The authors are indebted to the members of the Analytical Center of the Kyoto University for the microanalyses and Miss I. Uchida for measurement of the infrared spectra. They are also grateful to Mr. H. Koyama for his technical assistance in this work.

Summary

1) 7-Substituted 2-methyl- or 2-phenyl-oxazolo[4,5-*c*]pyridines were prepared from 3,5-dinitro-4-hydroxypyridine and some of them were hydrogenated over palladium-carbon or Raney nickel.

2) By examination of the hydrolysis products of 2-methyl-7-nitro(or bromo)-oxazolo-[4,5-*c*]pyridine monomethiodide and 2-methyloxazolo[4,5-*b*]pyridine monomethiodide, it was confirmed that methyl iodide combined with nitrogen atom in the pyridine ring of oxazolo-pyridine system.

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65. Sadae Tsutsumi : Analytical Studies on Antileprous Drugs. IV.*2 On the Metabolic Substances of Human and Rabbit Urine after Administration of 4,4′-Diaminodiphenyl Sulfone and 4,4′-Diaminodiphenyl Sulfoxide, with Special Reference to Labile N-Conjugates.

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In previous reports of this series,[1] electrophoretic method for the separation of 4,4′-diaminodiphenyl sulfone (DDS), 4,4′-diaminodiphenyl sulfoxide (DDSO) and 4,4′-diamino-diphenyl sulfide (DDSD) was reported. Applying this method, metabolites of DDSO excreted in human and rabbit urine were examined,[2] with special reference to intact N-acetyl conjugation of DDSO and unchangeability of DDSO to DDS or DDSD. As the second step, labile conjugates of DDS and DDSO were investigated in the present series of work. As reported by Bushby, *et al.*,[3] mono-N-glucuronide of DDS (DDSG) was detected in the urine of rabbit treated with DDS and also the mono-N-glucuronide of DDSO (DDSOG) in the urine of rabbit treated with DDSO. Both DDSG and DDSOG were also proved in human urine, but in this case, other metabolites of DDS and DDSO were found on the chromatogram.

In the case of DDS, the metabolite was found to be identical on chromatography with the synthesized potassium DDS mono-N-sulfamate (DDSS), while, in the case of DDSO, it was difficult to prove it, as the synthesis of mono-N-sulfamate of DDSO was not possible. However, it may be possible to consider that the mono-N-sulfamate of DDSO is metabolized in the same manner as DDS, because Rf value of the metabolite of DDSO is identical with that of DDSS and also there is no chromatographic difference between DDS and DDSO, DDSG and DDSOG, nor between the mono-N-acetylate of DDS and that of DDSO.[2]

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*1 Higashimurayama, Kitatama-gun, Tokyo (堤 貞衛).

*2 Part III : La Lepro, **29**, 96 (1960).

1) S. Tsutsumi : *Ibid.*, **28**, 268 (1959).

2) *Idem* : *Ibid.*, **29**, 88 (1960).

3) S. R. M. Bushby, A. J. Woiwod : Biochem. J., **63**, 406 (1956).

Materials and Methods

I. Preparation of Standards

Sodium 4,4′-Diaminodiphenyl Sulfone Mono-N-glucosiduronate (DDSG)——Synthesized by Ôgiya, Hashimoto, and Takitani's method.[4] m.p. 165~173° (decomp.).

Sodium 4,4′-Diaminodiphenyl Sulfoxide Mono-N-glucosiduronate (DDSOG)——A mixture of 2.7 g. of DDSO, m.p. 175~177° (decomp.), dissolved in 5.4 cc. of dimethylformamide containing 0.03 cc. of AcOH and added with 0.3 g. of sodium glucuronate in 4 cc. of ethylene glycol was placed in an incubator at 37° for 8 hr. After centrifugation, the precipitate was washed with Me_2CO and dried (0.3 g.). By chromatography, this precipitate was proved to contain DDSOG, a trace of DDSO, and disodium-DDSO N,N′-diglucosiduronate. This crude mixture dissolved in 20 cc. of developing solvent (PrOH-BuOH-0.2*N* NH_4OH=2:1:1) was passed through a cellulose powder column*3 (20 × 120 mm.) and developed with the same solvent. Effluent from the first 70 cc. contained DDSO, 70~120 cc. contained DDSOG, and that of 180~240 cc. contained diconjugate.

