Blue
<i>N</i> ¹ -Methylsulphadimethoxine	0.83	0.82	0.85	q*	None	Purple	None
<i>N</i> ¹ -Methylsulphadimethoxines	Streaks in all solvents			q*	None	Purple	None
Sulphanilamide	0.62	0.72	0.77	Violet	None	Red	
Sulphanilic acid	0.11	0.57	0.32	Violet	None	Red	None
Barbituric acid	0.03	0.35	0.48	q*	None	None (purple overnight)†	None
<i>N</i> -Methylbarbituric acid	0.09	0.50	0.65	q*	None	None (purple overnight)†	None
4-Amino-3-hydroxybenzene- sulphonic acid	0.19	—	0.06	Violet	Deep orange	Red	None
4-Amino-2-hydroxybenzene- sulphonic acid	0.27	—	0.08	Violet	Orange-red	Red	None

* Quenches background fluorescence of paper.

† Purple spot on leaving paper overnight.

‡ Hydrolysed to free amine in this solvent.

Chromatography

The R_F values of sulphadimethoxine and other relevant compounds are shown in Table 1. The following tests were used.

***p*-Dimethylaminocinnamaldehyde test.** *p*-Dimethylaminocinnamaldehyde was dissolved in 2N-HCl (0.2 g./100 ml.) and diluted with ethanol (4 vol.). On spraying, aromatic amines showed up immediately as red-purple spots. Weak *N*-conjugates such as the N^4 -glucuronide did not react immediately but a purple spot developed within 1–5 min. N^4 -Acetyl conjugates, barbituric acid and *N*-methylbarbituric acid showed up as purple spots on keeping the chromatogram overnight.

Naphtharesorcinol test. The paper was sprayed with 1% (w/v) naphtharesorcinol in acetone to which 10% phosphoric acid (4:1, v/v) was added just before use. On heating at 140°, the N^4 -glucuronide showed up as a bright-blue spot. Both the synthetic N^1 -glucuronide and the biosynthetic glucuronide isolated from human urine gave very weak blue-brown spots.

Ferric chloride test. The phenols gave red, brown or green colours when sprayed with aq. 0.1% FeCl_3 solution.

Diazotized sulphanilic acid (Elliott, 1959). Phenols gave red-pink spots with this reagent.

Gibbs reagent (see Elliott, 1959). Certain phenols gave red-blue spots with this reagent.

Ultraviolet light. Chromatograms were examined under ultraviolet light (254 m μ) from a Hanovia Chromatolite lamp (Engelhard Industries Ltd., Slough, Bucks.). Some compounds such as sulphanilic acid and sulphanilamide showed a weak purple fluorescence, but others such as sulphadimethoxine quenched the background fluorescence of the paper and appeared as dark spots.

Infrared and ultraviolet spectra

These were determined with the Perkin-Elmer Infracord spectrophotometer and the Unicam SP.500 spectrophotometer respectively.

Quantitative estimations

Free and total aromatic amines. Free amine was determined by using the method of Bratton & Marshall (1939) directly. Total aromatic amine was estimated after hydrolysis of the sample with an equal volume of 2N-HCl in a water bath for 20 min. (recoveries were 99.5–100%).

Glucuronides. These were determined by the method of Paul (1951).

Isolation of sulphadimethoxine glucuronide from human urine

Two male volunteers each took 2 g. of sulphadimethoxine orally and their urine (3 l.) was collected for 24 hr. This was evaporated in 500 ml. batches under reduced pressure at 50° to a syrup (75 ml.). An equal volume of ethanol containing 5% (v/v) of 2N-NH₃ solution was added and the precipitated inorganic material was filtered off and discarded. A strongly basic anion-exchange resin column (2 cm. $\times$ 35 cm.) [De-Acidite FF (100–200 mesh; 2–3% cross-linked); British Drug Houses Ltd.] was loaded with the filtrate and washed

