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66. Sadae Tsutsumi : Analytical Studies on Antileprous Drugs. V.[*1] On the Metabolic Difference of Promin and 4,4′-Diaminodiphenyl Sulfone in the Urine and Plasma of Rabbits

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Leprous patients showing resistance to chemotherapy with Promin (disodium 4,4′-diaminodiphenyl sulfone N,N′-diglucose sulfonate) have frequently been encountered and many clinicians[1a,b] reported that 4,4′-diaminodiphenyl sulfone (DDS) was effective in such cases. This clinical fact may suggest that Promin and DDS act on the leprous lesions in a different way. As reported in Part IV,[*1] DDS is metabolized to mono-N-conjugates, such as mono-N-acetylate and mono-N-glucuronide (DDSG) in the rabbit and man. On the other hand, the metabolic pattern of the intraveneously injected Promin (30 w/v% disodium-DDS N,N′-diglucose sulfonate in 5% glucose solution) is still uncertain, because the Promin injection is unstable and highly difficult to obtain it in its pure form. In the present work investigations were made on the metabolic difference of Promin and DDS in the urine and plasma of rabbits.

Experimental

I. Paper Chromatography of Commercial Promin Injection——Investigation was primarily undertaken with the object of examining the purity of commercial Promin injection (a) and it was found that this injection is a mixture of DDS, crude glucoside of DDS (c), and purified Promin (b).

(c) synthesized by Hecht's method[2] showed two Rf values on chromatogram. The lower one corresponded to (d) and the colored band with higher Rf value correspounded to (e). (e) decolorized gradually on repeated purification with MeOH-Et_2O. When purifying (c) by precipitation of (d) from MeOH solution with Et_2O, the precipitate was collected by refrigerated centrifugation, because (c) and (d) were highly labile to heat and were deliquescent in the air. (d) which was faintly yellow powder in cold Et_2O became insoluble pasty precipitate in cold MeOH.

II. Isolation of DDS N,N′-Diglucoside from the Crude Promin Powder——A cellulose powder column (15 × 300 mm.) was prepared as described in Part IV[*1] using solvent A. A small mass of cellulose powder was soaked in 1.5 g. of crude Promin powder prepared by the method of Jain, *et al.*[3] with 5 cc. of 0.2*N* NH_4OH and dried *in vacuo*. The dried powder was packed in a column and developed with solvent A. The effluent was fractionated every 5 cc. from the moment when it became positive to *p*-(dimethylamino)benzaldehyde test paper[4] and each fraction of the effluent was examined by paper chromatography using solvent A.

Effluent from 35～55 cc. was a mixture of a trace of DDS and (e) which showed Rf value of 0.60～0.62 (A) and that from 55～75 cc. was a mixture of (e) and (d). Each fraction was diluted with an equal volume of Et_2O and extracted with 0.2*N* NH_4OH. Aqueous extract was immediately freeze-dried below 25° and the residue was again purified by cellulose powder column, repeating the same procedure until majority of the effluent showed Rf value corresponding to (e).

Yield of (e) was 20 mg., which was hermetically sealed in a desiccator and preserved in a deep-freezer. Slightly yellow powder, m.p. 62～66°, decomposed above 130°. Soluble in MeOH, Me_2CO, and H_2O, insoluble in Et_2O, and rendered pasty with AcOEt. *Anal.* Calcd. for $C_{24}H_{32}O_{12}N_2S$: N, 4.89. Found : N, 5.02.

The molecular ratio of glucose/DDS measured by the Tsuda and anthrone reagents was 2.29. This showed Rf value corresponded to (d) + (e), even though it had showed Rf value corresponding

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[*1] Part IV. S. Tsutsumi : This Bulletin, **9**, 432 (1961).

[*2] Higashimurayama, Kitatama-gun, Tokyo (堤 貞衛).

1) *a*) R. Yajima, S. Kobayashi : Nihon Iji-shimpō, **1824**, 34 (1959). *b*) S. Hirako : Personal Communication.

2) P.B. Report, **248**, 14.

3) B.C. Jain, B.H. Iyer : Science and Culture, **11**, 568 (1946) (C.A., **40**, 4687 (1946)).

4) K. Kakemi, T. Uno, Y. Ikegami : Yakugaku Zasshi, **76**, 1331 (1956).

