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The Interaction of the Nucleoside Analogues, Formycins A and B, with Xanthine Oxidase and Hepatic Aldehyde Oxidase

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SUMMARY

Formycin B, an analogue of inosine, has been found to be oxidized to oxoformycin B, the xanthosine analogue, by hepatic aldehyde oxidase (EC 1.2.3.1). The K_m was 2.0×10^{-4} M. Formycin B and formycin A, the adenosine analogue, are not substrates but are competitive inhibitors for the related enzyme, xanthine oxidase, with K_i values of 1.3×10^{-5} M. Neither of these enzymes has previously been known to interact with purine nucleosides or close structural analogues of nucleosides. The effects of several related purine base and nucleoside analogues on hepatic aldehyde oxidase, bovine milk xanthine oxidase (EC 1.2.3.2), calf intestinal adenosine deaminase (EC 3.5.4.4), and human erythrocytic purine nucleoside phosphorylase (EC 2.4.2.1) are reported. A method for purifying aldehyde oxidase from rabbit liver is described.

INTRODUCTION

Formycin B [7-hydroxy-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine] was first discovered in Umezawa's laboratory in 1964 (1–4). It is an analogue of the natural purine ribonucleoside, inosine, and was shown to be identical with the antibiotic laurusin (5, 6) (Fig. 1).

This laboratory has been interested in formycin B and related compounds as possible inhibitors or substrates for enzymes of nucleoside metabolism. Formycin B has been found to be a competitive inhibitor of erythrocytic purine nucleoside phosphorylase, both with the isolated enzyme and with intact cells (7). Since formycin B is not a substrate for purine nucleoside phosphorylase (EC 2.4.2.1) and there is little evidence for the occurrence of an inosine kinase in animal tissues, which might be capable of converting formycin B to the monophosphate derivative, one might expect that formycin B would be metabolically inert.

However, we were informed by Drs. M. Hori and H. Umezawa that when formycin B was injected into rabbits and mice, both formycin B and a metabolite were excreted in the urine. This metabolite has been identified as an oxidized product of formycin B and has, therefore, been called oxoformycin B (8). This laboratory has confirmed the formation of this metabolite, and a preliminary report of part of this work has been presented (9). Since the presentation of this preliminary report, Tsukada *et al.* (10) have also concluded that liver aldehyde oxidase (EC 1.2.3.1) is the

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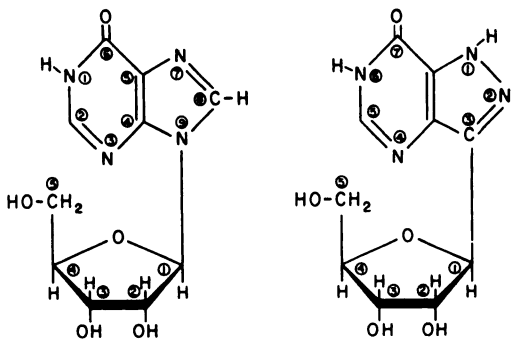

FIG. 1. *Structures of inosine and formycin B (laurusin)*

principal enzyme responsible for the oxidation of formycin B.

In addition, these results have led to the investigation of the metabolism of other compounds closely related in structure to formycin B, and of the interaction of the formycins with several enzymes that react with purines and their derivatives.

MATERIALS AND METHODS

Materials

Samples of formycins A and B were gifts from Dr. Hamao Umezawa of the Microbial Chemistry Research Foundation, Institute of Microbial Chemistry, Tokyo. Samples of formycins A and B and their 5′-phosphate mononucleotides were obtained from Dr. J. Frank Henderson of the McEachern Laboratory, University of Alberta, Edmonton. 7-Deazainosine [4-hydroxy-7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine] and 7-deazathioinosine [4-thio-7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine], both of which are derivatives of tubercidin (11), and 7-hydroxypyrazolo[4,3-*d*]pyrimidine and 7-hydroxy-3-methylpyrazolo[4,3-*d*]pyrimidine were gifts from Dr. R. K. Robins, University of Utah, Salt Lake City. A preparation of purified human erythrocytic purine nucleoside phosphorylase was provided by Dr. R. P. Agarwal of this laboratory. Frozen young rabbit livers were purchased from Pel-Freez Biologicals, Inc., and N^1-methylnicotinamide chloride and adenosine deaminase (from calf intestine) were purchased from the Sigma Chemical Company. Xanthine oxidase (from milk) (EC 1.2.3.2) was obtained from the Worthington Biochemical Corporation. All other compounds used were of the highest purity commercially available.

