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N-METHYLFORMYCINS

REACTIVITY WITH ADENOSINE DEAMINASE, INCORPORATION INTO INTRACELLULAR NUCLEOTIDES OF HUMAN ERYTHROCYTES AND L1210 CELLS AND CYTOTOXICITY TO L1210 CELLS*

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Abstract—The *N*-methyl derivatives of the C-nucleoside, formycin (7-amino-3(β-D-ribofuranosyl)pyrazolo[4, 3-*d*]pyrimidine) were compared to formycin and adenosine with regard to their substrate activity with human erythrocytic adenosine deaminase (ADA), their ability to form intracellular nucleotides and their cytotoxicity to L1210 cells. Only 2-methylformycin (K_m = 6.1 mM, relative V_{max} = 396) and N^7-methylformycin (K_m = 0.1 mM, relative V_{max} = 3) showed substrate activity with ADA (corresponding kinetic parameters for adenosine were: K_m = 0.025 mM, relative V_{max} = 100). In contrast to previous hypotheses, these results suggest that the conformation (either *syn* or *anti*) of an adenosine analog is not a major factor in determining substrate activity with ADA. Neither 4-methylformycin nor 6-methylformycin formed their corresponding nucleotides when incubated with human erythrocytes, whereas both 1-methylformycin and 2-methylformycin formed large amounts of their corresponding mono-, di- and triphosphate nucleotides. Inhibition of ADA by pretreatment of the erythrocytes with the potent ADA inhibitor, 2′-deoxycoformycin, had no effect on the incorporation of 1-methylformycin into erythrocytic nucleotides but greatly increased the incorporation of 2-methylformycin and N^7-methylformycin. The conversion of both 1-methylformycin and 2-methylformycin into nucleotides was almost complete after 18 hr of incubation (in the presence of 2′-deoxycoformycin in the case of 2-methylformycin), whereas that of N^7-methylformycin was only partially complete in the presence of 2′-deoxycoformycin. With both 1-methylformycin and N^7-methylformycin, transient accumulation of the corresponding nucleoside 5′-monophosphate derivative was observed prior to the accumulation of the triphosphate nucleotide. Results, qualitatively similar to those found with erythrocytes, were obtained when the effects of 2′-deoxycoformycin on the incorporation of 1-methyl- and 2-methylformycins into the nucleotide pools of L1210 cells *in vitro* were examined. Compounds capable of forming analog nucleotides in human erythrocytes or L1210 cells if deamination is prevented either by the molecular structure of the analog or by pretreatment of the cells with 2′-deoxycoformycin, also showed marked cytotoxicity to L1210 cells in culture, i.e. 1-methyl-, 2-methyl- and N^7-methylformycin exhibited ID_{50} values of 0.5 to 2 μM, whereas 4-methyl- and 6-methylformycin were not significantly growth inhibitory. The potential usefulness of the various *N*-methyl derivatives of formycin (alone or in combination with an ADA inhibitor) as cytotoxic or antiviral agents is discussed.

One of the most interesting adenosine analogs is the C-nucleoside, formycin§, which was isolated as an antibiotic from the rice mold, *Nocardia interforma* [1–3]. This compound possesses weak, but definite, antitumor, antibacterial, antifungal and antiviral activity in various test systems [4, 5]. Furthermore, this adenosine analog and its nucleotide derivatives are active as substrates for a variety of anabolic and catabolic enzymes of purine nucleoside, nucleotide, and nucleic acid metabolism [4, 5]. Formycin is a potent substrate for the degradative enzyme, adenosine deaminase (ADA), from a variety of sources, including the human erythrocyte [6]. This enzyme deaminates formycin, producing the inosine analog, formycin B, which, in turn, may be converted to the xanthosine analog, oxoformycin, by hepatic aldehyde oxidase (see Fig. 1) [7–9]. Both formycin B and oxoformycin have shown little or no biological activity and low toxicity in experimental animals [4, 5]. The rapid inactivation of formycin by animal tissues that contain ADA has limited investigations of therapeutic activities of this potentially useful antimetabolite. When formycin is incubated with human erythrocytes lacking ADA due to a genetic deficiency [10] or after treatment with a tight-binding ADA inhibitor such as coformycin or 2′-deoxycoformycin (K_i values 10^{-10} to 10^{-12} M) [11,12], rapid incorporation into the nucleotide pools occurs with the formation of high concentrations of formycin 5′-triphosphate [10, 13]. Therefore, chemically modi-

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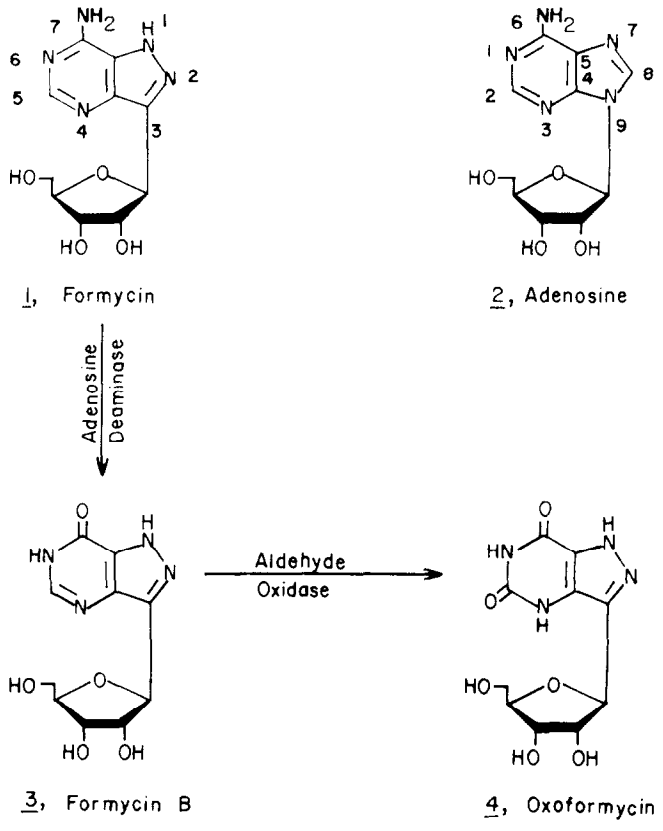

Fig. 1. Metabolic conversions of formycin. Numbering systems used for formycin and adenosine.

fied formycins were synthesized [14, 15] in an attempt to decrease or abolish the activity of the formycin molecule with ADA, while retaining the activity with adenosine kinase and other key enzymes of adenine nucleotide metabolism.