TABLE I. Rf Value of Each Fraction of Promin Mixture on Paper Partition Chromatography

Fraction	Color Reaction[a]	Solvent System																
		A PrOH-BuOH-0.2N NH_4OH (3:2:3)						B Upper layer of PrOH-BuOH-0.2N NH_4OH (2:3:5)				C PrOH-BuOH-0.2N NH_4OH (2:2:1)				D BuOH saturated with 0.2N NH_4OH		
(a) Diluted solution of commercial Promin injection[c]	NH_2	0.90	0.61	(0.38)[b]	—	0.15	0.07	0.85	0.57	0.22	0.06	0.83	0.56	—	0.10	0.81	0.45	0.06
	G	—	0.61	(0.37)	0.25	(0.15)	0.07	—	—	—	—	—	—	—	—	—	—	—
(b) Freshly prepd. Promin powder purified 6 times from H_2O-Me_2CO	NH_2	—	—	—	—	0.15	0.07	—	—	—	0.08	—	—	—	0.08	—	—	0.07
	G	—	—	—	—	0.15	0.07	—	—	—	—	—	—	—	—	—	—	—
(c) Crude glucoside prepared from DDS[d]	NH_2	—	0.61	0.36	—	—	—	—	0.56	0.24	—	—	0.56	0.13	—	—	0.48	0.07
	G	—	0.61	0.37	—	—	—	—	0.57	0.23	—	—	0.56	0.13	—	—	0.47	0.07
(d) Glucoside of DDS[e]	NH_2	—	—	0.36	—	—	—	—	—	0.23	—	—	—	0.13	—	—	—	0.07
	G	—	—	0.36	—	—	—	—	—	0.22	—	—	—	0.12	—	—	—	0.07
(e) Unknown substance	NH_2	—	0.61	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	G	—	0.61	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
DDS	NH_2	0.90	—	—	—	—	—	0.86	—	—	—	0.83	—	—	—	0.82	—	—
Glucose (G)	G	—	—	—	0.25	—	—	—	—	—	—	—	—	—	—	—	—	—

Filter paper: Toyo Roshi No. 50 (2 × 40 cm.)

a) Tsuda reagent was used for the detection of NH_2 and 2-aminodiphenyl reagent*1 for glucose (G).

b) Parentheses indicate occasional discoloration of some ampules.

c) 1 cc. of commercial Promin injection was diluted to 30 cc. with 0.2N NH_4OH.

d) Single purification from MeOH-Et_2O.

e) Purified 6 times from MeOH-Et_2O.

TABLE II. Administration to Rabbits

Rabbit	Substance given	Method of dosing	Urine	Plasma
♂, 2.5 kg.	2.5 cc. of (a)	intraveneously from aural vein	Urine of rabbits given (a), about 70 cc. collected for 12 hr.	——
♂, 2.8 kg.	300 mg./kg. of (b) as 30% aq. solution	〃	Urine of rabbits given (b), about 180 cc. collected for 12 hr.	——
♂, 3.0 kg.	〃	〃	——	Plasma (about 40 cc.) of rabbits given (b), collected 2 hr. after dosing
♂, 3.0 kg.	100 mg./kg. of DDS	orally	——	Plasma (about 30 cc.) of rabbits given DDS, collected 3 hr. after dosing
♂, 3.0 kg.	——	——	——	Normal plasma (20 cc.) containing 2 mg. of DDSG
				Normal plasma (20 cc.) containing 2 mg. of (b)

to (e) before freeze-drying. This fact suggests that (d) and (e) are isomers and that DDS N,N′-diglucoside is a mixture of (d) and (e).

III. Antibacterial Activity——For testing the antibacterial activity of Promin and its metabolites labile in the culture medium, *Escherichia coli* (strain $0\sim8$)[*3] was used as it is relatively susceptible to sulfone drugs and the period of incubation is much shorter than acid-fast bacilli. Labile derivatives of DDS were dissolved in ammonium nutrient medium[5] (pH 7.4), filtered by Seitz filter, and prepared into two-fold dilutions from 2×10^{-3} to $2^{-5} \times 10^{-3}M$. Grade of their decomposition in nutrient fluid at incubation temperature was examined by paper chromatography. DDS N,N′-diglucoside and DDSG were decomposed markedly, but at the end of 48 hr., considerable portion was still found to remain unchanged. The method for the determination of antibacterial activity was as reported before.[5]

IV. Rabbit Experiment——As standard, 30% solution of purified Promin powder (b) was used but at the same time, commercial Promin injection (b) was also used for comparison.

All of the collected rabbit urine was centrifuged at 7000 r.p.m. for 20 min. under refrigeration. Each supernatant was freeze-dried immediately at a temperature below 30° and the residue was dissolved in a small quantity of Britton-Robinson's universal buffer[*1] for immediate use. For the collection of plasma, rabbits were killed by total bleeding from the carotid artery and blood was dispensed into several test tubes marked at 20 cc. and containing 1 cc. of 3.8% sodium citrate, and centrifuged at 1500 r.p.m. for 15 min.