with water (500 ml.), and the washings were discarded. It was then eluted with a solution of SO₂ (prepared by bubbling the gas through water for 4 hr.) and the first 350 ml. fraction retained after the first appearance of the sulphite in the eluate. The fraction was degassed under reduced pressure and passed through a strongly acidic cation-exchange column (2.5 cm. $\times$ 25 cm.) [Amberlite IR-120 (H⁺ form; –16 + 25 mesh); British Drug Houses Ltd.]. After a washing with water (250 ml.) it was eluted with 2N-NH₃ solution and the first 300 ml. fraction retained after the first appearance of NH₃ in the eluate. The fraction was evaporated to dryness under reduced pressure at 45° to give 120 mg. of crude ammonium sulphadimethoxine glucuronide. This material was about 40% pure, the impurities being mainly amino acids. The salt (60 mg.) in methanol (2 ml.) was banded on a thin-layer plate (30 cm. $\times$ 30 cm.) of cellulose (MN-cellulose powder 300G; Camlab Ltd., Cambridge) and developed in butan-1-ol-acetic acid-water (4:1:2, by vol.) against a reference spot of the original urine. The band corresponding to the glucuronide (identified by examining the plate under ultraviolet light and also by taking a print from the wet plate on to Whatman no. 4 paper and spraying with naphtharesorcinol and *p*-dimethylaminocinnamaldehyde) was scraped from the plate, suspended in water (10 ml.) and centrifuged at 2000g for 10 min. The supernatant was evaporated to dryness under reduced pressure to give the ammonium salt of sulphadimethoxine glucuronide (20 mg.), which was 90% pure. This material (5 mg.) was recrystallized from methanol and 3 mg. of the salt (m.p. 150–160°) was obtained, which was 99% pure. Further attempts to purify the salt resulted in decomposition. The 90%-pure material was therefore dissolved in water (2 ml.) and treated with solid *S*-benzylthiuronium chloride (50 mg.). On standing, yellow prisms of the *S*-benzylthiuronium salt of the glucuronide appeared (yield, 13 mg.), m.p. 164° (decomp.). On recrystallization from water (charcoal) the m.p. was raised to 179–180° (decomp.) (Found: C, 46.5; H, 4.8; N, 12.3. C₂₆H₃₂N₆O₁₀S₂·H₂O requires C, 46.6; H, 5.1; N, 12.5%). The glucuronide did not reduce Benedict's solution on warming.

The ammonium salt of the glucuronide (0.46 mg.; 99% pure) was hydrolysed with 2N-HCl (1 ml.) at 100° for 20 min., and the glucuronic acid and aromatic amine in the hydrolysate were determined. It contained 35% of glucuronic acid and 56% of sulphadimethoxine (estimated as sulphanilic acid), which gives a glucuronic acid/sulphadimethoxine molar ratio 1:0.

Hydrolysis of sulphadimethoxine glucuronide

With β -glucuronidase. The ammonium salt of sulphadimethoxine glucuronide isolated from human urine was dissolved in water (0.33 mg./ml.). The sample used was 90% pure and was free from sulphadimethoxine as indicated by paper chromatography. Three test tubes were set up each containing 0.3 ml. of the glucuronide solution and 0.1 ml. of 0.1M-sodium acetate buffer, the pH of the mixture being 4.2. To one tube there were added 15000 Fishman units (Talalay, Fishman & Huggins, 1946) of mollusc β -glucuronidase solution (0.1 ml.) (Koch-Light Ltd.), to the second the same amount of boiled enzyme and to the third 0.1 ml. of water. The tubes were plugged and incubated for 24 hr. at 38°. The contents of each tube were then chromatographed in solvents A, B and C. The experiment was repeated with

Table 2. *Hydrolysis products of sulphadimethoxine and its derivatives*

The compounds were hydrolysed with acid or alkali as described in the text. The hydrolysis products were identified by R_F values and colour reactions (see Table 1).