The present paper describes the effects which methylation of the individual nitrogen atoms of formycin have on (a) the activity as a substrate for ADA, (b) the capacity for incorporation into the nucleotide pools of human erythrocytes and (c) the cytotoxicity to L1210 cells. Portions of this work have been presented in preliminary form [16]. The structures of formycin and its *N*-methyl derivatives are shown in Fig. 2.

§ Abbreviations used in the text are as follows: formycin, 7-amino-3(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine; ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4); formycin B, 3(β-D-ribofuranosyl)pyrazolo[4.3-*d*]pyrimidin-7-one; oxoformycin, 5,7-dioxy-3(β-D-ribofuranosyl)pyrazolo[4, 3-*d*]pyrimidin-5,7-dione; coformycin, 3(β-D-ribofuranosyl)-6,7,8-trihydroimidazo[4,5-*d*][1,3]diazepin-8-(R)-ol; 2′-deoxycoformycin, (R)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol (Covidarabine®); adenylate kinase, ATP:AMP phosphotransferase (EC 2.7.4.3); adenosine kinase, ATP:adenosine 5′-phosphotransferase (EC 2.7.1.20); and tubercidin, 4-amino-7(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine.

A. F. Lewis and L. B. Townsend, manuscript in preparation.

MATERIALS AND METHODS

Methods for methylation of specific nitrogen atoms of formycin have been described [17, 18].¶ Formycin was generously provided by Professor H. Umezawa of the Institute for Microbial Chemistry, Tokyo, Japan, and also was obtained from Meiji Seika Kaisha, Co., Tokyo, Japan. The ADA inhibitor, 2′-deoxycoformycin, was a gift from Dr. H. W. Dion of Parke, Davis & Co., Detroit, MI. The human erythrocytic ADA was a preparation partially purified as described earlier [6]. The substrate activity of formycin and its *N*-methyl derivatives with human erythrocytic ADA was determined by spectrophotometric and ammonia liberation assay procedures described previously [6, 10].

Incorporation of formycin and its derivatives into the nucleotide pools of human erythrocytes was determined as follows: freshly drawn, washed, human erythrocytes (20–25% suspensions) were incubated at 37° in a shaking water bath with air as the gas phase in a medium consisting of 50 mM potassium phosphate buffer (pH 7.4), 75 mM NaCl, 2 mM $MgSO_4$, 10 mM glucose, penicillin (100 units/ml) and streptomycin (100 μg/ml). The *N*-methylformycin compounds were present at 1.0 mM. When 2′-deoxycoformycin (1 μg/ml) was used, cells were preincubated with this compound for 15 min before addition of the *N*-methylformycin derivative. At appropriate times, 0.4-ml aliquots of the incubation mixtures were added to 0.1 ml of ice-cold 20% perchloric acid, mixed well and allowed to stand at 4° for 15 min. Mixtures were centrifuged to remove denatured protein and 0.3-ml aliquots of the

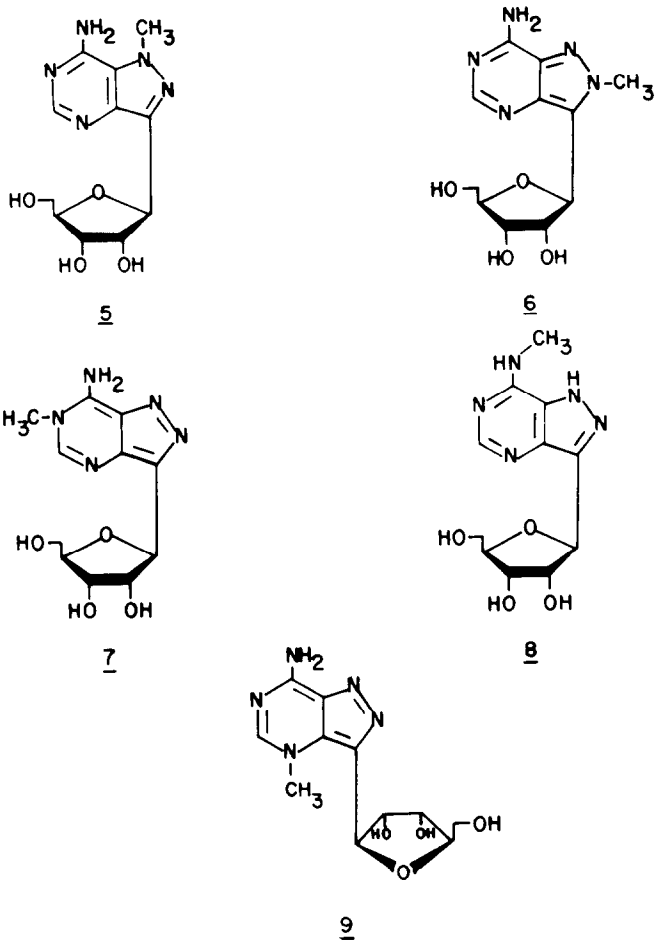

Fig. 2. Structural formulae of the various *N*-methyl derivatives of formycin. Key: 5 = 1-methylformycin, 6 = 2-methylformycin, 7 = 6-methylformycin, 8 = N^7-methylformycin, and 9 = 4-methylformycin.

supernatant fraction solutions were neutralized to pH 6.5 to 7.4 with KOH. After standing for 15 min at 4°, $KClO_4$ was removed by centrifugation and the resulting solutions were kept at −20° until analyzed by high-pressure liquid chromatography (h.p.l.c.).