Methods

Chromatographic separation of formycin B and oxoformycin B. The ascending technique was employed for both solvent systems, and Whatman No. 3MM chromatography paper was used.

1. Carter's isoamyl alcohol system (12): The solvent contained 2 volumes of 5% dibasic sodium phosphate to 1 volume of isoamyl alcohol. Chromatograms were developed for about 10 hr. The R_F values for formycin B and oxoformycin B were approximately 0.7 and 0.6, respectively.

2. Ammonium bicarbonate system: The solvent was an 85% saturated aqueous solution of ammonium bicarbonate (16 g/100 ml) equilibrated in the tank for at least 24 hr before use. Chromatograms were developed for about 2 hr. The R_F values for formycin B and oxoformycin B were approximately 0.62 and 0.52, respectively. Both compounds could be eluted with water and the solutions concentrated by flash evaporation or lyophilization.

Incubation of mouse liver homogenates with formycin B. Liver from a fasted mouse was homogenized in 3 volumes of 0.15 M KCl. Reaction mixtures were incubated for 60 min at 37° and contained 4.0 mg of formycin B and 0.8 ml of liver homogenate in a total volume of 2.0 ml of 0.9% NaCl. Other flasks were set up which contained, in addition, 0.1 mg of TPN^+, 0.1 mg of DPN^+, or 0.5 mg of allopurinol. Control incubations were denatured with perchloric acid at zero time, and the rest after 60 min. The supernatant fluids from these flasks were neutralized with potassium bicarbonate, and a small aliquot from each flask was spotted on No. 3MM paper and chromatographed in ammonium bicarbonate. Similar experiments were also set up in which the incubation flasks contained, in addition, 0.5 mg of allopurinol.

Purification of Rabbit Liver Aldehyde Oxidase.

The initial stages of the purification procedure were adapted from the method of Rajagopalan, Fridovich, and Handler (13).

Step 1: Preparation of the homogenate and heat denaturation. Frozen rabbit liver (900 g) was thawed slowly at 4° overnight and then homogenized in a Waring Blendor in 5 volumes of distilled water at room temperature. Aliquots of about 250 ml each were heated as rapidly as possible to 67–70° in a boiling water bath and then maintained at this temperature for 2 min. The homogenate was cooled quickly to 40° by the addition of ice and then placed in an ice bath until all the other aliquots had been treated in a similar manner. The precipitate was removed by centrifugation at 10,000 × *g* for 60 min, and the reddish-brown supernatant fluid was collected and stored at 4° overnight.

All steps from this stage onward were carried out at 4°, using 0.005 M potassium phosphate buffer, pH 7.8, containing 0.005% EDTA.

Step 2: Ammonium sulfate fractionation. The supernatant fluid from the previous step was brought to 50% saturation by adding solid ammonium sulfate (298 g/liter). After the solution had stood for 60 min, the precipitate was collected by centrifugation at 10,000 × *g* for 1 hr. Most of the red-colored substances remained in the supernatant fluid. The precipitate was dissolved in 0.005 M potassium phosphate buffer and dialyzed overnight against five changes of the same buffer (4 liters of buffer were used for each change). All dialysis tubing had previously been treated with boiling EDTA solution.

Step 3: Calcium phosphate gel-cellulose column chromatography. Whatman CFH cellulose (120 g, coarse fibers) and calcium phosphate gel (5 g, dry weight) (14) were packed in a 5.0 × 40 cm column and equilibrated with the above 0.005 M phosphate buffer. The dialyzed enzyme was loaded directly onto the column and washed with 200 ml of the same buffer. Elution was carried out with a linear potassium phosphate buffer (pH 7.8) gradient varying from 0.005 M to 0.4 M in a total volume of 1500 ml. Fractions (10 ml) were collected and assayed for activity both with N^1-methylnicotinamide and with formycin B. Ferricyanide was used as the electron acceptor with both substrates, so that the activities could be compared directly. The fractions containing the highest specific activity of aldehyde oxidase were pooled.

Step 4: Ammonium sulfate precipitation and dialysis. Step 2 was repeated as a means of concentrating the pooled enzyme in a suitable volume to load onto the DEAE-cellulose column.