Compound hydrolysed	Products of hydrolysis	
	With 2N-HCl	With 2.5N-NaOH
Sulphadimethoxine, N^4 -acetylsulphadimethoxine	Barbituric acid, sulphanilic acid, sulphanilamide*	Barbituric acid, sulphanilic acid, sulphanilamide
N^1 -Methylsulphadimethoxine	Barbituric acid, sulphanilic acid	Sulphanilic acid (trace),† sulphanilamide (trace)
N^{ring} -Methylsulphadimethoxine	N -Methylbarbituric acid, sulphanilic acid	Sulphanilic acid (trace),† sulphanilamide (trace)
Sulphadimethoxine N^1 -glucuronide ammonium salt (synthetic)	Barbituric acid, sulphanilic acid, sulphanilamide,* glucuronic acid, glucuronolactone	Barbituric acid, sulphanilamide, glucuronic acid
Sulphadimethoxine glucuronide ammonium salt (from human urine)	Barbituric acid, sulphanilic acid, glucuronic acid, glucuronolactone	Barbituric acid, sulphanilamide, glucuronic acid

* A minor product.

† The methylsulphadimethoxines appear to be relatively stable under these conditions of hydrolysis.

the ammonium salt of synthetic sulphadimethoxine N^1 -glucuronide. The first tube in both cases gave spots corresponding to glucuronic acid, sulphadimethoxine and unchanged sulphadimethoxine N^1 -glucuronide. The control tubes gave, in each case, only one spot corresponding to the unchanged glucuronide.

A sample (50 mg.) of the 40%-pure ammonium salt of sulphadimethoxine glucuronide isolated from human urine was dissolved in 0.2M-sodium acetate buffer, pH 4.3 (10 ml.). This sample of glucuronide was free of sulphadimethoxine. The solution was treated with 30000 Fishman units of β -glucuronidase and then incubated at 38° for 96 hr. The mixture was extracted with ethyl acetate and the extract was chromatographed as a band on Whatman no. 1 paper with the top layer of the solvent mixture butan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (4:1:5, by vol.). The band at R_F 0.4 was eluted with ethyl acetate. On evaporation, the eluate yielded pale-yellow crystals (1 mg.), which were identified as sulphadimethoxine (m.p. 200° and mixed m.p. 201°) by infrared spectrum, chromatography in solvents *A*, *B* and *C*, and its electrophoretic properties at pH 1 (0.1N-HCl) and pH 9 (0.1M- NaHCO_3 -NaOH).

With acid and alkali. Aqueous solutions (0.02%) of the biosynthetic or synthetic sulphadimethoxine N^1 -glucuronide were hydrolysed by heating at 100° for 1 hr. with an equal volume of 2N-HCl or 2.5N-NaOH. The hydrolysates were chromatographed in solvents *A* and *C* (Table 1). Aqueous suspensions (0.5%) of sulphadimethoxine, N^4 -acetylsulphadimethoxine, N^1 -methylsulphadimethoxine and the mixture of N^{ring} -methylsulphadimethoxines were similarly hydrolysed and chromatographed. The results are shown in Table 2. The products of the hydrolyses were identified by R_F values and colour reactions.

Hydrolysis of sulphadimethoxine

The drug (0.25 g.) in N-HCl (10 ml.) was heated on a boiling water bath for 1 hr. A 2 ml. portion of hydrolysate was put on an alumina (Whatman standard-grade powder) column (2 cm. $\times$ 20 cm.) and eluted with solvent *A*. The eluate was

collected in 50 ml. portions and the fifth eluate was evaporated to yield 5 mg. of sulphanilamide, which on recrystallization from water had m.p. 163–164° and mixed m.p. 162–164°. Its identity was checked by its infrared spectrum. The rest of the hydrolysate (8 ml.), on being kept overnight, yielded 30 mg. of colourless crystals, which were identified as barbituric acid (m.p. 247–249°, mixed m.p. 246–248°).