For the h.p.l.c. analyses reported here, a Varian LCS-1000 liquid chromatograph equipped with a Reeve–Angel Partisil-10 SAX (25 cm × 4.6 mm) column (Whatman, Inc., Clifton, NJ) was utilized. The low concentrate eluant was 0.002 M KH_2PO_4 (pH 4.5) and the high concentrate eluant was 0.5 M KH_2PO_4 (pH 4.5). The starting volume of low concentrate eluant in the mixing chamber was 25 ml and flow rates were 50 ml/hr for both column and gradient pumps. Using these pump settings, a concave gradient is established in which the flow of high concentrate eluant into the mixing chamber is balanced by the flow of gradient solution through the column. For all h.p.l.c. analyses, 20 μl aliquots of the neutralized perchloric acid extracts were used.

To facilitate detection of nucleotides of the formycin compounds, a variable wavelength Schoeffel SF 770 Spectroflow Monitor was placed in series with the standard fixed wavelength u.v. detector (254 nm). This combination of detectors permits the determination of absorbancy of h.p.l.c. effluents at two wavelengths, i.e. at 254 nm, which records the natural cellular nucleotides, and at a second wavelength of choice, e.g. formycin, 295 nm; 1-methylformycin, 303 nm; 2-methylformycin, 307 nm; and N^7-methylformycin, 284 nm.

Characterization of nucleotides of the 1-methyl- and 2-methylformycins was performed in two ways. First, a peak-shift technique was employed to convert nucleoside triphosphates to the corresponding nucleoside diphosphates. An aliquot (200 μl) of neutralized perchloric acid extracts of erythrocytes incubated with either 1-methyl- or 2-methylformycin in the presence of 2′-deoxycoformycin for 4 hr was treated with hexokinase (8 units, Sigma Chemical Co., St. Louis, MO) and glucose (10 mM) for 1 hr at room temperature in a medium consisting of Tris–HCl buffer (0.1 M, pH 7.5), $MgCl_2$ (20 mM) and KCl (0.1 M) in a total volume of 400 μl. The reaction was stopped by adding 200 μl of 6% PCA and the extract was neutralized with KOH. An aliquot of this neutralized extract was analyzed by h.p.l.c. (see above) and the resultant chromatogram was compared with that of the untreated sample (i.e. no hexokinase–glucose). This procedure converts ATP and its structural analogs to the corresponding diphosphate nucleotides. For the second characterization technique, 1.0 ml aliquots of the neutralized perchloric acid extracts used for the peak-shift experiment

were subjected to h.p.l.c. analysis as noted above. Fractions (1.0 ml) of the h.p.l.c. effluents were collected and those corresponding to the mono-, di- and triphosphate nucleotides of the 1-methyl- and 2-methylformycins were pooled separately. The ultraviolet spectrum of each of the pooled materials was determined at pH 4.5 in potassium phosphate buffer (0.5 M) on a Perkin–Elmer model 402 recording spectrophotometer. The spectrophotometric tracings so obtained were compared with that of the appropriate authentic methylformycin.

Incorporation of 1-methyl- and 2-methylformycins into the nucleotide pools of L1210 cells was examined as follows: L1210 cells were removed from $B_6D_2F_1$ mice 14 days after implantation of 10^4 cells. The cells were washed twice with 0.9% NaCl solution and once with the incubation medium used for the erythrocyte incubation studies (see above). The cells (3% suspension) were then incubated with the appropriate methylformycin (1.0 mM) in the presence and absence of 2′-deoxycoformycin (1 μg/ml), and neutralized perchloric acid extracts for h.p.l.c. analysis were prepared in a manner identical to that used for the erythrocyte studies.

For the cytoxicity studies reported here, L1210 cells were grown in static suspension culture as described previously [19]. The ID_{50} values were estimated from plots of average growth rates (from two or more experiments) vs $\log_{10}$ of the concentration of the test compound.

RESULTS

Substrate activities with adenosine deaminase. The reactivity of formycin and its *N*-methyl derivatives as substrates for human erythrocytic ADA is presented in Table 1, along with the natural substrates, adenosine and 2′-deoxyadenosine. Formycin has a V_{max} about 8-fold greater than that of adenosine, whereas 1-methyl-, 4-methyl- and 6-methylformycins displayed no substrate activity with this enzyme. It is noteworthy that 2-methylformycin has a V_{max} value about 4-fold greater than that of adenosine and a K_m value of about 6.0 mM.

Table 1. Kinetic parameters with human erythrocytic adenosine deaminase*

Compound	K_m (mM)	Relative V_{max}
Adenosine	0.025+	100+
2′-Deoxyadenosine	0.007+	60+
Formycin	1.0+	750–850+
2-Methylformycin	6.1	396
N^7-methylformycin	0.1	3

* 1-Methyl-, 4-methyl- and 6-methylformycins showed no activity with ADA. Kinetic parameters for all compounds except N^7-methylformycin were determined by both spectrophotometric and ammonia liberation assay procedures as described previously [6, 10]. For N^7-methylformycin a spectrophotometric assay was used which measured the change in absorbance at 305 nm during the conversion of N^7-methylformycin to formycin B. The molar absorbance change (ΔA) for this conversion in 50 mM potassium phosphate buffer (pH 7.5) was $11.9 \times 10^3\ M^{-1}\ cm^{-1}$.

\+ Values taken from Agarwal *et al.* [6].

N^7-methylformycin shows a V_{max} value of about 3 per cent of that of adenosine and a K_m (0.1 mM) which is about 4-fold greater than that of adenosine.

