Purine Nucleoside Phosphorylase from Human Erythrocytes

III. Inhibition by the Inosine Analog Formycin B of the Isolated Enzyme and of Nucleoside Metabolism in Intact Erythrocytes and Sarcoma 180 Cells[1]

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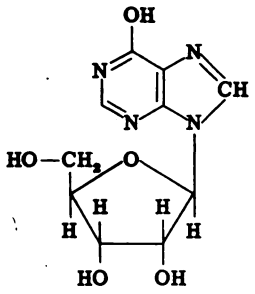
(Received January 23, 1968)

SUMMARY

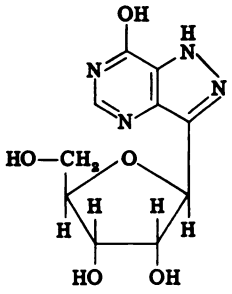
Formycin B, an analog of inosine, is a potent competitive inhibitor of purified erythrocytic purine nucleoside phosphorylase. The inhibition constant, K_i (1×10^{-4} M) is of the same order of magnitude as the K_m for inosine (5×10^{-5} M). Formycin B also inhibits the degradation of nucleosides by intact and hemolyzed human erythrocytes and by Sarcoma 180 ascites cells.

INTRODUCTION

In 1964, Umezawa's laboratory discovered, in culture filtrates of *Nocardia interforma*, the formycins, a new class of antibiotics (1–4). These interesting compounds are analogs of natural ribonucleosides which have pyrazolo[4,3-*d*]pyrimidine moieties replacing the purine bases, and therefore have a carbon-carbon bond instead of the nitrogen-carbon nucleosidic linkage (5). For example, formycin B (7-hydroxy-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine), is an analog of inosine as may be seen in the following structural formulas.



Inosine



Formycin B

Formycin A, the adenosine analog, inhibits the growth of sensitive Ehrlich ascites cells, where the mono-, di-, and triphosphates of formycin A are formed and *de novo* purine biosynthesis is inhibited (6, 7). Also, formycin A can be deaminated by adenosine deaminases to the inosine analog, formycin B (4).

This laboratory has recently been studying the molecular properties and reaction mechanism of erythrocytic purine nucleoside phosphorylase (8–10), and has had occasion to examine the behavior of the inosine analog, formycin B. As might be expected, since it does not have a normal nucleosidic linkage, formycin B is not a substrate, but rather is a potent competitive inhibitor of the enzyme. This finding is expecially intriguing in view of the reported low toxicity of formycin B. The LD_{50} for mice was roughly estimated at 1000 mg/kg (4). It seems possible that this relatively nontoxic substance might have useful effects in disorders of purine metabolism such as gout, or that it might alter the toxicity and/or antitumor behavior of

[1] This investigation was supported by United States Public Health Service Research Grant CA 07340 from the National Cancer Institute and by American Cancer Society Grant T-94.

other analog-containing nucleosides such as 6-thioguanosine or 6-mercaptopurine ribonucleoside. These latter analog-nucleosides, which tested in whole animal systems, were shown to behave very much like the respective free bases, suggesting that they are rapidly degraded to the free bases by the purine nucleoside phosphorylase of erythrocytes or other tissues (11–15).

Thus it became of immediate interest to learn whether the inhibition of purine nucleoside phosphorylase by formycin B would be reflected in an inhibition of the degradation of nucleosides by intact cells. Washed, free human erythrocytes and Sarcoma 180 ascites cells were used in these studies. A preliminary report of part of this work has been presented (16).

MATERIALS AND METHODS

Materials. Samples of formycin B were gifts from Dr. Hamao Umezawa, of the Microbial Chemistry Research Foundation, Institute of Microbial Chemistry, Tokyo, Japan, and from Dr. J. Frank Henderson, of the McEachern Laboratory, University of Alberta, Edmonton, Alberta, Canada. The formycin B preparations were approximately 96% pure, the principal contaminant being the adenosine analog formycin A. 6-Mercaptopurine ribonucleoside was obtained from the Sigma Chemical Company, and inosine from Boehringer, Mannheim. Xanthine oxidase was purchased from the Worthington Biochemical Corporation. CD 1 mice weighing 20–22 g were obtained from the Charles River Breeding Laboratories in North Wilmington, Massachusetts. Purine nucleoside phosphorylase was purified by the procedure of Kim, Cha, and Parks (8, 9). Isotonic 0.1 M sodium phosphate buffer, pH 7.4, was prepared by adding 20 ml of 1 N HCl to a solution containing 17.8 g of $Na_2HPO_4 \cdot 2H_2O$, then diluting to 1 liter. Freshly drawn blood was obtained from human volunteers. All reagents were of the highest purity commercially available.