Stability of sulphadimethoxine N^1 -glucuronide at various pH values

The ammonium salt of the synthetic glucuronide (5 mg.) was dissolved in 1 ml. of N-HCl and various buffers of pH 3, 5, 7 and 9. The solutions were shaken at 37° for various times up to 24 hr. Samples at intervals of 1, 3, 5 and 24 hr. were chromatographed on Whatman no. 1 paper in solvent *A*, and the spots corresponding to the N^1 -glucuronide and sulphanilic acid were eluted with water and analysed by the Bratton & Marshall (1939) method. The glucuronide was stable at pH 3, 5, 7 and 9, but at pH 0 it was slowly hydrolysed and in 24 hr. 92% had disappeared. About 73% was converted into sulphanilic acid.

DISCUSSION

The major metabolite of sulphadimethoxine in urine in man is a glucuronide (Koechlin *et al.* 1959; Bridges *et al.* 1964), and this has now been isolated and characterized. A perusal of the formula of sulphadimethoxine (I) shows that several structures could be postulated for this glucuronide. If it were an *O*-glucuronide, it could be derived from a sulphadimethoxine hydroxylated in the benzene ring or position 5 of the pyrimidine ring, or demethylated at one of the methoxyl groups in the pyrimidine ring. These types of structures can be eliminated because hydrolysis of the glucuronide with β -glucuronidase yields sulphadimethoxine and with

acid sulphanilic acid and some sulphanilamide. No hydroxysulphanilic acid or hydroxysulphanilamide was detected (see Table 2). Sulphadimethoxine may not be readily demethylated *in vivo*, for we found that when it was incubated *in vitro* at 38° with the rabbit-liver *O*-demethylating system described by Takemori & Mannering (1958) no formaldehyde was produced during 1 hr. The drug was not hydroxylated or demethylated by the ascorbic acid-iron system described by Brodie, Axelrod, Shore & Udenfriend (1954).

The glucuronide must therefore be an *N*-glucuronide, and four of these are possible (II–V). The *N*⁴-glucuronide (II) can be readily eliminated. Aromatic amine *N*-glucuronides are labile, reduce alkaline copper reagents and are not hydrolysed by β -glucuronidase (Bridges & Williams, 1962). A sample of sulphadimethoxine *N*⁴-glucuronide was synthesized and found to be labile, to reduce Benedict's solution and Fehling's solution, and to give a colour slowly with *p*-dimethylaminocinnamaldehyde (see Table 1). These properties did not agree with the urinary glucuronide, which is stable, non-reducing and gives a colour immediately with *p*-dimethylaminocinnamaldehyde. The *R_f* values were also different (Table 1). The urinary glucuronide was similar in all respects to a sulpha-

dimethoxine glucuronide synthesized from sodium sulphadimethoxine and methyl 2,3,4-tri-*O*-acetyl-1-bromoglucuronate (see Tables 1–4). The *S*-benzylthiouronium salt of the urinary glucuronide was identical with that of the synthetic glucuronide.

A glucuronide synthesized as above could have one of three structures (III, IV or V) as a result of the tautomerism of the sulphanilamidopyrimidines. A basis for distinguishing the amido structure (III) from the imido structures (IV) and (V) has been suggested by Uno, Machida, Hanai, Ueda & Sasaki (1963), who showed that the amido and imido forms of pyridine and thiazole sulphonamides could be distinguished by their SO₂ symmetrical stretching frequencies. In the imido forms this frequency occurs in the range 1120–1145 cm.⁻¹, whereas in the corresponding amido forms it occurs in the range 1145–1170 cm.⁻¹. Methyl derivatives of sulphadimethoxine and 4-methoxy-6-sulphanilamidopyrimidine were synthesized containing both these structures. The structures of the methyl derivatives containing the amido link were proved by synthesis, i.e., 4-methoxy- and 2,4-dimethoxy-6-(*N*¹-methylsulphanilamido)pyrimidines and their *N*⁴-acetyl derivatives. The structures of the corresponding *N*^{ring}-methyl derivatives were then deduced by inference. For sulphadimethoxine, the routes were as shown in Scheme 1. The SO₂ symmetrical stretching frequencies of these compounds are shown in Table 3, and on this type of evidence it is clear that the synthetic and urinary sulphadimethoxine glucuronides have the amido structure (III).