Pooled plasma thus prepared was freeze-dried below 30° and the residue was extracted with 10 cc. of MeOH. Insoluble substances were removed by centrifugation at 3000 r.p.m. for 10 min. The supernatant was evaporated *in vacuo*, the residue was reextracted with a small quantity of 80% MeOH containing Britton-Robinson's buffer, and used immediately.

Results

I. Comparison of Glucoside of DDS (d) with Purified Promin (b) and Sodium DDS Mono-N-glucosiduronate (DDSG)——(d) was decomposed to DDS and glucose, likewise DDSG to DDS and glucuronic acid, when examined by ascending chromatography with solvent H[*1] (BuOH-AcOH-H_2O=12.5:3:12.5), but (b) was gradually decomposed and showed marked tailing.

On paper electrophoresis, as described previously,[*1] (b) and DDSG migrated to the anode and (d) to the cathode. The reduction of copper reagent[6] was observed with (d) but not with (b).

II. Metaboic Difference of Promin and DDS

i) Paper Electrophoresis of Matabolic Substances——As shown in Fig. 1, the metabolites in the urine of rabbit injected with (b) (Ub) migrated to cathode as did (c) and DDS, while (b) was found to migrate to the anode and (a) revealed colored bands which migrated

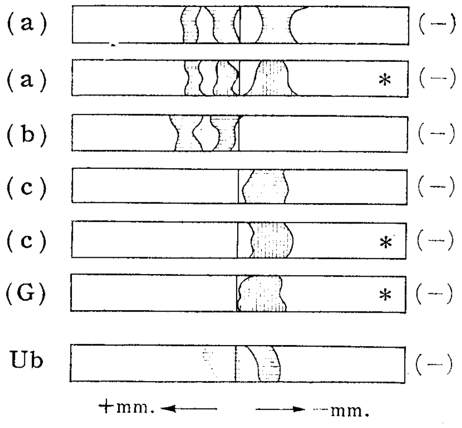


Fig. 1.

Comparison of the Electrophoretic Patterns of the Metabolites and Respective Pure Substances

Buffer : Sörensen's buffer ($M/15$ Na_2HPO_4-$M/15$ KH_2PO_4, pH 7.4).
Current : 0.75 mA/cm., $98\sim85$ v., 3 hr.
Filter paper : Toyo Roshi No. 50 (2 × 18 cm.).
Migration distances (mm.) : (a), −18 +9 +26, (a)*, −16 +8 +25; (b), +7 +28; (c), −16; (c)*, −19; (G)*, −14; Ub, −18 +7.

* Revealed by 2-aminodiphenyl reagent, all the others by Tsuda reagent.

[*3] Donated from Dr. R. Nakaya of the National Institute of Health, Tokyo.

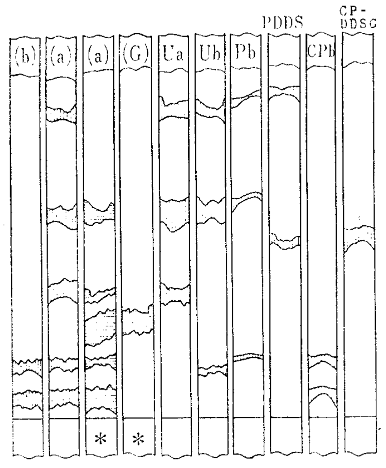
5) S. Tsutsumi : La Lepro, **29**, 75 (1960).

6) N. Nelson : J. Biol. Chem., **153**, 375 (1944); M. Somogyi : *Ibid.*, **160**, 61, 74 (1945).

both to the cathode and anode. In view of such a finding, it can be said that components of Ub is N-glucoside, DDS, or mono-N-acetyl-DDS which migrated to the cathode like DDS under the same condition,⁷⁾ and that N-glucuronide is not present. Further, (b) still showed two migrations to the anode which may be attributed to $OSO_2^-Na^+$.