DDSOG was isolated by the addition of an equal volume of Me_2CO to the second fraction. Colorless powder, m.p. 165~173° (decomp.). *Anal.* Calcd. for $C_{18}H_{19}O_7N_2NaS$: N, 6.51. Found : N, 6.34.

Disodium 4,4′-Diaminodiphenyl Sulfoxide N,N′-Diglucosiduronate——Out of the effluent from the cellulose powder column used for the separation of DDSOG, the portion corresponding to the third fraction (180~240 cc.) was collected, diluted with Et_2O, this dilution was extracted with 0.2*N* NH_4OH, and this extract was freeze-dried immediately. Molar ratio of DDSO/sodium glucuronate quantitatively determined on this freeze-dried residue by the usual diazo-coupling method (Tsuda reagent) and naphthoresorcinol picrate method[5] was found to be 0.53. This residue showed Rf value of 0.12 when developed with PrOH-BuOH-0.2*N* NH_4OH (3:2:3) (A) and 0.06 when the ratio was 2:3:5 (B)[3] (upper layer).

Potassium 4,4′-Diaminodiphenyl Sulfone Mono-N-sulfamate (DDSS)——To the ice-cold solution of 5 g. of DDS in 25 cc. of pyridine, 2 g. of $ClSO_3H$ was added dropwise. After leaving for 1 hr. at room temperature, 40 cc. of AcOEt was added and the solvent mixture was removed by decantation. Sticky residue was triturated with AcOEt, dissolved in 15 cc. of 20% K_2CO_3 solution, and 80 cc. of AcOEt was added to it. After leaving in a refrigerator for 3 hr., this mixture formed three layers and the separated middle layer was saturated with K_2CO_3. The precipitate was dissolved in 0.2*N* NH_4OH. After filtration, cellulose powder was soaked in dried *in vacuo*, and packed in a cellulose powder column (20 × 300 mm.). Developing solvent used was (C) PrOH-BuOH-0.2*N* NH_4OH (2:2:1) and the effluent was examined by paper chromatography. The results were as follows :

Fraction No. 1 (60~105 cc.)	Mixture of DDS and DDSS
Fraction No. 2 (105~155 cc.)	DDSS (Colorless precipitate appeared in the solvent when preserved in a deep-freezer)
Fraction No. 3 (155~225 cc.)	Mixture of DDSS and another substance which showed lower Rf values when developed by solvent systems A, B, and C

Fraction No. 2 was diluted with Et_2O, extracted with 0.2*N* NH_4OH, and the aqueous layer was soaked into cellulose powder and repurified by the same procedure as above. Final aqueous extract was freeze-dried below 30°. The residue was washed with Et_2O and dried. Colorless powder, m.p. 62~66°, decomposed at 102°, and the yield was 1.2 g. Soluble in H_2O and MeOH, insoluble in Et_2O and benzene, and slightly soluble in Me_2CO. *Anal.* Calcd. for $C_{12}H_{11}O_5N_2KS$: N, 7.84. Found : N, 7.65.