Formation of analog nucleotides in erythrocytes. Incorporation of these nucleosides into the nucleotide pools of human erythrocytes was evaluated from h.p.l.c. profiles of extracts of cells incubated for various times with analogs (1 mM) in 20% erythrocytic suspensions. Under these conditions, only small quantities of analog nucleotides were detected after 18 hr of incubation with formycin [10, 13], whereas with the 4-methyl- or 6-methyl derivatives, no analog nucleotide formation was detectable. A small amount of the 5′-triphosphate nucleotide of N^7-methylformycin was observed after 18 hr. On the other hand, substantial quantities of analog nucleotides were formed in cells incubated with 1-methyl- and 2-methylformycins.

Figures 3 and 4 present h.p.l.c. profiles of extracts of human erythrocytes after 0, 1 and 18 hr of incubation with 1-methylformycin and 2-methylformycin respectively. With both compounds, the formation of the analog mono-, di- and triphosphate nucleotides was readily detected. 1-Methylformycin appeared to be converted to nucleotides more completely than 2-methylformycin after 18 hr of incubation, as shown by the almost complete absence of an ultraviolet absorbing peak in the nucleoside region (retention time 3–5 min) of the 18 hr h.p.l.c. profile for 1-methylformycin but a sizable peak in the profile for 2-methylformycin, some of which may represent the deamination product of 2-methylformycin. Figures 3 and 4 (right side of blocks) also show the effects of inhibition of erythrocytic ADA by preincubation with 2′-deoxycoformycin (1 μg/ml) on the incorporation of 1-methyl- and 2-methylformycins into erythrocytic nucleotide pools. Nucleotide profiles obtained after 18 hr of incubation show that inhibition of ADA has little effect on the incorporation of 1-methylformycin (Fig. 3). In contrast, ADA inhibition has a marked effect on the incorporation of 2-methylformycin where pronounced increases in analog nucleotide formation occurred (Fig. 4). Almost complete conversion of 1-methyl- and 2-methylformycins to nucleotides took place during the 18 hr of incubation in the presence of 2′-deoxycoformycin.

Figure 5 shows the results of similar experiments with N^7-methylformycin. As noted above, even after 18 hr of incubation, only small quantities of N^7-methylformycin nucleotides appear to have accumulated. When the erythrocytic ADA was inhibited by pretreatment of the cells with 2′-deoxycoformycin, significantly larger quantities of analog nucleotides were formed from N^7-methylformycin in the 2′-deoxycoformycin-treated cells than in control cells. These results can be explained in part by the reactivity of N^7-methylformycin with ADA as noted above (see Table 1).

Comparison of the patterns of incorporation of 1-methyl- and 2-methylformycins into erythrocytic nucleotide pools reveals qualitative as well as quantitative differences. With 1-methylformycin (Fig. 3), after 1 hr of incubation, a large peak of analog 5′-monophosphate nucleotide was observed in both the 254- and 303-nm tracings, whereas only a small peak was detected in the 303-nm tracing in a position consistent with the formation of analog-5′-triphosphate, i.e. with a retention time of about 6 min less than that of ATP. This observation is consistent with the rapid synthesis of large quantities

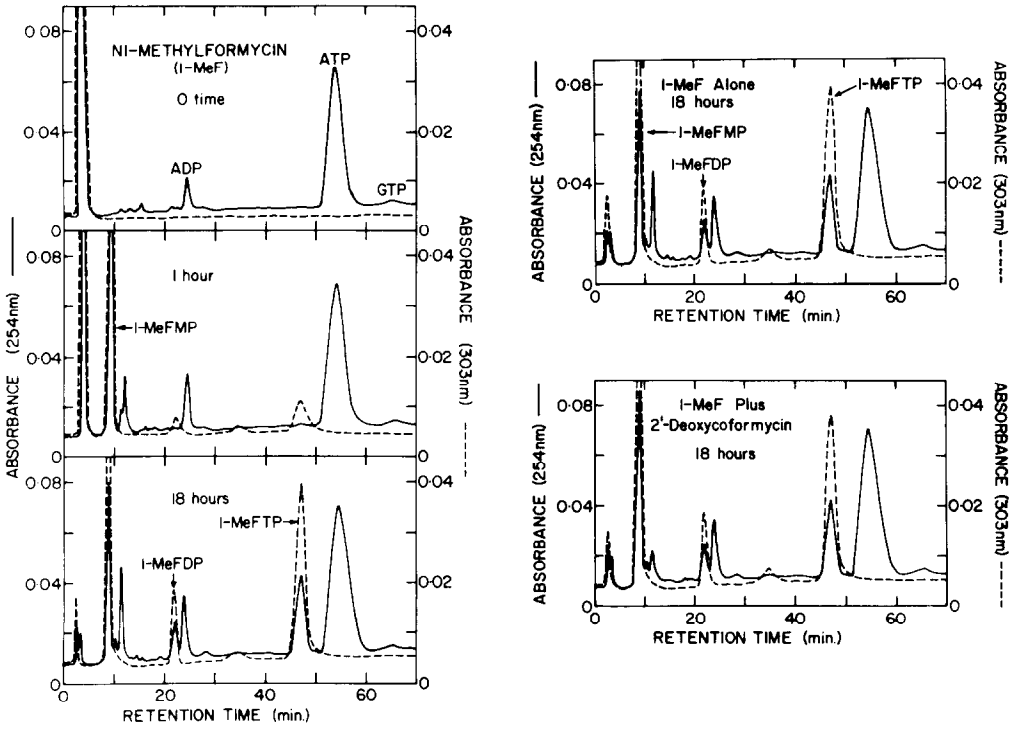

Fig. 3. High-pressure liquid chromatographic profiles of human erythrocytes incubated with 1-methylformycin in the absence and presence of the ADA inhibitor 2′-deoxycoformycin. Fresh, washed human erythrocytes (20–25 per cent suspensions) were incubated with 1-methylformycin (1.0 mM) at 38° in a shaking water bath. At the times indicated, aliquots of the incubation mixture were extracted with perchloric acid, and aliquots (20 μl) of the neutralized extracts were subjected to high-pressure liquid chromatography (h.p.l.c.). Details of the procedures used for incubation, extraction and h.p.l.c. are described in Materials and Methods. When 2′-deoxycoformycin (1 μg/ml) was present, cells were preincubated with this compound for 15 min before the addition of 1-methylformycin. The three profiles on the left of this figure represent erythrocytes incubated, in the absence of 2′-deoxycoformycin, with 1-methylformycin for 0, 1 and 18 hr (top, middle and bottom profiles respectively). On the right are shown profiles which represent erythrocytes incubated with 1-methylformycin for 18 hr in the absence (top) and presence (bottom) of 2′-deoxycoformycin. The solid line represents absorbance of h.p.l.c. column effluents monitored at 254 nm and the dashed line represents absorbance monitored at 303 nm.