Step 5: DEAE-cellulose column chromatography. The dialyzed enzyme was loaded directly onto a DEAE-cellulose phosphate column (5.0 × 35 cm) equilibrated with the 0.005 M phosphate buffer. The column was washed with 500 ml of the same buffer, and then a linear gradient was started from 0.005 M to 0.4 M phosphate buffer to a total volume of 2 liters; 10-ml fractions were collected, assayed, and pooled as in step 3.

Step 6: Bio-Gel P-200 column chromatography. After storage overnight, enzyme from 100 ml of the 235-ml pool from step 5 was precipitated with 50% ammonium sulfate, collected by centrifugation, and dissolved in as small a volume as possible of 0.05 M phosphate buffer (about 0.2 ml). This sample was then loaded onto a Bio-Gel P-200 column (2.2 × 65 cm) in equilibrium with 0.05 M phosphate buffer. The enzyme was eluted with the same buffer.

Other Methods

Measurement of protein concentration. The protein concentration was measured by the method of Waddell (15). The difference between the absorbance at 215 and 225 mμ was multiplied by a factor of 144 to give the protein concentration in micrograms per milliliter.

Spectrophotometric assays. A Beckman DU spectrophotometer equipped with a Gilford optical density converter and recorder was used.

Assay of aldehyde oxidase activity. Two assay systems were employed, both adapted from the methods of Rajagopalan and Handler (16), which used arbitrary absorbance units. In both systems, the full range of the

Gilford scale was set at 0.1 absorbance. Enough aldehyde oxidase was added to the cuvette to give a reasonable velocity.

1. Ultraviolet absorption method at 300 mμ: The reaction mixture for the standard assay contained 0.1 ml of 0.1 M N^1-methylnicotinamide chloride dissolved in 0.05 M potassium phosphate buffer (pH 7.8), aldehyde oxidase, and 0.05 M potassium phosphate buffer to make a total volume of 1.0 ml. The increase in absorbance per minute at 300 mμ was recorded.

2. Assay with ferricyanide as electron acceptor: The contents of the cuvette were the same as for the ultraviolet absorption method, with the addition of potassium ferricyanide at a final concentration of 1 mM. The decrease in absorbance per minute as the ferricyanide becomes reduced was recorded at 420 mμ.

Spectrophotometric assay with formycin B as substrate. The activity of aldehyde oxidase with formycin B as substrate could be assayed by slight modification of the two methods described for N^1-methylnicotinamide. In both assays 0.1 ml of the 10 mM formycin B replaced the N^1-methylnicotinamide as substrate. The ultraviolet assay recorded the increase in absorbance per minute at 310 mμ as formycin B was oxidized to oxoformycin B. Because the molar absorbance change is smaller with formycin B than with N^1-methylnicotinamide, about twice as much aldehyde oxidase was required when formycin B was the substrate. The ferricyanide assay was unaltered except for the change of substrate.

Spectrophotometric assays for purine nucleoside phosphorylase and xanthine oxidase. The methods employed were the same as those of Kim, Cha, and Parks (17).

Spectrophotometric assay of adenosine deaminase activity. The decrease in absorbance at 307 mμ as formycin A was deaminated to formycin B was measured. Each cuvette contained the following in 1 ml: 0.05 M potassium phosphate buffer (pH 7.8), formycin A in concentrations ranging from 0.2 to 0.033 M, and enough adenosine deaminase to give a reasonable velocity when the full scale of the Gilford recorder was 0.13 absorbance unit (this represents a conversion of 2 mμmoles of substrate to product). The temperature was held constant at 30°.

RESULTS

Studies of formycin B metabolism in vivo and identification of metabolite. When formycin B was injected into mice and the urine was subjected to paper chromatography, there appeared on the chromatograms, besides formycin B, a new compound which was not present in the urine from untreated mice. The compound fluoresced on paper when exposed to ultraviolet light and ran behind formycin B (which absorbs ultraviolet light) in both solvent systems. In Carter's isoamyl alcohol system the R_f values for formycin B and for the fluorescent compound were approximately 0.7 and 0.6, respectively, and in ammonium bicarbonate the values were 0.62 and 0.52, respectively. The fact that formycin B absorbs and the metabolite fluoresces aids greatly in distinguishing the two compounds. No evidence was seen for more than one metabolite in either of these solvent systems. In order to obtain enough metabolite for studies on the structure of this compound, the urine from several mice treated as above was collected over a 24-hr period, pooled, and concentrated by flash evaporation. The concentrate was applied to chromatography paper and run in the ammonium bicarbonate system. The bands of metabolite were eluted with hot water, and the solution obtained was flash-evaporated to dryness.