The *N*^{ring}-methylsulphadimethoxine obtained by methylation of sulphadimethoxine appeared to be a mixture of two compounds, which we failed to separate. This conclusion was based on the infrared

(I)

(II)

(III)

(IV)

(V)

Table 3. SO_2 symmetrical stretching frequencies of sulphadimethoxine and related compounds

Infrared spectra in KBr pellets were determined in a Perkin-Elmer Infracord spectrometer.

Compound	SO_2 stretching frequency (cm^{-1})
Sulphanilamide	1150
2,4-Dimethoxy-6-sulphanilamidopyrimidine (sulphadimethoxine)	1150
6-($N^{4'}$ -Acetylsulphanilamido)-2,4-dimethoxypyrimidine	1150
Sulphadimethoxine glucuronide ammonium salt (from urine)	1160
Sulphadimethoxine N^1 -glucuronide ammonium salt (synthetic)	1160
2,4-Dimethoxy-6-($N^{1'}$ -methylsulphanilamido)pyrimidine	1150
6-(N^4 -Acetyl- N^1 -methylsulphanilamido)-2,4-dimethoxypyrimidine	1155
4-Methoxy-6-sulphanilamidopyrimidine	1155
4-Methoxy-6-($N^{1'}$ -methylsulphanilamido)pyrimidine	1150
6-($N^{4'}$ -Acetyl- $N^{1'}$ -methylsulphanilamido)-4-methoxypyrimidine	1155
2,4-Dimethoxy-1- and 2,4-dimethoxy-3-methyl-6-sulphanilamidopyrimidine*	1125
4-Methoxy-1- or 4-methoxy-3-methyl-6-sulphanilamidopyrimidine†	1130
6-($N^{4'}$ -Acetylsulphanilamido)-4-methoxy-1- or 6-($N^{4'}$ -acetylsulphanilamido)-4-methoxy-3-methylpyrimidine†	1130

* This is a mixture of the two possible N^{ring} -methyl forms.† These are single compounds, but the position of the N -methyl group in the pyrimidine ring is either 1 or 3.

spectrum, which showed extensive doubling of the absorption bands. Methylation of 4-methoxy-6-sulphanilamidopyrimidine appeared to give only one N^{ring} -methyl derivative, in which the doubling of infrared-absorption bands was absent. Acid hydrolysis of the N^{ring} -methylsulphadimethoxine yielded N -methylbarbituric acid, whereas that of the N^1 -methyl derivative gave barbituric acid. Acid hydrolysis appears to demethylate all the methoxypyrimidine derivatives (see Table 2). The products of hydrolysis of the methylated 4-methoxy-6-sulphanilamidopyrimidines could not be identified.

It has been claimed that ultraviolet spectra can be used to distinguish the N^1 -methyl and N^{ring} -methyl derivatives of sulphapyridine and sulphathiazole (Shepherd *et al.* 1942; Sheinker, Postovskii-Voronina & Kushkin, 1957; Sheinker & Kuznetzova, 1957). The N^1 -methyl (amido) forms in ethanol shows one absorption peak, whereas the N^{ring} -methyl (imido) forms show two peaks, the major of which occurs at longer wavelengths than the single peak of the amido forms. The absorption peaks (above $220\text{m}\mu$) of a number of sulphanilamido-

