METABOLISM OF FORMYCIN AND FORMYCIN B *IN VIVO*

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Formycin and formycin B are excreted into urine in their metabolized form which was isolated. This metabolized product was found to be an oxidized product of formycin B.

As reported in a previous paper[1], formycin is phosphorylated by EHRLICH carcinoma cells and its transportation into cells is stimulated by the phosphorylation. On the other hand, formycin is deaminated to formycin B by the cells, and most of the formycin in the medium is deaminated to formycin B. Therefore, it was considered that most of the formycin injected into animals would be excreted as formycin B. However, as reported in this paper, formycin B is further oxidized and excreted in urine.

Methods and Materials

Formycin was prepared by the fermentation of *Nocardia interforma* and recrystallized three times from distilled water. Formycin B was obtained by chemical deamination of formycin. ^{3}H-Formycin was prepared by Dr. KOMAI, Isotope Division, National Institute of Health, Tokyo. It showed the same antibacterial activity to *Xanthomonas oryzae* as formycin.

Formycin, formycin B and the metabolized product were assayed by the high voltage paper electrophoresis (Savant Inst. Inc.). The buffer was a mixture of formic acid, acetic acid and water (25 : 75 : 900 v/v, pH 1.8) and the electrophoresis was done at 2,500 V for 90 minutes. Formycin, formycin B and the metabolized product were detected by the inhibitory activity against *X. oryzae* or by radioactivity. The radioactivity was measured by a liquid scintillation counter (Beckman Inst. Inc. CPM-200). The activity of formycin was also determined by the cylinder plate method, using *X. oryzae* as the test organism.

Results

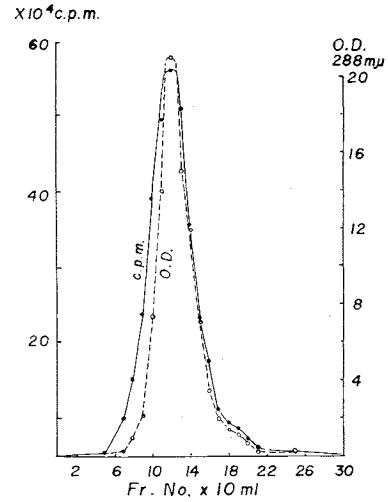
When formycin or formycin B was injected into mice, their concentration in urine was unexpectedly small, as determined by the activity against *X. oryzae*. Therefore, the urine was examined by high voltage electrophoresis. In addition to formycin B, a spot inhibiting growth of *X. oryzae* was found in the region close to formycin B. It showed slightly less mobility, toward the cathode than formycin B. This spot was cut out and the ultraviolet spectrum of the material was different from formycin B. After Dowex -1 chromatography with water, it appeared in effluent earlier than formycin or formycin B and gave a yellow fluorescence. This substance was found to be a metabolized product of formycin by examination of mouse urine after the injection of tritiated formycin (^{3}H-formycin).

Table 1. Excretion of formycin (FM) by the rabbit.

Rabbit 2.7 kg
Formycin 50 mg/kg subcutaneously
FM 134 mg, ^{3}H-FM 3 mg) 2.52 ml of saline

Time	Vol.	FM conc.		
		cpm ($\times 10^5$)	mg in total	Activity on *X. oryzae* (mcg in total)
30 min	7.6	0.02	0.17	—
1 hr	9.6	12.2	10.11	432.0
2	11.6	16.6	13.71	1,013.0
4	18.6	16.3	13.46	630.0
6	10.0	7.5	6.24	300.0
8	20.0	0.4	0.31	—
Total		53.02	44.0 mg	2.37 mg
Recovery		31.97%		1.72 %

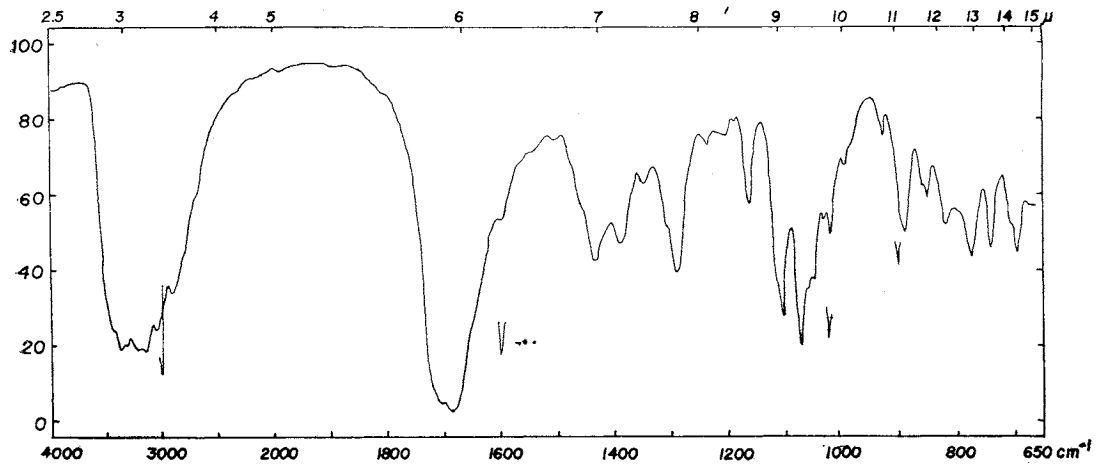
Fig. 1. Chromatography of oxoformycin B on Dowex 1X4



In order to isolate the metabolized product, 50 mg/kg of formycin was subcutaneously injected in a rabbit weighing 2.7 kg and the urine collected for 8 hours after the injection. The formycin injected was a mixture of 3 mg of ^{3}H-formycin and 134 mg of formycin, dissolved in 2.52 ml of distilled water. Total cpm of formycin injected was 16.5×10^6. The radioactivity and the activity against *X. oryzae* were examined on urine taken at 30 minutes, 2 hours, 4 hours, 6 hours and 8 hours with the result shown in Table 1. The excreted amount shown by the radioactivity is about 20 times larger than the amount shown by the disc method using *X. oryzae* as the test organism and formycin as the standard.