of 1-methylformycin-5′-monophosphate with the much slower formation of 1-methylformycin-5′-triphosphate. After 18 hr of incubation, however, sizable peaks were observed in both the 254-nm and 303-nm tracings of the chromatographs with retention times consistent with the formation of the 5′-mono-, di- and triphosphate analog nucleotides. The slow formation of di- and triphosphate nucleotides, however, indicates that 1-methylformycin-5′-monophosphate is a relatively poor substrate for the enzyme adenylate kinase. In comparison with these results, in the absence of ADA inhibition the incorporation of 2-methylformycin into the nucleotide pools was much slower and less extensive (Fig. 4). In contrast to the results with 1-methylformycin, marked accumulation of analog nucleotide in the 5′-monophosphate region of the chromatograph was not observed and the formation of the analog di- and triphosphate nucleotides proceeded readily in a ratio that approximated the ATP:ADP ratio. This suggests that 2-methylformycin-5′-phosphate is a better substrate for adenylate kinase than is 1-methylformycin-5′-phosphate. Significantly, when erythrocytic ADA was inhibited by 2′-deoxycoformycin, almost complete incorporation of both 1-methyl- and 2-methylformycin into erythrocytic nucleotides occurred. Again, these observations emphasize the importance of ADA in the control of human erythrocytic metabolism of adenosine and its analogs [20].

The incorporation of N^7-methylformycin into erythrocytic nucleotide pools (Fig. 5) appears to be qualitatively similar to that seen with 1-methylformycin, i.e. N^7-methylformycin-5′-monophosphate appears to accumulate with the slower formation of N^7-methylformycin di- and triphosphates. On a quantitative basis, however, a greater percentage of 1-methylformycin than of N^7-methylformycin is converted into analog nucleotides. These data may reflect (a) the poor reactivity of N^7-methylformycin with erythrocytic adenosine kinase, and (b) the relatively poor reactivity (compared to 2-methylformycin-5′-monophosphate) of N^7-methylformycin-5′-monophosphate with the enzyme, adenylate kinase.

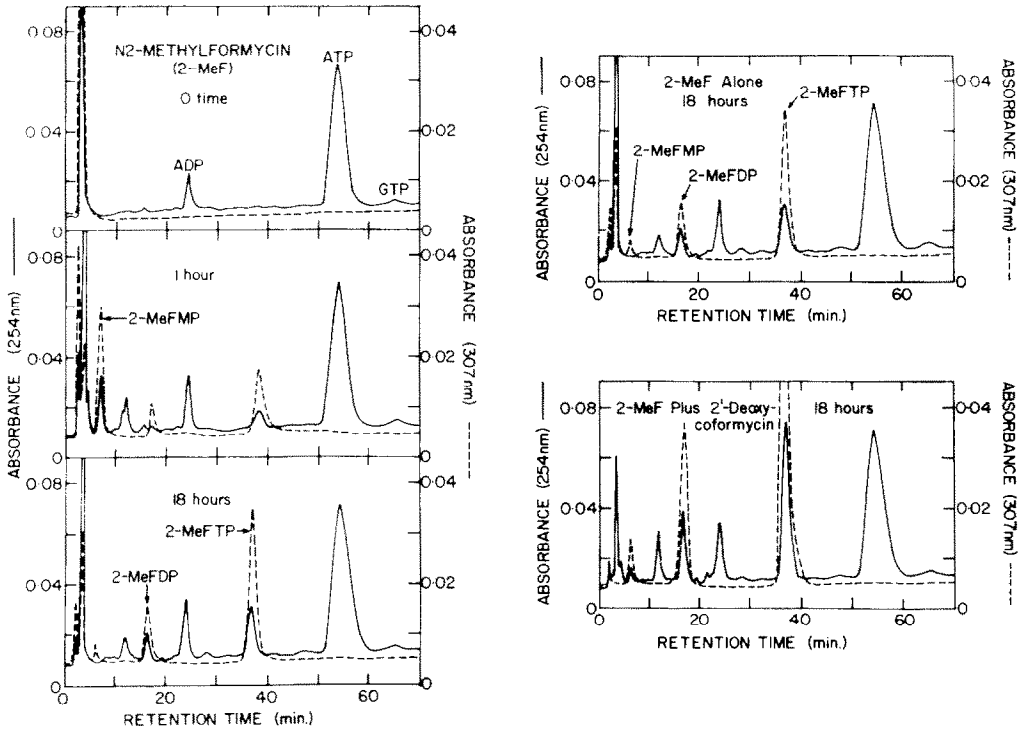

Fig. 4. High-pressure liquid chromatographic profiles of human erythrocytes incubated with 2-methylformycin in the absence and presence of 2′-deoxycoformycin. Experimental details are identical to those given in the legend to Fig. 3, except that 1-methylformycin is replaced by 2-methylformycin. The solid line represents absorbance of h.p.l.c. column effluents monitored at 254 nm and the dashed line represents absorbance at 307 nm.

