METABOLIC CONVERSION OF FORMYCIN B TO FORMYCIN A AND TO OXOFORMYCIN B IN *NOCARDIA INTERFORMA*

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(Received for publication January 26, 1968)

A strain of *Nocardia interforma* having low productivity of formycin A was selected. Washed cells of this strain convert formycin B to oxoformycin B (reaction I), while those of the original strain collected during formycin A-producing phase convert formycin B to formycin A (reaction II). Reaction I is inhibited by allopurinol, an inhibitor of xanthine oxidase. Reaction II is inhibited by hadacidin, an inhibitor of adenylosuccinate synthetase, but not by azaserine. As intermediates of reaction II, formycin B-monophosphate and formycin A-monophosphate were detected in the nucleotide pool of the cell. No other organisms than *Nocardia interforma* perform reaction II except *Xanthomonas oryzae*. Besides *Nocardia interforma*, *Pseudomonas fluorescens* and *Streptomyces kasugaensis* perform reaction I.

Formycin A and formycin B were isolated from the culture filtrate of *Nocardia interforma*[1,2] and identified as 7-amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine and 3-(β-D-ribofuranosyl)pyrazolo[4,3-d]6(H)-7-pyrimidone, respectively[3]. They are analogs of adenosine and inosine with unusual C-riboside linkages. Oxoformycin B, a xanthosine analog of the formycin family, was first found in the urine of a rabbit injected with formycin A or formycin B[4]. Since oxoformycin B was proved to be biologically inert and no other metabolite appeared in the urine, this compound was assumed to be an end product by detoxication mechanism of the rabbit[4].

We observed recently that, in *Nocardia interforma*, formycin B was converted to formycin A as well as to oxoformycin B. In this connetion, studies were made to clarify the biochemical events which would be involved in these metabolic conversions and it was found that amination of formycin B to formycin A took place on the level of their phosphorylated forms, probably catalyzed by adenylosuccinate synthetase[5]. This amination process is likely to be involved in the biosynthesis of formycin A in *Nocardia interforma*.

Materials and Methods

Washed-cell suspension and reaction process:

Cells of *Nocardia interforma* which had been grown under conditions for optimum production of formycin A[1], were collected at various days of growth. The cells were washed once in physiological saline by suspension and centrifugation, and the resulting

cell-paste was suspended at 4 % (v/v) in 25 ml of glucose-CZAPEK medium containing 1 mg of ^{3}H-formycin B (5 μc). Reaction proceeded at 27°C for 3 hours with reciprocal shaking and was terminated by chilling in an ice bath. The cells were separated from the medium by centrifugation, washed twice in physiological saline containing 10^{-4} M of NaF (no NaF for Procedure II, see below) and submitted to analysis of labeled products which accumulated in intracellular nucleotide pool (Procedure I). The medium and washings were combined and submitted to analysis of the labeled products which were released in the medium (Procedure II). No significant amount of labeled material other than ^{3}H-formycin A, ^{3}H-formycin B or ^{3}H-oxoformycin B was detected in the medium under these conditions.

Procedure I (identification of ^{3}H-formycin A-monophosphate and ^{3}H-formycin B-monophosphate):