It should be noted that this metabolite was produced in relatively large quantities. When 10 mg of formycin B per mouse were injected (approximately 500 mg/kg), about half of the injected dose was excreted as the metabolite. This is in accord with the findings of Ishizuka *et al.* (8), who showed that most of the formycin B injected into rabbits was excreted as a metabolite when doses of 50 mg/kg were given.

While our studies were in progress a report appeared by Ishizuka *et al.* (8) which identified the metabolite of formycin B isolated from rabbit urine as the xanthosine analogue, which they call oxoformycin B. Our findings indicate that the metabolite isolated from mouse urine is identical with

that reported by Ishizuka *et al.* Ishizuka and this laboratory both recognized that the ultraviolet spectrum of the formycin B metabolite very closely resembles that of 5, 7-dihydroxypyrazolo [4,3-*d*] pyrimidine, which was first synthesized in 1888 by Behrend and called "isoxanthine" (18, 19). Elemental analysis obtained by Ishizuka *et al.* (8) on the purified metabolite gave fair agreement with the proposed structure. These workers interpreted the loss of a hydrogen (demonstrated by NMR) to be in agreement with the concept that formycin B had been oxidized at position 5. Infrared spectra were obtained for formycin B and the metabolite isolated from mouse urine. The spectrum of the metabolite was in agreement with that of oxoformycin B reported by Ishizuka *et al.* (8), particularly as it related to the band at 1100 cm^{-1}.

Studies with rabbit liver aldehyde oxidase. Preliminary experiments with homogenates and cell fractions demonstrated that the oxidation of formycin B takes place principally in the high-speed supernatant fraction of mouse or rabbit liver and that the reaction occurs in the absence of either TPN^+ or DPN^+. Xanthine oxidase was eliminated as the responsible enzyme when it was shown that administration of the potent xanthine oxidase inhibitor, allopurinol, did not inhibit the formation of the metabolite either *in vivo* or *in vitro*. As will be discussed below, formycin B is an inhibitor and not a substrate for purified milk xanthine oxidase.

We then turned our attention to the possibility that the enzyme hepatic aldehyde oxidase might catalyze the conversion of formycin B to oxoformycin B. Aldehyde oxidase was purified from rabbit liver, because this enzyme is known to have a particularly low specificity for substrates (20–23). The activity of aldehyde oxidase was assayed with N^1-methylnicotinamide as substrate. Almost as soon as purification was started it became obvious that N^1-methylnicontinamide could be replaced by formycin B as substrate, either with or without ferricyanide as electron acceptor. Figure 2 demonstrates that in all three column methods used to purify hepatic aldehyde oxidase the ratio of activities of this enzyme

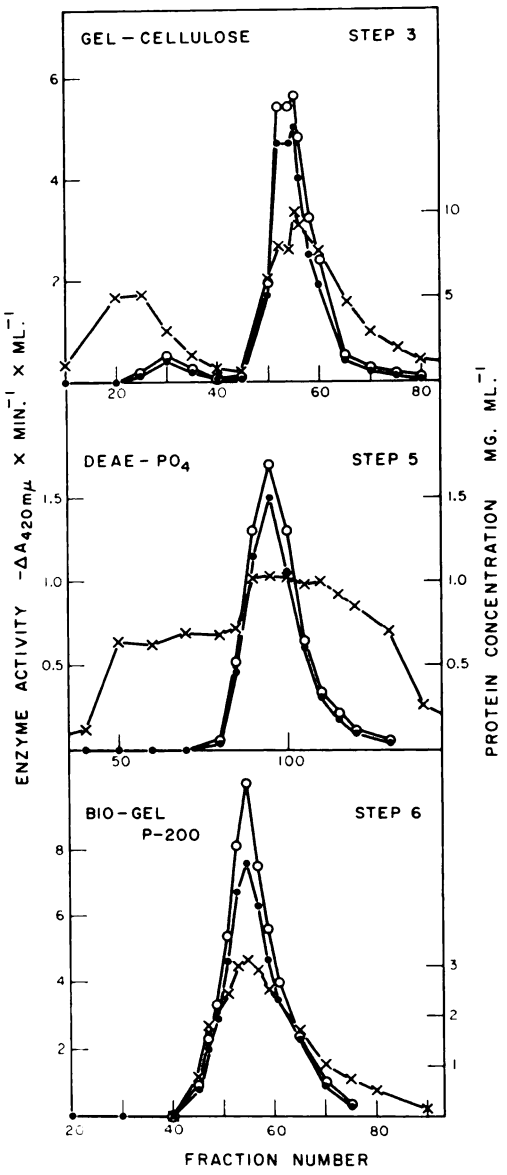

FIG. 2. *Elution profiles in the purification steps of aldehyde oxidase which demonstrate the parallel activities with formycin B and N^1-methylnicotinamide.*