For detection of mono-N-acetyl-DDS from the urine of rabbit given (a)(Ua) and Ub, the alkaline urine was extracted with ethyl acetate and the evaporated residue was electrophoresed, employing Clark-Lubs' buffer (*N*/5HCl-*M*/5KCl, pH 1.2) as reported in Part II.⁷⁾ Also, mono-N-acetyl-DDS was not clearly detectable from Ub and only faintly detectable from Ua.

ii) **Buffered Chromatography of the Metabolic Substances**——Pictures of buffered chromatography developed by A (Fig. 2.) showed that the main metabolite of both Ua and Ub was (e) and, as other metabolites, there were faintly colored bands corresponding to DDS and (d) in the case of Ua and those corresponding to DDS and one of the two colored bands of (b) in the case of Ub. When developed by other solvent systems, Ua showed a colored bands corresponding to DDS, (e), and (d), at Rf 0.86, 0.57, and 0.21 in the case of B, 0.83, 0.56, and 0.11 in C, and 0.82, 0.46, and 0.07 in D.



Solvent System : PrOH-BuOH-Buffer (3:2:3)

	Rf Values					
(b)	—	—	—	—	0.15	0.06
(a)	0.90	0.61	0.37	—	0.15	0.07
(a)*	—	0.61	0.37	0.24	0.15	0.05
(G)*	—	—	—	0.25	—	—
Urine of rabbit given (Ua)	0.90	0.62	0.37	—	—	—
Urine of rabbit given (Ub)	0.90	0.62	—	—	0.14	—
Plasma of rabbit given (Pb)	0.93	0.64	—	—	0.16	—
Plasma of rabbit given DDS (PDDS)	0.94	—	0.48	—	—	—
Plasma from control rabbit added with (b) (CPb)	—	—	—	—	0.15	0.08
Plasma from control rabbit added with DDSG (CPDDSG)	—	—	0.48	—	—	—

* Revealed by 2-aminodiphenyl reagent, others by Tsuda reagent.

Fig. 2. Comparison of the Chromatographic Patterns of Metabolites and Respective Pure Substances

In view of the results obtained, it was presumed that Promin was metabolized by stepwise decomposition like desulfonation and, in the case of DDS, as reported by Bushby, *et al.*,⁸⁾ DDSG was detected even in the blood plasma not to speak of urine.

Discussion

Metabolic difference between Promin and DDS may not be the only reason for the fact that DDS is effective in the treatment of Promin-resistant patients. In this connection, investigations were made on the antibacterial activity of those two drugs and their metabolites. Previously, Sweet, *et al.*⁹⁾ made an inference that Promin is probably present mainly as DDS mono-N-glucose sulfonate *in vivo* and its single free NH_2 radical is playing an important rôle in the treatment of tuberculosis and other bacterial infections.

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7) S. Tsutsumi : La Lepro, **29**, 88 (1960).
8) S. R. M. Bushby, A. J. Woiwod : Biochem. J., **63**, 406 (1956).
9) L. A. Sweet, E. H. Payne : Intern. J. Leprosy, **18**, 254 (1950).

On the other hand, DDS N,N′-diglucoside and DDS N,N′-digalactoside[2)] were once used as bacteriostatic drugs like sulfonamides under the names of AMGL and Tibatin, respectively, but experiments made with *Escherichia coli*, which is relatively susceptible to sulfone drugs, showed no antibacterial activity in the fluid medium except DDS. The molecular inhibitory dilutions of the test were as follows: DDS, 0.5×10^{-3} (0.25×10^{-3}); purified Promin, (10^{-3}); DDS N,N′-diglucoside, (10^{-3}); DDSG, (10^{-3}); DDSS,[*1] (10^{-3}) (Parentheses indicate incomplete inhibition).

Antibacterial activity of DDS on tubercle bacilli, however, is known to be much inferior to other antituberculosis drugs. These facts make it extremely difficult to explain the absence of cross-resistance between Promin and DDS only by their antibacterial activity except to say that Hansen's bacilli have some particular specific drug susceptibility. At any rate, it might be admitted on the basis of the analysis of metabolites excreted into urine, that they work on Virchow's lepra cells, in which Hansen's bacilli can survive and multiply, as two different kinds of metabolites, namely, as mono-N-conjugates such as DDSG, and mono-N-acetyl-DDS and as desulfonated substances. As for the main metabolite of Promin (e), it was found to be an isomer of DDS N,N′-diglucoside rather than the DDS mono-N-glucoside desulfonated from DDS mono-N-glucose sulfonate inferred by Sweet, *et al.*[9)] However, studies will be made on further details of this isomer as well as on the difference in Promin metabolism between resistant and non-resistant patients.

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Summary

Metabolic difference between Promin and 4,4′-diaminodiphenyl sulfone was investigated using urine and plasma of rabbits. It was clarified that Promin is metabolized mainly to N-glucoside of 4,4′-diaminodiphenyl sulfone, receiving desulfonation. Further, N-conjugation was not clearly proved, being different from 4,4′-diaminodiphenyl sulfone which was metabolized to mono-N-conjugates.

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