pyrimidines were determined (see Table 4). It is clear that, in this series of compounds, ultraviolet spectra cannot be used to distinguish amido and imido forms. Most of the compounds in Table 4 show one peak, and there is no separation of the absorptions due to the sulphanilamide and pyrimidine portions of the molecules. The N^1 -methyl and N^{ring} -methyl derivatives of 2,4-dimethoxy- and 4-methoxy-6-sulphanilamidopyrimidines have similar spectra, showing an inflexion in the $255\text{--}265\text{m}\mu$ region and a peak in the $275\text{--}285\text{m}\mu$ region. These amido and imido forms are thus not readily distinguishable by ultraviolet spectra. The sulphadimethoxine glucuronide isolated from human urine showed a spectrum identical with that of synthetic sulphadimethoxine N^1 -glucuronide (Table 4).

Sulphadimethoxine N^1 -glucuronide could be an α - or β -glycoside. Specific optical rotations (tri-acetyl methyl ester, $+87^\circ$ in methanol; ammonium salt, $+4.5^\circ$ in water; S -benzylthiuronium salt, -13° in water) gave no clear indication of the configuration. The glucuronide, however is slowly hydrolysed by β -glucuronidase and the hydrolysis is inhibited by saccharo-(1 $\rightarrow$ 4)-lactone (J. W.

Table 4. *Ultraviolet spectra of 6-sulphanilamidopyrimidines*

The solvent used was ethanol. ~ indicates inflexion. Wavelengths are given to the nearest $m\mu$ and $\epsilon_{\max}$ to the nearest 100.

Compound	$\lambda_{\max}$ ($m\mu$)	$10^{-3} \times \epsilon_{\max}$
Sulphanilamide	273	17.4
2,4-Dimethoxy-6-sulphanilamidopyrimidine (sulphadimethoxine)	261, 273	19.0, 23.0
Sulphadimethoxine glucuronide ammonium salt (from urine)	269	21.4
Sulphadimethoxine <i>N</i> ¹ -glucuronide ammonium salt (synthetic)	269	21.8
2,4-Dimethoxy-6-(<i>N</i> ¹ -methylsulphanilamido)pyrimidine	~265, 274	19.4, 21.7
4-Methoxy-6-sulphanilamidopyrimidine	275	19.9
4-Methoxy-6-(<i>N</i> ¹ -methylsulphanilamido)pyrimidine	~255, 279	12.9, 22.4
2-Methoxy-6-sulphanilamidopyrimidine	268-280	20.0
5-Methoxy-6-sulphanilamidopyrimidine	272	19.3
2,5-Dimethoxy-6-sulphanilamidopyrimidine	270, 295	16.0, 16.8
4,5-Dimethoxy-6-sulphanilamidopyrimidine	275	22.3
2,4-Dimethyl-6-sulphanilamidopyrimidine	272	20.2
4-Methoxy-2-methyl-6-sulphanilamidopyrimidine	274	19.8
6-Amino-2,4-dimethoxypyrimidine	253	6.7
2,4-Dimethoxy-1- and 2,4-dimethoxy-3-methyl-6-sulphanilamidopyrimidine	~265, 284	19.6, 24.5
4-Methoxy-1- or 4-methoxy-3-methyl-6-sulphanilamidopyrimidine	~259, 278	14.8, 22.0

Bridges, M. R. Kibby & R. T. Williams, unpublished work), a known inhibitor of β -glucuronidase (Levy, 1952). This suggests that the compound has β -configuration and can be tentatively described as 1-deoxy-1-[*N*^{1'}-(2'',4''-dimethoxypyrimidin-6''-yl)-sulphanilamido- β -D-glucosid]uronic acid.

The glucuronide showed only about one-hundredth of the antibacterial activity of the parent drug against *Staphylococcus aureus* Oxford (tested by Dr McFazean of May and Baker Ltd., Dagenham, Essex).