Spectrophotometric assays of phosphorolytic activities. The assay for purine nucleoside phosphorylase with inosine as the substrate employed the coupled xanthine oxidase method of Kalckar (17). This is based on the measurement of the increase in absorbancy at 293 mμ due to the formation of uric acid. A similar system was used for 6-mercaptopurine ribonucleoside where formation of thiouric acid was measured by the increase in absorbancy at 348 mμ. For 6-mercaptopurine ribonucleoside to thiouric acid at 348 mμ, the molar absorbancy change was 24.5×10^3. The molar absorbancy change for inosine to uric acid was 12.5×10^3 at 293 mμ. In whole-cell studies the high absorption of formycin B at 293 mμ interfered with the estimation of uric acid and measurements were made at 302 mμ. The molar absorbancy change for inosine to uric acid at 302 mμ was 7.64×10^3. Beckman D.U. and Zeiss spectrophotometers with Gilford optical density converters and recorders were used.

Measurement of phosphorolytic activity in human erythrocytes. When human erythrocytes were incubated in an isotonic phosphate medium containing inosine (or 6-mercaptopurine ribonucleoside), the liberated free base could be measured with xanthine oxidase at the appropriate wavelength (302 mμ or 348 mμ). Human blood, freshly drawn into a heparinized syringe, was centrifuged, and the plasma and buffy coat were removed. The erythrocytes were washed twice with 0.9% NaCl. They were then diluted to approximately 4% with saline, and incubated with 6-mercaptopurine ribonucleoside or inosine and formycin B. When very high concentrations of erythrocytes were studied certain technical difficulties were encountered. 6-Mercaptopurine and 6-mercaptopurine ribonucleoside were found to bind to the acid-denatured proteins of the erythrocyte. This binding could be reversed or prevented by the addition of Cleland's reagent (dithiothreitol) to the reaction mixtures. This binding phenomenon was not a significant factor with the concentrations of erythrocytes employed in these experiments, nor did it occur in the studies with Sarcoma 180 cells.

The total volume of all incubation mixtures was 2.0 ml. This was composed as follows: 1.0 ml of erythrocytes diluted with

saline as indicated in the legend of each figure, 0.5 ml of ribonucleoside substrate dissolved in isotonic 0.1 M phosphate buffer, and 0.5 ml of formycin B also made up in 0.1 M phosphate buffer (or 0.5 ml of 0.1 M phosphate buffer only, when formycin B was absent). The final phosphate concentrations in all incubations was therefore 0.05 M. The incubations were carried out in air in stoppered flasks in a Dubnoff metabolic shaker at 37°. The reaction was halted by pipetting a 1.2-ml aliquot of the incubation mixture into 0.4 ml of ice-cold 1.5 M perchloric acid. Blanks, or zero-time controls, contained 0.6 ml of red cell suspension denatured with 0.4 ml of perchloric acid plus 0.3 ml of ribonucleoside solution plus 0.3 ml of formycin B (or phosphate buffer). Both blank and test samples were centrifuged, and 1.0 ml of the supernatant was neutralized with 0.5 ml of 0.78 M potassium bicarbonate. After centrifugation, 0.8 ml of the supernatant fluid was assayed for hypoxanthine or 6-mercaptopurine with xanthine oxidase in 0.2 ml of 1 M potassium phosphate buffer, pH 7.4. The optical density was recorded when the reaction had gone to completion (for inosine the optical density was read at 302 mμ due to the large absorption of formycin B at 293 mμ). Preliminary experiments were carried out to determine conditions where the formation of purine base was proportional to the time of incubation, i.e., the experiments were performed under "zero order" conditions. The quantity of the purine base formed over a fixed period of time was therefore a measure of the velocity of the purine nucleoside phosphorylase activity in the erythrocytes.