Enzyme activity with both formycin B and N^1-methylnicotinamide was assayed using ferricyanide as the electron acceptor, so that the activities could be compared directly. The steps noted on the figure correspond to those described under *Methods*. ●——●, enzyme activity with N^1-methylnicotinamide as substrate; ○——○, enzyme activity with formycin B as substrate; ×——×, protein concentration.

with either substrate was constant in all fractions. In addition, Table 1 shows that throughout purification the ratio of activity with both substrates remained essentially constant, whichever assay procedure was used. This was strong evidence that aldehyde oxidase is the enzyme responsible for the oxidation of formycin B, particularly as the most highly purified fraction of aldehyde oxidase obtained by this method was less than 2 times lower in specific activity than the purest preparations reported previously (13).

The product formed when formycin B reacted with purified rabbit liver aldehyde oxidase was isolated and was shown by paper chromatography and by spectroscopy to be the same as the compound isolated from the urine of mice.

Independently, Tsukada *et al.* (10) uncovered evidence which also indicates that hepatic aldehyde oxidase converts formycin B to oxoformycin B.

Kinetic studies of aldehyde oxidase with formycin B. Formycin B is an excellent substrate for aldehyde oxidase, comparable in activity to N^1-methylnicotinamide. The K_m for formycin B with hepatic aldehyde oxidase was determined by plotting the reciprocal velocity against the reciprocal substrate concentration according to the method of Lineweaver and Burk (24). Several samples of aldehyde oxidase of different specific activity were used, with and without ferricyanide as electron acceptor. In all cases the K_m was in the order of 2×10^{-4} M. The K_m for aldehyde oxidase with N^1-methylnicotinamide is also in this range of concentration, at 2.7×10^{-4} M. The V_{max} values for aldehyde oxidase with formycin B and N^1-methylnicotinamide could be compared directly when ferricyanide was used as the electron acceptor, and assays were carried out at saturating levels of substrate. Table 1 shows that with the ferricyanide assay, the V_{max} when formycin B was the substrate was slightly higher than when N^1-methylnicotinamide was the substrate, the average ratio being approximately 1.1:1.

TABLE 1

Purification of rabbit liver aldehyde oxidase

Fraction	Total volume	Total protein	Total activity[a]	Specific activity	Yield	Purification[b]	Ratio of formycin B to N^1-methylnicotinamide activity	
							Ultraviolet assay	Ferricyanide assay
	ml	*mg*	*units*	*units/mg*	%	*-fold*		
Supernatant from heat-denatured homogenate	5,200	30,100	1,131	0.038	100	5	0.16	1.12
Ammonium sulfate, 0–50%	76	3,800	910	0.24	81	31.5	0.37	1.04
Calcium phosphate gel-cellulose column	110	1,050	825	0.78	73	105	0.34	1.11
Ammonium sulfate, 0–50%	18.2	800	780	0.98	69	130	0.38	1.16
DEAE-cellulose (phosphate) column	235	280	540	1.93	48	255	0.43	1.10
Bio-Gel P-200 column (100 ml from DEAE-cellulose pool used)	11.5	46	150	3.2	21	325	0.42	1.20

[a] One unit of enzyme activity is the amount of enzyme producing a change at 300 mμ of 1 absorbance unit/min with 0.005 M N^1-methylnicotinamide as substrate in 0.05 M potassium phosphate buffer, pH 7.8, at 30° (16).

[b] The specific activity of a high-speed supernatant from the liver homogenate was about 0.008, so that the purification obtained by heat denaturation was approximately 5-fold.

Final proof that aldehyde oxidase was responsible for the oxidation of formycin B, and, furthermore, that the same catalytic site on the enzyme was involved, was obtained when formycin B was shown to be a competitive inhibitor of the oxidation of N^1-methylnicotinamide (Fig. 3). The K_i value obtained for formycin B was 1.6×10^{-4} M, which is in close agreement with the value obtained for the Michaelis constant. In contrast, the adenosine analogue, formycin A, was neither a substrate nor an inhibitor of the enzyme.