The cells were extracted with 20 ml of cold 10 % perchloric acid and 20 ml×2 of cold 5 % perchloric acid. The extracts were combined, neutralized with 10 % KOH and filtered. The filtrate was charged on a charcoal (1 g) column. After washing off unadsorbed materials with water, 50 ml of ammoniacal ethanol (ethanol – conc. NH_4OH – water, 50 : 5 : 45) was passed through. The eluate was evaporated to dryness *in vacuo*, and the residue was dissolved in 4 ml of water. The solution was charged onto a column of Dowex 1×4 (100~200 mesh, Cl^- form, 3 ml of wet volume). After washing off unadsorbed materials (mostly intracellular ^{3}H-nucleosides) with water, nucleotides were eluted with 0.01 N HCl-0.02 N NaCl. The eluate was again applied to a charcoal (500 mg) column to remove unadsorbed inorganic salts. The nucleotide fraction eluted with ammoniacal ethanol was concentrated *in vacuo* to 1 ml and analyzed by (a) paper chromatography developed with a solvent system of *n*-butanol-ethanol-water (50 : 15 : 35)[6], (b) DEAE-cellulose paper chromatography developed with a solvent system of *tert*-amyl alcohol-formic acid-water (3 : 2 : 1)[7] and (c) high voltage paper electrophoresis at 3,500 volt for 15 minutes in a buffer system of formic acid-acetic acid-water (25 : 75 : 900). Authentic preparations of formycin A-monophosphate and formycin B-monophosphate which were prepared by chemical or enzymatic processes as described in a previous paper[8] were run as markers in each procedure. ^{3}H-Analogs were localized by inspection of the paper under ultraviolet light, appropriate areas were cut out and placed in counting vials, each of which contained 1 ml of water and 9 ml of scintillation mixture (PPO and naphthalene in dioxane), and the radioactivity was determined in a Beckman Liquid Scintillation System. For conclusive identification, a fraction containing each ^{3}H-nucleotide analog was treated with alkaline phosphatase (intestinal) to give the corresponding nucleoside whose identification procedure is given in Procedure II.

Procedure II (identification of ^{3}H-formycin A, ^{3}H-formycin B and ^{3}H-oxoformycin B):

The combined solution was charged onto a charcoal (1 g) column. After washing off unadsorbed materials with water, labeled products were eluted with 80 ml of 50 % aqueous acetone (pH 8.0). The eluate was concentrated to 2 ml, which was submitted to two analytical procedures: (a) 0.05 ml was spotted onto a paper strip (Toyo Roshi, No. 51A) for analysis by high voltage paper electrophoresis and the radioactivity in appropriate fractions was counted as stated above; (b) the rest of the concentrated solution was charged onto a column of Dowex 50×2 (H^+ type, 4 ml of wet volume) and eluted thoroughly with water. ^{3}H-Oxoformycin B appeared at the front followed by ^{3}H-formycin B with satisfactory separation. ^{3}H-Formycin A was eluted with 0.5 N NH_4OH. A suitable volume of each fraction was analyzed for radioactivity.

Since ^{3}H-oxoformycin B is lacking in ^{3}H at C-5 of the chromophore moiety, the rate of conversion to this compound on the basis of ^{3}H recovery was corrected by a factor of 0.58 which had been empirically obtained by a preliminary experiment.

Results

Productivity of an antibiotic is generally reduced after repeated passages and

prolonged storage of the producing organisms in agar slant cultures. On this basis, we purposely selected a strain of *Nocardia interforma*, designated here as Strain (a), which showed poor production of formycin A. The strain retaining high productivity of formycin A is designated here as Strain (b). As shown in Table 1, accumulation of formycin A by Strain (b) reached 112 mcg/ml at the 6th day of culture while Strain (a) accumulated only 15 mcg/ml at the 4th day of culture.

Table 1. Formycin A production by strain (a) and strain (b) of *Nocardia interforma* (Test organism : *Xanthomonas oryzae*)

Strain	Formycin A (mcg/ml) in culture broth					
	2 days	3 days	4 days	5 days	6 days	7 days
Low productivity (a)	4	9	15	8	7	5
High productivity (b)	11	27	39	47	112	96

When ^{3}H-formycin B was exposed to washed cells of these two strains, the reaction patterns as followed by ^{3}H-radioactivity were different. As shown in Table 2, the amination reaction, yielding formycin A, predominated in the metabolism of Strain (b) especially at the 5th day of the culture. It should be remembered that this strain showed the maximum rate of formycin A-production during the period from the 5th to the 6th day of the culture. In contrast, Strain (a) showed marked rates of oxidation, yielding oxoformycin B. Only limited rate of amination was observed with this strain throughout the cultivation. Such good correlation between the amination activity and the trend of formycin A-production, in respect to the type of antibiotic-producing strain as well as the time course phenomenon, led us to assume that metabolic processes which were responsible for the conversion of formycin B to formycin A should be involved in the *de novo* synthesis of formycin A.