Preparation of Sarcoma 180 ascites cells and measurement of the phosphorolytic activity. Sarcoma 180 ascites cells were inoculated intraperitoneally into CD 1 mice, and after 7 days the tumor cells were harvested and washed five times with several volumes of 0.9% NaCl, until the packed cells were without visible red cell contamination. The cells were suspended in 0.9% NaCl to about 6.0% (v/v), and phosphorolytic activity was measured by the method described above for erythrocytes.

RESULTS

Studies with Purified Erythrocytic Purine Nucleoside Phosphorylase

The human erythrocytic purine nucleoside phosphorylase preparations employed in these studies were purified about 2300-fold to a specific activity of 30–40 μmolar units per milligram of protein. The method of purification has been reported (8). Recently this laboratory has crystallized human erythrocytic purine nucleoside phosphorylase. The crystalline enzyme has a specific activity of about 96 μmolar units per milligram of protein and is homogeneous to Sephadex chromatography and disc electrophoresis (10).

Kinetic studies have revealed that, when inosine or deoxyinosine is employed as the substrate, there is apparent substrate activation at high concentrations; thus Lineweaver-Burk plots are linear at low concentrations of substrate, but are concave downward as the substrate concentration is increased. These observations are consistent with the concept that the enzyme is multivalent with the occurrence of cooperative interaction between the active sites (8). Therefore, in the study of inhibition, concentrations of inosine were chosen where Lineweaver-Burk plots are linear, in order that reproducible estimations of Michaelis constants and of inhibition constants could be obtained.

The apparent Michaelis constants for inosine and 6-mercaptopurine ribonucleoside were 5×10^{-5} M, and 7×10^{-5} M, respectively (Figs. 1 and 2). When formycin B was employed in this test system, it proved to be a competitive inhibitor with both inosine and 6-mercaptopurine ribonucleoside (Figs. 1 and 2). The K_i values estimated from replots, as shown in the insets to Figs. 1 and 2, are 1×10^{-4} M, and 3.3×10^{-5} M, when inosine and 6-mercaptopurine ribonucleoside were employed as the substrates, respectively. No evidence was obtained which indicated that formycin B was capable of serving as a substrate for purine nucleoside phosphorylase. No spectral shift in the region of 240–360 mμ was obtained upon prolonged incubation of for-

mycin B with purine nucleoside phosphorylase, either in the presence or absence of xanthine oxidase.

Studies with intact and hemolyzed human erythrocytes. Formycin B is a potent inhibitor of purine ribonucleoside degradation in intact erythrocytes; these findings are demonstrated in Figs. 3 and 4. The metabolism of both inosine (Fig. 3) and 6-mercaptopurine ribonucleoside (Fig. 4) is inhibited.

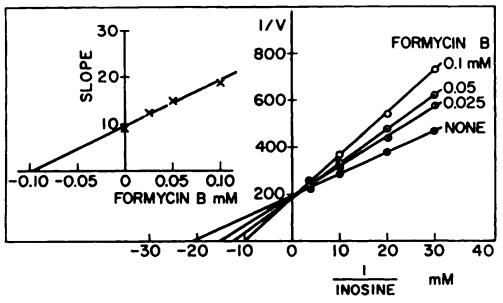


FIG. 1. *Inhibition by formycin B of purified purine nucleoside phosphorylase with inosine as substrate*

Plot of reciprocal of the initial velocity (v = μmoles/ml/min) *versus* reciprocal concentration of inosine (mM). Potassium phosphate buffer, pH 7.5, was held constant at 50 mM and xanthine oxidase at 0.03 μmolar units. Inosine and formycin B were added in the concentrations indicated. Specific activity of human erythrocytic purine nucleoside phosphorylase was 30 μmolar units/mg. The inset shows the replot of slope *versus* concentration of formycin B.