The excretion product of formycin in the urine was extracted and purified. The volume of the urine collected in 6 hours after the injection was 56 ml with a total radioactivity of 5.26×10^6 cpm. It was passed through a column (18 mm × 200 mm) of

Fig. 2. Infrared absorption spectrum of oxoformycin B (KBr pellet)



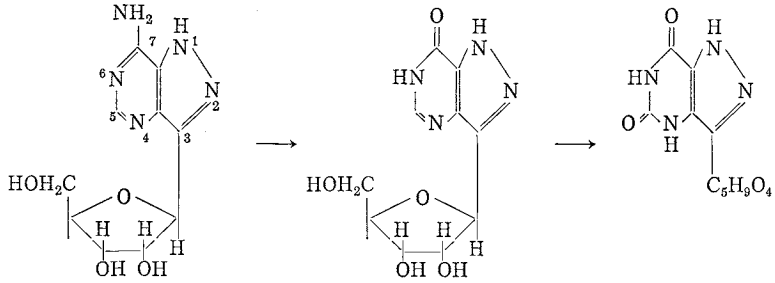
Dowex 1 X 4 resin (15 ml, in Cl^- form) and eluted with distilled water. The effluent was cut into 10 ml fractions, and examined for radioactivity and optical density at 288 mμ. The result is shown in Fig. 1. The total activity of fractions 3 to 20 (Fig. 1) was 4.4×10^6 cpm (recovery of the total radioactivity in urine: 83.7 %). Fractions 11 to 20 were combined, concentrated under vacuum to 3~5 ml and kept at 4°C overnight. Yellowish crystals appeared and were collected (42.5 mg, 3.0×10^4 cpm/mg). The crystals were dissolved in 10 ml of water and passed through a carbon column (8 mm × 140 mm; 3 ml of carbon). The column was washed with 100 ml distilled water. The metabolized product was eluted with 100 ml of acetone-1/100 N HCl (1 : 1 v/v). The radioactive fractions eluted were combined (35 ml) and concentrated under vacuum and cooled to give 13 mg of crystals. The specific activity of the crystals was 3.01×10^4 cpm/mg. Similar crystals were obtained by the same procedure after injecting cold formycin.

The crystals obtained from urine after injection of cold formycin were white, melting at 274°C with decomposition. Calcd. for $C_{10}H_{12}O_6N_4$: C 42.25, H 4.25, N 19.27, O 33.78; found: C 41.69, H 4.46, N 18.43, O 33.32. The infrared spectrum shown in Fig. 2 indicates the existence of two carbonyl groups at 1,690 and 1,710 cm^{-1}. The ultraviolet spectrum gave in H_2O, λ_{max} at 288 mμ ($E^{1\%}_{1cm}$ 395); 1/10 N HCl, λ_{max} at 288 mμ ($E^{1\%}_{1cm}$ 220); in 1/10 N NaOH, λ_{max} at 304 mμ ($E^{1\%}_{1cm}$ 168). The signal of 8.02 ppm in n. m. r. of formycin B is not found in n. m. r. of oxoformycin B. As reported in another paper, the structure of the base part of oxoformycin B assumed to be 5,7-dihydroxy-pyrazolo-[4,3-*d*] pyrimidine. This metabolized product of formycin or formycin B is designated oxoformycin B. On electrophoresis, oxoformycin B moves toward the cathode 2~4 cm and under the same conditions formycin moves 32 cm and formycin B 4~6 cm.

The specific activity of the ^{3}H-oxoformycin B crystals obtained from urine was 3.01×10^4 cpm/mg, or 8.55×10^6 cpm/mmole, since the molecular weight of ^{3}H-oxoformycin B is 284. The molecular weight of ^{3}H-formycin monohydrate is 285, and the specific activity of formycin monohydrate injected was 1.21×10^5 cpm/mg, or 3.45×10^7 cpm/mmole. This result indicates that about 75 % of the tritium in ^{3}H-formycin was on C-5 of the formycin nucleus, the position which is oxidized.

At 200 mcg/ml, oxoformycin B does not inhibit growth of *S. aureus*, *M. flavus*, *Sar. lutea*, *E. coli*, *Sal. enteritidis*, *Sh. flexneri*, *Pro. vulgaris*, *Ps. aeruginosa*, *Kleb. pneumoniae*, *Ser. marcescens*, *B. cereus*, *B. subtilis*, *Myc.* 607, *Myc. phlei* and *Ps. fluorescens* when tested on nutrient agar medium. At 200 mcg/ml, it showed no inhibition of *Piricularia oryzae*, *Candida albicans*, *Candida pseudotropicalis*, *Ophiobolus miyabeanus*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Trichophyton asteroides* on 1 % glucose nutrient agar medium. It inhibited *X. oryzae* on 1 % glucose nutrient agar medium at 25 mcg/ml.

When formycin B was injected in mice, the urine was shown by high voltage paper electrophoresis to contain mainly oxoformycin B. Thus, metabolism of formycin or formycin B *in vivo* can be shown as follows:



Formycin Formycin B Oxoformycin B

References

1) Umezawa, H.; T. Sawa, Y. Fukagawa, I. Homma, M. Ishizuka & T. Takeuchi: Studies on formycin and formycin B in cells of Ehrlich carcinoma and *E. coli*. J. Antibiotics, Ser. A (in press)