Table 2. Metabolic conversion of formycin B to formycin A and to oxoformycin B

Strain	Conversion to	$\frac{^{3}H \text{ as Formycin A or Oxoformycin B}}{^{3}H \text{ recovered in the medium}} \times 100(\%)$				
		3 days	4 days	5 days	6 days	7 days
Low productivity (a)	Formycin A	6.7	3.0	1.1	—	0
	Oxoformycin B	65.4	73.0	73.7	—	28.4
High productivity (b)	Formycin A	5.7	42.1	73.0	17.6	—
	Oxoformycin B	2.1	11.6	16.1	8.7	—

Considering our earlier unpublished observation as well as the observation made by OHTAKE *et al.*[9] that the accumulation of formycin B did not precede but followed the accumulation of formycin A, it is likely that the *de novo* synthesis of formycin A involves formycin B-monophosphate, but not formycin B itself. Exogenously added formycin B should first be converted to this key intermediate. This possibility was examined by the next two

Table 3. Effect of inhibitors on the metabolic conversion of formycin B to formycin A Test organism : Strain (b) of *Nocardia interforma*.

Inhibitor	Concentration (mcg/ml)	Conversion* (%)
None (control)		26.8
Hadacidin	40	0.6
Azaserine	20	26.8

* $\frac{^{3}H \text{ recovered as formycin A}}{^{3}H \text{ added as formycin B}} \times 100$

experiments. Because of the close similarity in structure, we suspected that amination of formycin B-monophosphate, the proposed intermediate, could be catalyzed by adenylosuccinate synthetase by analogy of conversion of inosine-5'-monophosphate to adenylosuccinate. As shown in Table 3, the amination reaction was effectively blocked by hadacidin, an inhibitor of this enzyme[10], while azaserine, a glutamine antagonist[11], was without effect.

For a better demonstration of this metabolic scheme, an attempt was made to detect formycin B-monophosphate and formycin A-monophosphate in the nucleotide pool of the cell. Adenylosuccinate synthetase is one of the most vulnerable enzymes which are involved in the nucleic acid metabolism, and NaF was reported to inhibit it in a specific manner[12]. When the cells were labeled with ^{3}H-formycin B in the presence of NaF and $CuSO_4$, accumulation of ^{3}H-formycin B-monophosphate in the nucleotide pool was evident while that of ^{3}H-formycin A-monophosphate was limited, as shown in Fig. 1 (a). The rate of conversion of ^{3}H-formycin B to ^{3}H-formycin A was severely inhibited. $CuSO_4$ was used because of its possible stimulation of some phosphorylating enzyme[13]. Another advantage in using NaF and $CuSO_4$ in combination was that both of them were inhibitors of phosphatase and therefore could protect those phosphorylated intermediates from the attack of the enzyme. When NaF and $CuSO_4$ were added to the system 60 minutes after the reaction was initiated, that is, after the conversion of ^{3}H-formycin B to ^{3}H-formycin A was allowed to proceed to some extent, an appreciable amount of ^{3}H-formycin A-monophosphate as well as ^{3}H-formycin B-monophosphate was detected, as shown in Fig. 1 (b).

Oxidation of formycin B to oxoformycin B was inhibited by allopurinol, 4-hydroxypyrazolo(3,4-d)pyrimidine, an inhibitor of xanthine oxidase[14]. One may

Fig. 1. Electrophoretic patterns of the nucleotide pool labeled with ^{3}H-formycin B.

Test organism :
Strain (b) of *Nocardia interforma*.

(a) : NaF and $CuSO_4$ were added to 10^{-3} M at 0 minute together with ^{3}H-formycin B. Under this condition, only 1.5 % of ^{3}H-radioactivity, originally added as formycin B, was recovered as formycin A. Total radioactivity recovered from the paper was 1,295 cpm.

(b) : ^{3}H-Formycin B was added at 0 minute while the inhibitors were added at 60 minutes. Under this condition, 4.6 % of ^{3}H-radioactivity, originally added as formycin B, was recovered as formycin A. Total radioactivity recovered from the paper was 1,040 cpm.