The method of measuring phosphorolytic activity in the intact erythrocytes made it necessary to use concentrations of substrate which were ten times greater than in the isolated enzyme studies. At these high concentrations (about 1.0 mM) the isolated enzyme studies with both inosine and 6-mercaptopurine ribonucleoside showed concave downward Lineweaver-Burk plots (8). It seemed possible that similar effects would be seen in whole cell studies. With inosine (Fig. 3) this concave downward pattern was demonstrated. However, with 6-mercaptopurine ribonucleoside, the curves were concave upward when formycin B was present (Fig. 4). When the data were

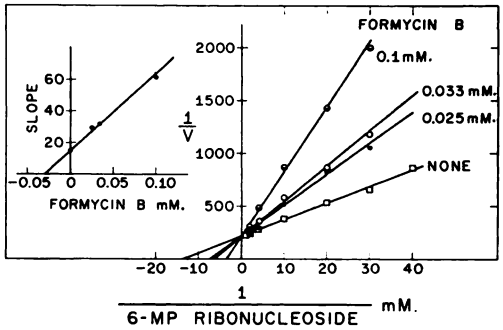


FIG. 2. *Inhibition by formycin B of purified purine nucleoside phosphorylase with 6-mercaptopurine ribonucleoside (6-MP ribonucleoside) as substrate*

Plot of reciprocal of the initial velocity (v = μmoles/ml/min) *versus* reciprocal concentration of 6-mercaptopurine ribonucleoside (mM). Potassium phosphate buffer, pH 7.5, was held constant at 100 mM and xanthine oxidase at 0.2 μmolar units. 6-Mercaptopurine ribonucleoside and formycin B were added in the concentrations indicated. Specific activity of human erythrocytic purine nucleoside phosphorylase was 40 μmolar units/mg. The inset shows the replot of slope *versus* concentration of formycin B.

plotted according to Dixon (18), upward curvatures were seen with both inosine and 6-mercaptopurine ribonucleoside.

Paterson and co-workers have produced evidence that some inhibitors of ribo-

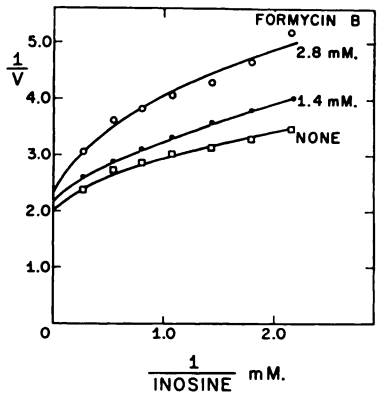


FIG. 3. *Inhibition of phosphorolysis of inosine by formycin B with intact human erythrocytes*

Plot of the reciprocal velocity (v = μmoles of hypoxanthine formed per milliliter of incubation mixture per 25 min at 37°) *versus* reciprocal of the inosine concentration (mM). Erythrocytic suspension was 2.0% (v/v).

nucleoside metabolism are effective only in whole cells, their action being to block the nucleoside transport system across the cell wall (19–21). The concave upward curvature could then be explained if formycin B inhibited the transport of 6-mercaptopurine ribonucleoside into the cell or if there is a difference in the rate of uptake of the compounds by the red cell. To test these possibilities, intact erythrocytes were preincubated for 30 min with varying concentrations of formycin B, and a parallel

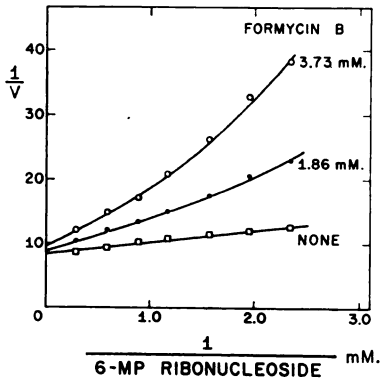


FIG. 4. *Inhibition of the phosphorolysis of 6-mercaptopurine ribonucleoside (6-MP ribonucleoside) by formycin B with intact human erythrocytes*

Plot of the reciprocal velocity (v = μmoles of 6-mercaptopurine formed per milliliter of incubation mixture per 25 min at 37°) *versus* reciprocal of the 6-mercaptopurine ribonucleoside concentration (mM). Erythrocytic suspension was 1.7% (v/v).

experiment was set up where no preincubation was employed. Essentially identical inhibitory patterns were seen in both cases. Also if a transport mechanism is involved then intact and disrupted cells should behave differently. Figure 5 shows Dixon plots (18) for formycin B inhibition of 6-mercaptopurine ribonucleoside breakdown in intact and hemolyzed cells. In both cases an upward curvature was seen. A transport phenomenon did not therefore appear to be involved.