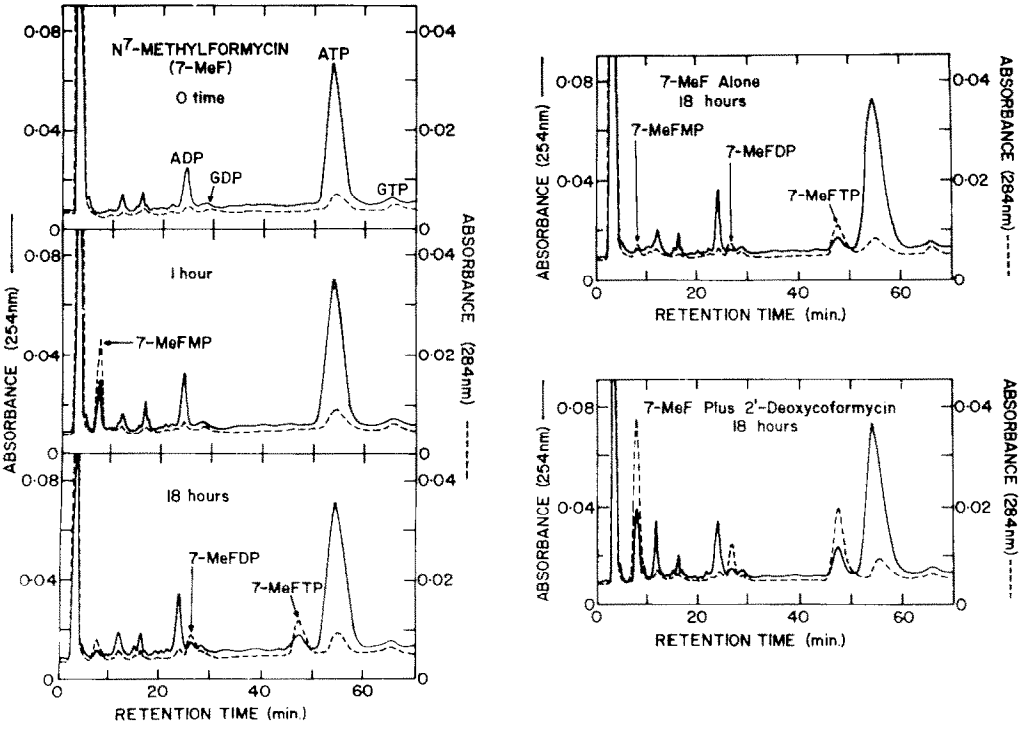

Fig. 5. High-pressure liquid chromatographic profiles of human erythrocytes incubated with N^7-methylformycin in the absence and presence of 2′-deoxycoformycin. Experimental details are as described in the legend to Fig. 3 except that 1-methylformycin is replaced by N^7-methylformycin. The solid line represents absorbance of h.p.l.c. column effluents monitored at 254 nm and the dashed line represents absorbance monitored at 284 nm.

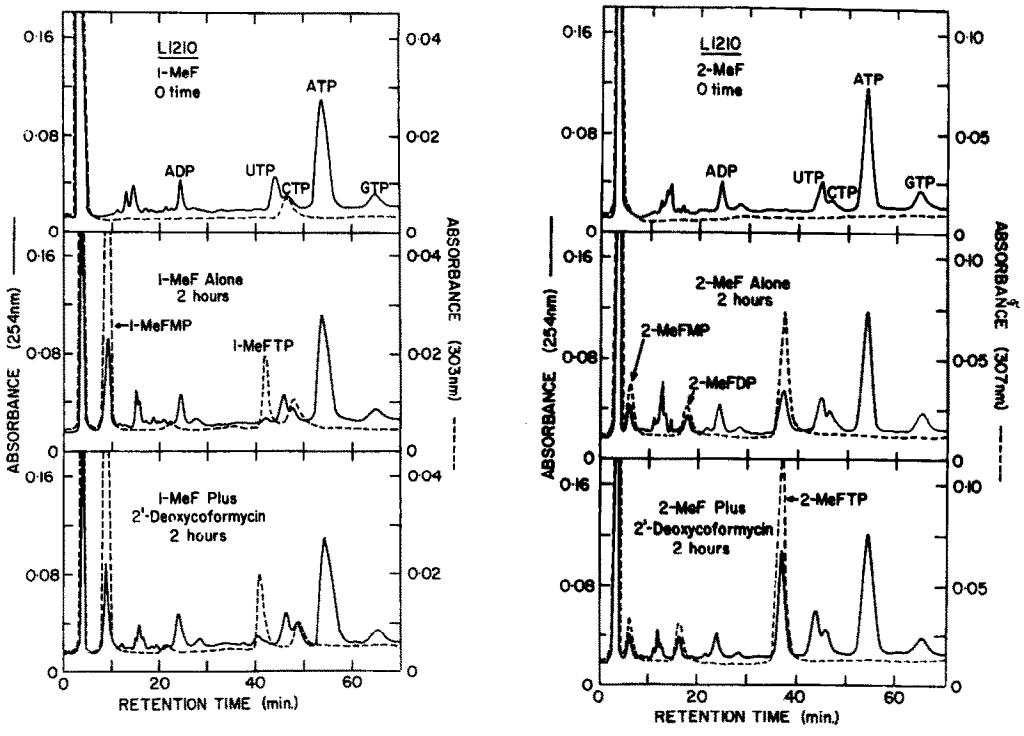

Fig. 6. High-pressure liquid chromatographic profiles of L1210 cells incubated *in vitro* with 1-methylformycin (left side) or 2-methylformycin (right side). L1210 cells (3% suspension) were incubated with 1.0 mM analog for 0 time (top profiles) or for 2 hr in the absence (middle profiles) or presence (bottom profiles) of 2′-deoxycoformycin (1 μg/ml). Details of incubation, extraction, etc. are given in the legend to Fig. 3. The solid lines represent absorbance of the h.p.l.c. column effluents monitored at 254 nm and the dashed lines represent absorbance monitored at the λ_{max} of the compound under study (303 nm for 1-methylformycin and 307 nm for 2-methylformycin). High-pressure liquid chromatographic sample size was 100 μl.