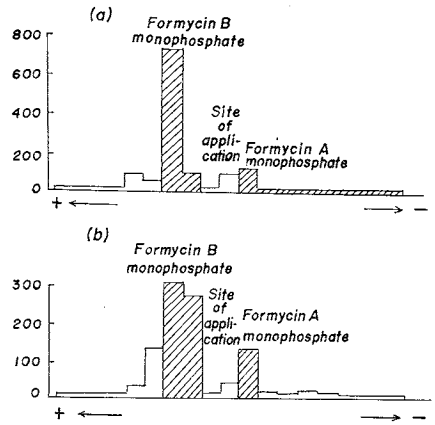

Table 4. Effect of inhibitors on the metabolic conversion of formycin B to oxoformycin B

Test organism :
Strain (a) of *Nocardia interforma*

Inhibitor	Concentration (mcg/ml)	Conversion* (%)
None (control)		21.1
Hypoxanthine	200	10.6
Xanthine	200	14.5
Allopurinol	80	5.1

$$* \frac{^3\text{H recovered as oxoformycin B}}{^3\text{H added as formycin B}} \times 100$$

To block the alternative pathway to formycin A, hadacidin was added to each system at 40 mcg/ml which had caused no effect on this oxidation process.

assume that this reaction is catalyzed by xanthine oxidase which is an omnipresent enzyme with low substrate specificity. However, contradictory to such assumption, either hypoxanthine or xanthine which could compete for xanthine oxidase with formycin B caused only a partial inhibition of this reaction even at 200 mcg/ml. These results are shown in Table 4. In addition, a purified preparation of xanthine oxidase (milk; Sigma Co. Ltd.) failed to oxidize formycin B. This observation is consistent with the recent report by LETTRÉ *et al.*[15] that 9-ribosyl purines did not serve as substrates for xanthine oxidase. To elucidate these questions, studies are in progress to isolate and identify the enzyme (or enzymes) responsible for oxidation of formycin B.

A variety of organisms other than *Nocardia interforma* were examined for their metabolic activity on formycin B and the results are summarized in Table 5. No organism converted formycin B to formycin A except *Xanthomonas oryzae* which had been reported to be slightly active[16]. It was interesting to find that several organisms were active in converting formycin B to oxoformycin B.

Table 5. Metabolic conversion of formycin B to formycin A and to oxoformycin B by various organisms

Organisms		Metabolic conversion to	
		oxoformycin B	formycin A
Bacteria	*Bacillus subtilis* NRRL-558	−	−
	Escherichia coli K-12	−	−
	Pseudomonas fluorescens	++	−
	Staphylococcus aureus FDA 209P	−	−
	**Xanthomonas oryzae*[16]	++	+
Actinomycetes	*Nocardia interforma*	++ (Strain a)	++ (Strain b)
	Streptomyces griseus NIHJ-4(S-4)	−	−
	Streptomyces kasugaensis	+	−
Mammalian cells	*Mouse (*in vivo*)[18]	+	−
	*Rabbit (*in vivo*)[4]	+	−

To prepare the cell suspensions of bacteria and actinomycetes, 2-day-old and 5-day-old cell mats on agar slants were used respectively. Other conditions were as given under Materials and Methods.
* Previous observations.

Discussion

Formycin B is accumulated in the culture broth of *Nocardia interforma* after the formycin A-producing stage in parallel with concomitant decrease in formycin A content. Apparently formycin B is derived from formycin A by catalytic action of adenosine deaminase. Accordingly, the terminal steps of the biosynthetic route of formycin family should be as the following scheme.

(?) ⟶ Formycin B-monophosphate ⟶ Formycin A-monophosphate ⟶
Formycin A ⟶ Formycin B ⟶ Oxoformycin B

Oxoformycin B seems to be an irreversible end product, since it was found stable to metabolism of various organisms including *Nocardia interforma*.

Acknowledgment

The authors are grateful to Prof. HAMAO UMEZAWA for his interest in this work and for many fruitful discussions.

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