A concave upward Dixon plot would also have been observed if the product, in this case 6-mercaptopurine, was being metabo-

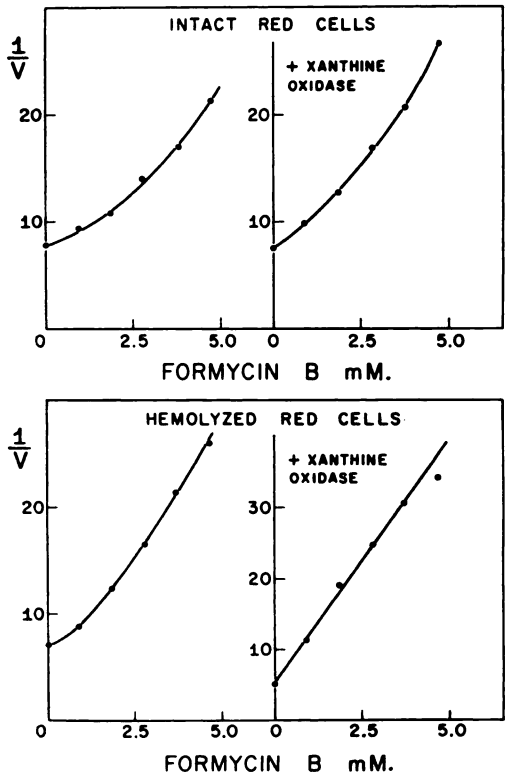


FIG. 5. *Inhibition of phosphorolysis of 6-mercaptopurine ribonucleoside by formycin B*

For whole-cell preparations the velocity v is the number of micromoles of 6-mercaptopurine formed per milliliter of incubation mixture per 20 min at 37°. For hemolyzates the velocity v is the number of micromoles of 6-mercaptopurine formed per milliliter of incubation mixture per 10 min at 37°. Plots of the reciprocal velocity v *versus* formycin B concentration are shown. Cell suspensions were 2.5% (v/v). Hemolyzates were made by adding the cells to water to the equivalent of 2.5% (v/v) instead of saline. 6-Mercaptopurine ribonucleoside concentration was held constant at 0.85 mM. Xanthine oxidase concentrations were 0.2 μmolar units per incubation mixture where indicated.

lized to the nucleotide and was thus made unavailable for measurement in our test system. Also, if 6-mercaptopurine acted as a product inhibitor a similar pattern would be seen. Both of these effects might be prevented by adding xanthine oxidase to the test system. Xanthine oxidase would remove 6-mercaptopurine as soon as it was formed in the incubation medium. Figure 5

shows these results. With intact cells where xanthine oxidase cannot penetrate the cell membrane and is only partially effective in removing 6-mercaptopurine, the curvature was less pronounced. However, when xanthine oxidase was added to hemolyzed cells, essentially linear reciprocal velocity *vs* formycin B concentration plots were observed. It is possible, then, that 6-mercaptopurine either is being metabolized to the nucleotide or is acting as a product inhibitor.

Studies with intact Sarcoma 180 cells. In order to learn whether formycin B could inhibit the metabolism of nucleosides in intact cancer cells, as well as in erythrocytes, Sarcoma 180 ascites cells were examined. As may be seen in Fig. 6,

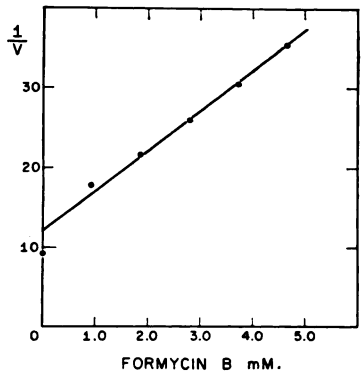


FIG. 6. *Inhibition of phosphorolysis of 6-mercaptopurine ribonucleoside by formycin B with intact Sarcoma 180 ascites cells*

Plot of reciprocal velocity ($v = \mu$moles of 6-mercaptopurine formed per milliliter of incubation mixture per 25 min at 37°) *versus* formycin B concentration (mM). Cell suspension was 3.0% (v/v). 6-Mercaptopurine ribonucleoside concentration was held constant at 0.85 mM.

formycin B inhibited the degradation of 6-mercaptopurine ribonucleoside in Sarcoma 180 cells. In contrast to the finding with intact erythrocytes, the Dixon plot was linear.