There have been reported in the literature several types of *N*-glucuronides that are formed *in vivo*. These include the labile *N*-glucuronides of aromatic amines, the stable *N*-glucuronides of carbamates of which two are known, namely those of meprobamate (2-methyl-2-propylpropane-1,3-diol dicarbamate) and of ethinamate (1-ethynylecyclohexyl carbamate; Valamin) (Tsukamoto, Yoshimura & Tatsumi, 1963*a,b*), the *N*^{ring}-glucuronide of sulphisoxazole (Uno & Kono, 1962) and the *N*¹-glucuronides of sulphonamide drugs of which two are known, namely those of sulphathiazole (Uno & Ueda, 1963) and of sulphadimethoxine (this paper).

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REFERENCES

- Baddiley, J. & Topham, A. (1944). *J. chem. Soc.* p. 678.
 Bollenback, G. N., Long, J. W., Benjamin, D. G. & Lindquist, J. A. (1955). *J. Amer. chem. Soc.* **77**, 3310.
 Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.
 Bredereck, H., Kupsch, G. & Wieland, H. (1959). *Chem. Ber.* **92**, 583.
 Bretschneider, H. & Kloetzer, W. (1955). *U.S. Patent* 2703800.
 Bridges, J. W., Kibby, M. R. & Williams, R. T. (1964). *Biochem. J.* **91**, 12*r*.
 Bridges, J. W. & Williams, R. T. (1962). *Biochem. J.* **83**, 27*r*.
 Brodie, B. B., Axelrod, J., Shore, P. A., & Udenfriend, S. (1954). *J. biol. Chem.* **208**, 741.
 Brown, D. J. (1955). *J. appl. Chem., Lond.*, **5**, 358.
 Elliott, D. C. (1959). In *Data for Biochemical Research*, pp. 221-229. Ed. by Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. Oxford: The Clarendon Press.
 Hull, R. (1951). *J. chem. Soc.* p. 2214.
 Koehlin, B. A., Kern, W. & Engelberg, R. (1959). *Antibiot. Med., Brit. Ed.*, **6** (suppl.), 22.
 Levy, G. A. (1952). *Biochem. J.* **52**, 464.
 Okumura, K. (1962). *Yakugaku Kenkyu*, **34**, 36. Cited in *Chem. Abstr.* (1963), **58**, 6915*d*.
 Paul, J. (1951). Ph.D. Thesis: University of Glasgow.
 Randall, L. D., Bagdon, R. F. & Engelberg, R. (1959). *Toxicol. appl. Pharmacol.* **1**, 28.
 Schnitzer, R. J., DeLorenzo, W. F. & Grunberg, E. (1959). *Proc. Soc. exp. Biol., N.Y.*, **99**, 421.
 Semenitz, E. (1954). *Z. Immunforsch.* **111**, 386.
 Sheinker, Y. N. & Kuznetsova, I. K. (1957). *J. phys. Chem., Moscow*, **31**, 2656.
 Sheinker, Y. N., Postovskii, I. Y., Voronina, N. M. & Kushkin, V. W. (1957). *J. phys. Chem., Moscow*, **31**, 1745.
 Shepherd, R. G., Bratton, A. C. & Blanchard, K. G. (1942). *J. Amer. chem. Soc.* **64**, 2532.
 Takemori, A. E. & Mannering, G. J. (1958). *J. Pharmacol.* **123**, 171.
 Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
 Tsukamoto, H., Yoshimura, H. & Tatsumi, K. (1963*a*). *Chem. pharm. Bull., Tokyo*, **11**, 1134.
 Tsukamoto, H., Yoshimura, H. & Tatsumi, K. (1963*b*). *Life Sci.* **2**, 382.
 Uno, T. & Kono, M. (1962). *J. pharm. Soc. Japan*, **82**, 1660.
 Uno, T., Machida, K., Hanai, K., Ueda, M. & Sasaki, S. (1963). *Chem. pharm. Bull., Tokyo*, **11**, 704.
 Uno, T. & Ueda, M. (1963). *Chem. pharm. Bull., Tokyo*, **11**, 709.
 Winkelman, W. (1927). *J. prakt. Chem.* **115**, 292.