$BaSO_4$ precipitated when the aqueous solution of this colorless powder was warmed with 5% $BaCl_2$ in 1*N* HCl, and by heating with 1*N* HCl, DDS (m.p. 175~177°) was isolated after neutralization with NaOH. The infrared absorption spectra of DDS and DDSS are shown in Fig. 1,*4 and their absorptions were as follows : DDS : ν_{max}^{KBr} cm^{-1} $\nu_{as}(SO_2)$ 1275, $\nu_s(SO_2)$ 1130; DDSS : $\nu_{as}(SO_2)$ 1250, $\nu_{as}(SO_3^-)$ 1145, $\nu_s(SO_2)$ 1105, $\nu_s(SO_3^-)$ 1042, $\nu_{N\text{-}S}$ or $\nu_{C\text{-}S}$ 890.*5

*3 Toyo Roshi Cellulose Powder (100~200 mesh) was suspended in Me_2CO, plugged uniformly, Me_2CO was replaced by the developing solvent, allowed to stand overnight.

4) Y. Hashimato, S. Takitani : Personal Communication; S. Ôgiya, M. Suzuki : Yakugaku Zasshi, **81**, 349 (1961).

5) M. Ishidate, T. Nambara : This Bulletin, **5**, 515 (1957).

*4 Kōken Model DS-301 spectrophotometer with NaCl prism was used for infrared spectra and samples were run as KBr disks and corrected with polystyrene.

*5 Thanks are due to Dr. B. Nakazawa and Miss S. Hara, National Institute of Health, Tokyo, for infrared analysis and also to Dr. G. Chihara, Tokyo University Hospital, and Dr. T. Hino, University of Tokyo, for their kind advices in the assignment of absorption spectra.

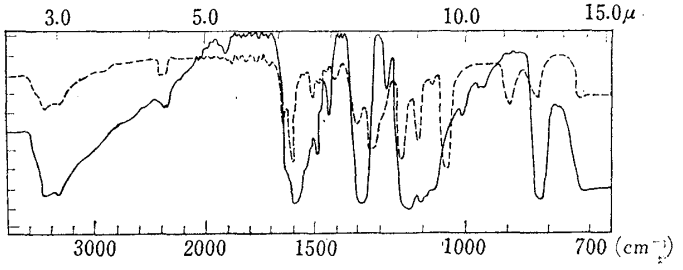

Fig. 1.
Infrared Absorption Spectra of DDS and DDSS
—— 4,4′-Diaminodiphenyl sulfone
------ Potassium 4,4′-diaminodiphenylsulfone mono-N-sulfamate

The substance with lower Rf value contained in fraction No. 3 is considered to be dipotassium DDS N,N′-disulfamate. Its Rf values were 0.20 (A), 0.11 (B), and 0.06 (C), and it showed a migration distance to the anode larger than DDSS on paper electrophoresis.

II. Determination of Metabolites

Paper Chromatography——Toyo Roshi No. 50 (2 × 40 cm.) was used for one-dimensional method and Toyo Roshi No. 51 (40 × 40 cm.) for the elution of pure materials and for two-dimensional method. Materials were all developed by the ascending procedure. In the case of metabolites, buffered chromatography was used. In this case, filter papers were pretreated with solvent buffer mixture and dried in the air protected from dust. Buffer used was Britton-Robinson's universal buffer ($0.04M$ (AcOH-H_3BO_3-H_3PO_4) + $0.2N$ NaOH, pH 7.4).[6]

Paper Electrophoresis——To avoid generation of heat, short filter papers (Toyo Roshi No. 50, 2 × 18 cm.) were used in ice-cold condition. Original line was 9 cm. from the terminal. The apparatus was horizontal type (Kobayashi). The buffer used was Sörensen's buffer ($M/15$ Na_2HPO_4-$M/15$ KH_2PO_4, pH 7.4).

Detection on Filter Paper——For amino radicals, Erlich's reagent (1% *p*-(dimethylamino)benzaldehyde in 1% MeOH-HCl) and Tsuda reagent were used.