DISCUSSION

The above studies clearly demonstrate that formycin B, an inosine analog in which a carbon-carbon bond replaces the nitrogen-carbon nucleosidic linkage, is a potent competitive inhibitor of nucleoside phosphorylase. Further evidence is also offered which indicates that formycin B inhibits purine nucleoside phosphorylase both in intact human erythrocytes and in intact murine Sarcoma 180 cells. The apparent Michaelis constants measured for inosine and 6-mercaptopurine ribonucleoside are in the same order of magnitude as the inhibition constants measured for formycin B, a finding which suggests that the formycin B structure resembles that of normal purine nucleosides so closely that they apparently have very similar binding effects with the active site on the enzyme. However, since it is the nucleosidic linkage which is split by this enzyme, the carbon-carbon bond in formycin B is not susceptible to degradation.

A particularly intriguing aspect of formycin B is that it appears to have relatively low toxicity (4). There is little evidence for the occurrence in animal tissues of an inosine kinase which would be capable of converting inosine to IMP, and therefore it seems unlikely that formycin B can be converted to the monophosphate derivative. One might expect that it would be excreted largely unchanged in the urine. However it has been observed by Hori and Umezawa (personal communication) and confirmed by us that, in addition to unchanged formycin B, a metabolite is excreted in the urine of mice (22). Studies are currently under way to identify this metabolite and to determine its metabolic origin.

It seems possible that use might be made of the capacity of formycin B to inhibit purine nucleoside phosphorylase *in vivo*, and that formycin B will prove a useful tool in evaluating the role and importance of purine nucleoside phosphorylase in animal tissues. Also, it is likely that certain of the analog-containing nucleosides such as 6-thioguanosine are rapidly degraded by purine nucleoside phosphorylase, and this degradation may also be inhibited by formycin B. If so, formycin B administration might potentiate the antitumor effects of nucleosides such as 6-thioguanosine. Studies to test this possibility are currently being performed in this labora-

tory. It is of interest to speculate that the normal degradation of nucleosides might be inhibited by formycin B, possibly slowing down the production of uric acid in conditions such as gout. In view of the findings of Umezawa and colleagues that formycin B has low toxicity in mice, but causes leukopenia in dogs at doses of 50 mg/kg,[2] it is apparent that effects selective for both species and tissues may occur. It is also possible that specific effects might be seen in certain disease states.

Consideration should be given to compounds of the formycin B type as possible chemotherapeutic agents. Analogs containing 6-mercaptopurine or 6-thioguanosine might prove especially interesting. If, owing to the lack of the appropriate nucleoside kinase, they prove to be metabolically inert in animal tissues, they might in turn be selectively inhibitory to the growth of any invading organism or tissue which is capable of converting formycin B or related compounds (such as the mercaptopurine analog) to nucleotides.

In the important studies of Paterson and colleagues, evidence is offered for the occurrence of a nucleoside transport mechanism by which nucleosides such as inosine are transported across cell membranes. A number of nucleoside analogs inhibit this process in whole cells such as intact erythrocytes, but do not inhibit the metabolism of nucleosides in disrupted cells (18–20). Formycin B, on the other hand, gave similar inhibition patterns whether the intact erythrocytes were preincubated or not, and whether the cells were lysed or not. This indicates that its principal effect is not upon the nucleoside transport mechanism, but rather on some component which does not depend upon the integrity of the cell wall. The studies with highly purified purine nucleoside phosphorylase strongly suggest that the component inhibited in the intact cells is in fact purine nucleoside phosphorylase.

[2] Chronic toxicity tests of formycin B in dogs showed that doses of 50 mg/kg caused marked leukopenia on the second or third day, with recovery in 7–10 days (H. Umezawa, personal communication).

ACKNOWLEDGMENT

The authors would like to express their appreciation to Dr. Sungnan Cha for his help in the preparation of this manuscript.

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