Since it was established that aldehyde oxidase could oxidize formycin B to oxoformycin B, it was of interest to re-examine the question of the possible oxidation of inosine by this enzyme. Neither inosine nor xanthosine served as substrate for aldehyde oxidase when tested at 0.1 mM concentrations with amounts of enzyme increased 20-fold over the amount used in routine assays. Furthermore, neither of these natural nucleosides inhibited hepatic aldehyde oxidase.

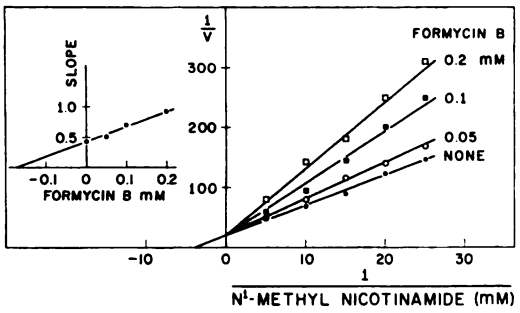

FIG. 3. *Inhibition by formycin B of purified rabbit liver aldehyde oxidase with N^1-methylnicotinamide as substrate*

Plot of the reciprocal of the initial velocity (v = ΔA_{290}/ml/min) against reciprocal millimolar concentration of N^1-methylnicotinamide. The oxidation of N^1-methylnicotinamide was recorded at 290 mμ because the simultaneous oxidation of formycin B did not interfere with velocity measurements at this wavelength. Potassium phosphate buffer, pH 7.8, was held constant at 0.05 M. N^1-Methylnicotinamide and formycin B were added in the concentrations indicated. Specific activity of rabbit liver aldehyde oxidase was 2.3 units/mg. The inhibition constant, K_i, of formycin B as the competitor of N^1-methylnicotinamide, estimated by the replot shown in the inset, was 1.6×10^{-4} M.

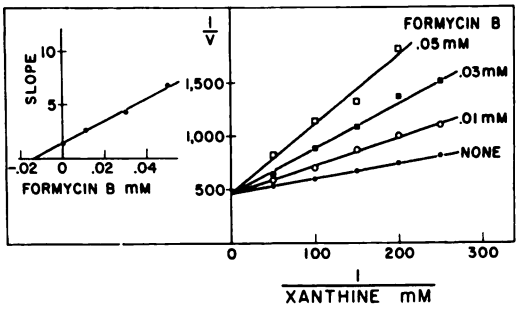

FIG. 4. *Inhibition by formycin B of purified milk xanthine oxidase with xanthine as substrate*

Plot of the reciprocal of the initial velocity (v = μmoles/ml/min) against reciprocal millimolar concentration of xanthine. Potassium phosphate buffer, pH 7.8, was held constant at 0.05 M. Xanthine and formycin B were added in the concentrations indicated. Specific activity of xanthine oxidase was 0.12 μM units/mg of protein. The inhibition constant, K_i, of formycin B as the competitor of xanthine, estimated by the replot shown in the inset, was 1.3×10^{-5} M.

Kinetic studies of xanthine oxidase with formycins A and B. Since xanthine oxidase is related to aldehyde oxidase in molecular properties and reaction mechanism and several compounds are common substrates for both enzymes (25), the possible interaction of the formycins with xanthine oxidase was reinvestigated. Both formycins A and B were found to be inhibitors of xanthine oxidase competitive with xanthine; the results of the experiment with formycin B are shown in Fig. 4. The K_i values calculated from replotting the slopes against the inhibitor concentrations were identical, 1.3×10^{-5} M.

These results have led us to examine the substrate activity of the base of formycin B, 7-hydroxypyrazolo[4,3-*d*]pyrimidine and 7-hydroxy-3-methylpyrazolo [4, 3-*d*]pyrimidine (Table 2). Both these compounds were found to be rather poor substrates for aldehyde oxidase when ferricyanide was used as electron acceptor, and were not substrates for xanthine oxidase. As shown in Table 2, 7-deazainosine and 7-deazathioinosine, both derivatives of the antibiotic tubercidin, were inactive with xanthine oxidase and

TABLE 2

Kinetic parameters for formycin B and related compounds

All K_m values were evaluated according to the method of Lineweaver and Burk (24). K_i values with aldehyde oxidase and xanthine oxidase were obtained by methods similar to that described for N^1-methylnicotinamide inhibited by formycin B. Reaction of xanthine oxidase with the purine analogues was measured with ferricyanide as electron acceptor at 420 mμ. Substrate and inhibition studies of purine nucleoside phosphorylase were performed as described elsewhere (7, 14).