Formation of analog nucleotides from 1-methyl- and 2-methylformycins in L1210 cells in vitro. Results qualitatively similar to those seen with human erythrocytes were obtained when 1-methyl- and 2-methylformycins were incubated with L1210 cells *in vitro* in the presence and absence of 2′-deoxycoformycin. Figure 6 illustrates h.p.l.c. profiles of L1210 cells treated with 1-methylformycin (left side) or 2-methylformycin (right side) for 0 hr (top frames) and for 2 hr in the absence (middle frames) and presence (bottom frames) of 2′-deoxycoformycin. Thus, both analogs form mono-, di- and triphosphate nucleotides in L1210 cells after 2 hr of incubation. Again, 1-methylformycin appears to accumulate primarily at the monophosphate nucleotide level. Conversion of the monophosphate nucleotide to the analog triphosphate nucleotide appears to be relatively slow compared to that seen with 2-methylformycin. Pretreatment of the cells with 2′deoxycoformycin has no apparent effect on the incorporation of 1-methylformycin into the nucleotide pools of L1210 cells. In comparison, the incorporation of 2-methylformycin into the intracellular nucleotide pools is enhanced by pretreatment of the cells with 2′-deoxycoformycin.

Characterization of analog nucleotides. Figure 7 and Table 2 present the results of experiments designed to characterize the nucleotides which accumulate in erythrocytes incubated for 4 hr with 1-methylformycin and 2-methylformycin in the presence of 2′-deoxycoformycin. Figure 7 shows h.p.l.c. profiles of aliquots of neutralized perchloric acid extracts of such treated cells before (top profiles) and after (bottom profiles) incubation with a combination of hexokinase and glucose. This enzyme is highly specific for ATP or its close structural analogs. After hexokinase–glucose treatment of extracts of both 1-methylformycin- (left side) and 2-methylformycin- (right side) treated cells, peaks corresponding to ATP and analog triphosphate nucleotides disappear and corresponding increases in the ADP and analog diphosphate nucleotides occur. Because of the high specificity of hexokinase for ATP or its close analogs, the results of this peak-shift experiment are a strong indication that the peaks on the h.p.l.c. profiles in Fig. 7 (and in Figs. 3 and 4) labeled 1-MeFTP and 2-MeFTP correspond to the 5′-triphosphates of 1-methylformycin and 2-methylformycin respectively.

To obtain more definitive identification of the analog nucleotides in question, large (1.0 ml) volumes of the same extracts used for the peak-shift experiment were subjected to high-pressure liquid chromatography as outlined in Materials and Methods. Pools made from the fractions corresponding to the mono-, di- and triphosphates of 1-methyl- and 2-methylformycins were scanned for ultraviolet spectra. Table 2 lists the λ_{max} values of the various pools compared with those of the authentic analog nucleosides. As Table 2 shows, all of the peaks scanned exhibit λ_{max} values virtually identical

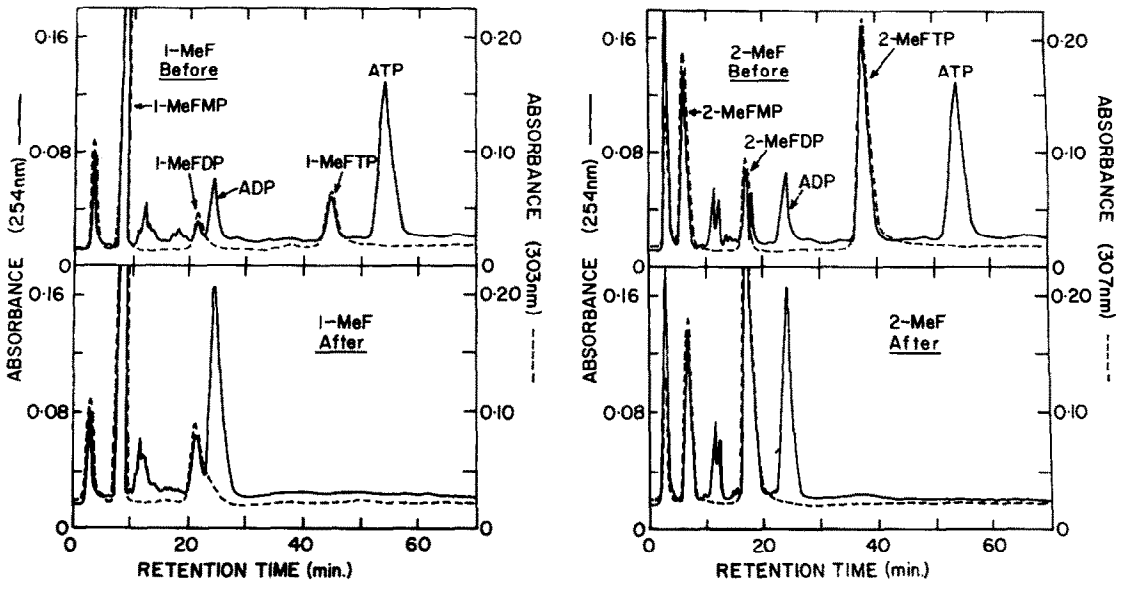

Fig. 7. Characterization of analog nucleoside triphosphates by an enzymic peak-shift technique. High-pressure liquid chromatographic profiles of neutralized PCA extracts of erythrocytes incubated with 1-methylformycin (left side) or 2-methylformycin (right side) in the presence of 2′-deoxycoformycin (1 μg/ml) for 4 hr. For details of incubation and extraction conditions see legend to Fig. 3. The top profiles in each case represent aliquots of extracts before the peak-shift, the bottom profiles represent aliquots of the same extracts after treatment with hexokinase and glucose. The peak-shift was performed by incubating 200 μl of the neutralized PCA extracts with hexokinase (8 units) and glucose (10 mM) in a medium composed of Tris–HCl (0.1 M, pH, 7.5), $MgCl_2$ (20 mM) and KCl (0.1 M) in a total of 400 μl. After 1 hr at room temperature, reactions were terminated by the addition of 200 μl of 6% perchloric acid. Extracts were neutralized with KOH as described in Materials and Methods. Solid lines represent absorbance of column effluents monitored at 254 nm; dashed lines represent absorbance at the λ_{max} of the compound under study (303 nm for 1-methylformycin and 307 nm for 2-methylformycin). High-pressure liquid chromatographic sample size was 100 μl.