For glucuronic acid, samples were heated at 105° for 5 min. after spraying 2-aminodiphenyl reagent which contains 1.69 g. of 2-aminodiphenyl, 0.9 g. of oxalic acid, 5 cc. of glycerol, 84 cc. of Me_2CO, and distilled H_2O per 100 cc. of the reagent.[7]

III. Administration of DDS and DDSO to Man and Rabbits——100 mg./kg. of DDSO was given orally to a rabbit (♂, 2.5 kg.), and total urine collected for 12 hr. was 85 cc. In the case of human beings, 6 mg./kg. of DDS or DDSO and 100 mg./kg. of $NaHCO_3$ were given at one time for the excretion of alkaline urine, and total urine for 2~6 hr. was 350 cc. (pH 7.6) when DDS was used, and 250 cc. (pH 7.8) when DDSO was used. Human and rabbit urines were centrifuged at 7000 r.p.m. for 20 min., separately under refrigeration, and the supernatant was immediately freeze-dried. The freeze-dried residues from human (2/3) and rabbit (1/3) urine and cellulose powder were separately mixed with $0.2N$ NH_4OH and each mixture was dried *in vacuo*. Each soaked cellulose powder was packed in a column and developed with the solvent of PrOH-BuOH-$0.2N$ NH_4OH (2:1:1). Rf values of the effluents are shown in Table I.

Results and Discussion

Metabolites of Rabbit Urine——As shown in Table I, two kinds of fraction (F-1 and F-2) were isolated from the DDSO urine, one showing Rf value corresponding to that of DDSO or mono-N-acetyl-DDSO and the other corresponding to DDSOG when developed with several solvent systems. After ascending paper chromatography, the latter fraction colored both by Tsuda reagent and 2-aminodiphenyl reagent.

On paper electrophoresis with Sörensen's buffer for 6 hours under the conditions of 0.75 mA/cm., 85~75 v., this fraction migrated 25 mm. towards the anode, while DDS and DDSO migrated 45 mm. towards the cathode and sodium glucuronate 50 mm. towards anode. Molar ratio (DDSO/sodium glucuronate) was also measured and it was 0.99 in the case of F-2. Further, when the urine of a rabbit administered with DDS was ascended by paper chromatography after freeze-drying, N-glucuronide was detected as reported by Bushby, *et al.*[3] and its Rf value was in agreement with that of F-2.

6) H. T. S. Britton, R. A. Robinson : J. Chem. Soc., **1931**, 1456; S. Ôgiya : Yakugaku Zasshi, **79**, 953 (1959).
7) T. E. Timell, C. P. J. Glaudemans, A. L. Currie : Anal. Chem., **28**, 1916 (1956).

Table I. Rf Values of the Components in the Effluent from Chromatography

Fraction	No.	(cc.)	Solvent System A	B	C	D	E	F	G
DDSO Urine of Rabbit	F-1	12~16	0.89	0.86	0.84	0.82	—	—	—
	F-2	24~28	0.46	0.17	0.09	0.14	0.75	0.16	0.62
DDS Urine of Human	F-3	15~30	0.89	—	—	—	—	—	—
	F-4	36~45	0.59	0.40	0.31	0.16	—	—	—
	F-5	51~57	0.46	0.18	0.09	0.14	—	—	—
DDSO Urine of Human	F-6	18~27	0.88	0.86	0.84	0.82	—	—	—
	F-7	33~42	0.61	0.40	0.31	0.14	—	—	0.81
	F-8	51~57	0.46	0.18	0.08	0.14	0.75	0.16	0.62
DDS			0.90	—	—	—	—	—	—
DDSO			0.90	0.86	0.83	0.82	—	—	—
DDSG			0.46	0.18	—	—	—	—	—
DDSOG			0.46	0.18	0.08	0.14	0.76	0.16	0.62
DDSS			0.61	0.40	0.31	0.14	—	—	—
Mono-N-acetyl-DDS			0.90	0.86	—	—	—	—	—
Mono-N-acetyl-DDSO			0.91	0.87	—	—	—	—	—

Cellulose powder column	Human Urine	Rabbit Urine
Internal Diameter	15 mm.	10 mm.
Length	150 mm.	90 mm.
Effluent Collected	every 3 cc.	every 2 cc.