Enzyme	Substrate	K_m	Relative V_{max}	Competitive inhibitor	K_i
		M	%		*M*
Rabbit liver aldehyde oxidase	Formycin A	No reaction			
	Formycin B	2×10^{-4}	110	Formycin A	No inhibition
	N^1-Methylnicotinamide	3×10^{-4}	100	Formycin B	1.6×10^{-4}
	N^1-Methylnicotinamide	3×10^{-4}	100	Inosine	No inhibition
	7-Hydroxypyrazolo[4,3-*d*]pyrimidine	4×10^{-3}	3.3		
	7-Hydroxy-3-methylpyrazolo [4,3-*d*]pyrimidine	4.5×10^{-3}	2.5		
	7-Deazainosine	6.5×10^{-2}	>1		
	7-Deazathioinosine	1×10^{-1}	>1		
	Formycin A 5′-phosphate	No reaction			
	Formycin B 5′-phosphate	No reaction			
Milk xanthine oxidase	Xanthine	1×10^{-6}		Formycin B	1.3×10^{-5}
	Xanthine	1×10^{-6}		Formycin A	1.3×10^{-5}
	Xanthine	1×10^{-6}		Inosine	No inhibition
	7-Hydroxypyrazolo[4,3-*d*]pyrimidine	No reaction			
	7-Hydroxy-3-methylpyrazolo [4,3-*d*]pyrimidine	No reaction			
	7-Deazainosine	No reaction			
	7-Deazathioinosine	No reaction			
Calf intestinal adenosine deaminase	Formycin A	5.7×10^{-2}			
Erythrocytic purine nucleoside phosphorylase	Inosine	5×10^{-5}		Formycin B	1×10^{-4}
	Inosine	5×10^{-5}		7-Deazainosine	3.3×10^{-4}
	Inosine	5×10^{-5}		7-Deazathioinosine	1.5×10^{-4}

showed weak substrate activity of questionable significance with aldehyde oxidase. However, both compounds were good inhibitors of erythrocytic purine nucleoside phosphorylase, with K_i values of the same order of magnitude as the K_m for inosine. Formycin A is deaminated to formycin B by calf intestinal adenosine deaminase, but the K_m for this reaction is relatively high, 5.7×10^{-2} M.

DISCUSSION

The above studies clearly demonstrate that the enzyme hepatic aldehyde oxidase is responsible for the oxidation of the inosine analogue, formycin B, to the xanthosine analogue, oxoformycin B (Fig. 5).

Formycin B is an excellent substrate for this enzyme, with a K_m of the same order, and a V_{max} slightly higher than the corresponding values for the commonly used substrate, N^1-methylnicotinamide. Also, both formycin B and formycin A, the adenosine analogue, are competitive inhibitors of purified milk xanthine oxidase ($K_i = 1.3 \times 10^{-5}$ M), an enzyme which is similar in many respects to the aldehyde oxidase.

Hepatic aldehyde oxidase is a complex enzyme, with a molecular weight of about 280,000, and contains the components of an internal electron transport system (13), i.e., 2 moles of FAD, 2 g atoms of molybdenum, 8 g atoms of non-heme iron, and 1 or 2 moles of a material tentatively identified as coenzyme Q_{10} per mole of enzyme. This enzyme has broad substrate specificity (20, 22, 23) and will react with such diverse compounds as quinine, N^1-methylnicotinamide, 6-methylthiopurine, and methotrexate. To our knowledge, however, the substrate activity of formycin B is the first example of a nucleoside-like compound that can react with aldehyde oxidase.

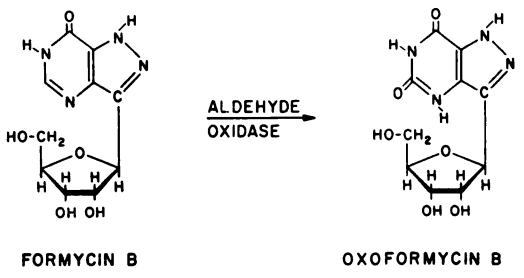

FIG. 5. *Oxidation of formycin B to oxoformycin B by aldehyde oxidase*

The interpretation of the above findings poses an intriguing problem. Formycins A and B closely resemble the natural nucleosides, adenosine and inosine, in their ability to interact with various enzymes of nucleoside metabolism (7, 26, 27). However, liver aldehyde oxidase neither oxidizes nor is inhibited by inosine; neither adenosine nor inosine reacts with or inhibits xanthine oxidase. On the other hand, formycin B, a close structural analogue of inosine, is an excellent substrate for liver aldehyde oxidase, and both formycins A and B are competitive inhibitors of milk xanthine oxidase, with inhibition constants of about 1.3×10^{-5} M. It is possible that these seemingly anomalous findings may be explained by subtle differences between the formycins and the natural nucleosides both in their steric conformations and in the electronic properties of the ring systems. X-ray crystallographic studies suggest that the structures of the formycins and the purine ribonucleosides are quite similar (28). The C—N glycosidic bond of adenosine has a length of 1.47 A whereas its replacement, the C—C bond of the formycins, is slightly longer, 1.55 A (29). Also, the plane of the sugar is at a dihedral angle of 64.2 degrees to the base, a little smaller than the values reported for normal nucleosides and nucleotides (28). However, if one examines molecular models of inosine and formycin B, it may be observed that the rotation of the ribose moiety about the carbon-carbon bond of formycin B is relatively unhindered and a rotation of almost 360 degrees is possible. In contrast, in molecular models of inosine the rotation of the ribose moiety about the C—N glycosidic bond is markedly hindered by contact of both the 3-nitrogen and the C-8 hydrogen of the purine ring with the C-2′ hydrogen of the ribose. In molecular models of formycin B the contact between the C-8 and C-2′ hydrogens does not occur because the carbon and hydrogen at position 8 are replaced by a nitrogen atom. Thus it seems possible that, because of their relatively unhindered rotation, the formycins can assume conformations that are not pos-

sible for the natural nucleosides, inosine and adenosine, and therefore can adapt themselves to the active sites of enzymes, such as liver aldehyde oxidase and xanthine oxidase, whereas such adaptations are not possible for the natural nucleosides because of steric hindrance.[2] However, it does not appear that these steric considerations alone can wholly explain the differences in substrate specificities of these enzymes. The base of formycin B and its 3-methyl derivative are relatively poor substrates for liver aldehyde oxidase (Table 2), whereas if steric factors alone were involved one might expect these compounds to rival formycin B in substrate activity. Thus it seems necessary to invoke additional factors if one wishes a more satisfying explanation of these discrepancies. In any case, it is apparent that the presence of the ribose moiety on formycin B facilitates both its binding capability and its reactivity with aldehyde oxidase.

The recent studies of Ward and Reich and their associates (26, 29, 33, 34) with nucleotides and polynucleotides that contain formycin A suggest that the freer rotation of the ribose moiety may explain some of the biochemical characteristics of these substances. The formycin compounds replace their natural adenosine counterparts in a number of enzymatic reactions. However, formycin polymers display anomalous behavior in several cases. In its reaction with bovine pancreatic ribonuclease A, polyformycin resembles polycytosine rather than polyadenosine (33). Although, when tested as a template for polypeptide synthesis (26), formycin codes like adenosine in copolymers, the homopolymer, polyformycin, does not mimic polyadenosine in coding for polylysine synthesis. Several physical properties of formycin-containing polymers, including ultraviolet hypochromism, fluorescence, and modified thermostability, suggest a greater ease of conformational transitions, which may result from the greater freedom of rotation about the C—C bond of formycin (29).

It is of interest that to date the chemical synthesis of oxoformycin B has not been achieved. Hence, the enzymatic approach may offer a practical means of synthesizing oxoformycin B in reasonable quantities, and the substitution of a hydroxyl group for the hydrogen on C-5 of the pyrazolo[4,3-*d*]pyrimidine ring may open a synthetic route for the preparation of other analogues of potential interest, such as the 6-thioguanosine analogue of the formycin series (7).

[2] While purine and pyrimidine nucleosides and nucleotides are found in a preferred *syn* or *anti* conformation in the crystalline state, studies have shown that nucleosides in the liquid state (in cyclohexane) have no exclusive *syn* or *anti* conformation (28, 30–32). Whether the mixture of *syn*, *anti*, and intermediate conformers persists in aqueous solutions has not been established. The low rotational energy barrier of the glycosidic bond makes it likely that mixtures of conformers will exist in solutions. To date physical measurements have not provided conclusive evidence to define the preferred steric conformers of nucleosides and their analogues in the aqueous state.

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