Solvent System

D : BuOH saturated with H_2O[a)]
E : PrOH-H_2O (5:3)
F : PrOH-pyridine-H_2O (3:2:3)
G : iso-PrOH-BuOH-H_2O (3:2:3)

a) Universal buffer in the case of buffered chromatography (F-1~8) and 0.2*N* NH_4OH in the case of normal chromatography.

Metabolites of Human Urine——Both the metabolites of DDS urine (F-5) and that of DDSO urine (F-8) were proved to be identical with DDSG and DDSOG, respectively, exactly in the same manner as rabbit urine. This finding was also confirmed by the two-dimensional chromatography. F-5 and F-8 were diluted separately with Et_2O and extracted with a small quantity of 0.2*N* NH_4OH. They were chromatographed with B as the first solvent system and H (BuOH-AcOH-H_2O=12.5:3:12.5)[8)] as the second. For example, results from F-8 were as follows :

	Rf (B)	Rf (H)	
	0.20	0.83	0.07
Ehrlich reagent		+	−
2-aminodiphenyl reagent		−	+

This indicates that the decomposition products of DDSOG contained in F-8 were DDSO and glucuronic acid by acidic solvent H.

Di-N-conjugate was not detected, unlike the rabbit urine, but the fractions with Rf value corresponding to DDSS were detected, namely, F-4 from DDS-urine and F-7 from DDSO-urine. In order to prove whether or not F-4 and F-7 were N-sulfamate, they were again repeatedly ascended by paper chromatography using solvent system B. The eluates from filter papers showed marked turbidity in contrast to the eluate from adjacent area of the filter paper, when they were added with 5% barium chloride in 1*N* hydrochloric acid and heated for a few minutes. On the other hand, Williams[8)] previously reported that in the human urine 4-aminobenzenesulfonamide is metabolized to 3-hydroxy-4-aminobenzenesulfonamide O-sulfate.

*6 Donated from Drs. S. Owari and E. Matsui of the Iatrochemical Institute, Pharmacological Research Foundation, Tokyo.

8) R. T. Williams : Biochem. J., **41**, 1 (1947).

In addition, the freeze-dried residue of human urine after administration of 1 mg./kg. of sodium hydrogencarbonate showed the main colored band corresponding to DDSS when developed with solvent system A or B, and the band, positive to 5% barium chloride in 1*N* hydrochloric acid, migrated to the anode on electrophoresis. This finding suggests that the main metabolite of OH-DDS is the ethereal O-sulfate conjugate. Thus, even if the Rf values of F-4 corresponded to DDSS, it was suspected that this metabolite could also be the O-sulfate conjugate of phenol formed from DDS by oxidation. For the elucidation of this problem, following test was carried out. Freeze-dried residue from about 100 cc. of human urine collected after administration of DDS or OH-DDS was extracted with methanol, and, after evaporation of methanol in vacuum, the residue was developed by solvent system B using Toyo Roshi No. 51 (40 × 40 cm.). Portion of the filter paper corresponding to DDSS was cut out and extracted with 10 cc. of 0.1*N* acetate buffer (pH 5.6). After incubation with 10 mg. of diastase at 38° for 5 hours, the solution was concentrated *in vacuo* and centrifuged. Supernatant thus obtained colored with salicylaldehyde reagent (1% salicylaldehyde in 50% ethanol-water containing 5% acetic acid). The results were as follows :

	No incubation	Addition of diastase (10 mg.)
0.1 cc. of 0.1% OH-DDS in acetate buffer	++	——
Eluate from DDS urine	——	−
Eluate from OH-DDS urine	——	+

Though the amount of the metabolite corresponding to DDSS was extremely small, absence of *ortho*-OH group might suggest that F-4 is N-sulfamate.

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Summary

Presence of N-glucuronide in the urine of man and rabbit was proved using both 4,4′-diaminodiphenyl sulfone and 4,4′-diaminodiphenyl sulfoxide. Further, presence of N-sulfamate was presumed in the human urine.

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