to those of the starting material, i.e. 1-methylformycin or 2-methylformycin. Therefore, the results presented in Fig. 7 and Table 2 clearly confirm the identity of peaks corresponding to analog nucleotides which are presented on h.p.l.c. profiles of cells incubated with 1-methyl- or 2-methylformycin.

Cytotoxicity to L1210 cells. The ID_{50} values for growth inhibition of L1210 cells in culture by formycin, formycin B and the *N*-methylformycins are shown in Table 3. Formycin, 1-methyl-, and 2-methyl- and N^7-methylformycin all show similar toxicity, with ID_{50} values in the range of 0.5 to 2.0 μM, whereas 4-methyl- and 6-methylformycin do not show significant growth inhibition even at 100 μM.

Table 2. Ultraviolet spectral data of analog nucleotides*

Compound or pool	λ_{max} (nm)†
1-Methylformycin	209, 293(s), 303, 314(s)
1-Methylformycin-5′-monophosphate (1-MeFMP)	209, 303, 314(s)
1-Methylformycin-5′-diphosphate (1-MeFDP)	209, 303, 314(s)
1-Methylformycin-5′-triphosphate (1-MeFTP)	209, 303, 314(s)
2-Methylformycin	210, 295(s), 307, 314(s)
2-Methylformycin-5′-monophosphate (2-MeFMP)	Not done
2-Methylformycin-5′-diphosphate (2-MeFDP)	209, 295(s), 307, 315(s)
2-Methylformycin-5′-triphosphate (2-MeFTP)	210, 307, 316(s)

* Aliquots (1.0 ml) of neutralized perchloric acid extracts of human erythrocytes incubated for 4 hr with either 1-methylformycin or 2-methylformycin (1.0 mM) in the presence of 2′-deoxycoformycin (1 μg/ml) were subjected to high-pressure liquid chromatography. For details of incubation, extraction, neutralization and h.p.l.c. analysis, see Materials and Methods. Effluents from the h.p.l.c. column were collected in 1.0 ml fractions. Fractions corresponding to the analog mono-, di- and triphosphates were separately pooled and ultraviolet absorption spectra of the pooled fractions were obtained at pH 4.5 in potassium phosphate buffer (0.5 M) using a Perkin–Elmer model 402 recording spectrophotometer.

† (s) = shoulder.

Table 3. Cytotoxicities to L1210 cells *in vitro*

Compound	ID_{50}* (μM)
Formycin	1.0
Formycin B	75
1-Methylformycin	0.8
2-Methylformycin	0.5
4-Methylformycin	
6-Methylformycin	130†
N^7-methylformycin	2.0

* Concentration required to reduce the relative growth rate to 50% of control. A dash indicates no effect on cell growth at 100 μM.

† The growth inhibition observed for 6-methylformycin may be due to the unavoidable presence of N^7-methylformycin in small amounts (approximately 1 per cent), since 6-methylformycin may undergo a rearrangement to form N^7-methylformycin (Ref. 17 and A. F. Lewis and L. B. Townsend, manuscript in preparation).

DISCUSSION

Recent reports have proposed a correlation between the conformations of adenosine analogs and their abilities to serve as substrates for ADA [18, 21–24]. It was postulated that adenosine analogs which exist predominantly in the *anti* conformation would be excellent substrates for ADA, whereas those in the *syn* conformation would have little or no substrate activity. This hypothesis predicted that 1-methylformycin, which should exist predominantly in the *anti* conformation (since there is no steric hindrance to rotation about the glycosyl bond), would be a good substrate for ADA. On the other hand, marked steric hindrance to rotation occurs with 2-methylformycin and, accordingly, this compound has been found to exist predominantly in the *syn* conformation in the crystalline state [25]. The above hypothesis consequently predicted that 2-methylformycin would be a poor substrate for ADA. The data in Table 1 invalidate this hypothesis. No substrate activity for ADA was detected with 1-methylformycin, a compound that is capable of readily assuming the *anti* conformation, whereas 2-methylformycin (presumably fixed in the *syn* conformation) displays substrate activity ($K_m \simeq 6.0$ mM, V_{max} about four times greater than that for adenosine) with erythrocytic ADA. Furthermore, 4-methylformycin, a compound in which the *syn* conformation should be blocked and the *anti* conformation highly favoured, displayed no substrate activity with erythrocytic ADA. On the other hand, N^7-methylformycin, which, like 1-methylformycin, should exist predominantly in the *anti* conformation, showed activity as a substrate with ADA (Table 1). These results suggest strongly that the conformation (either *syn* or *anti*) of an adenosine analog is not a major consideration in determining ADA substrate activity. In fact, it appears that the lack of substrate activity for certain adenosine analogs is due primarily to specific chemical modifications of the compound which result in a change of the electronic, steric and/or structural parameters, rather than the conformational aspects, at least for erythrocytic ADA.

The above results emphasize further the importance of the 7 position (purine ring structure) for the binding of adenosine and its analogs to the active site of ADA. For example, compounds of the tubercidin class, in which N-7 of the purine ring is replaced by a carbon atom, display neither substrate nor inhibitory activity with ADA [6]. However, it must be noted that the simple replacement of purine N-7 by a carbon atom does not, *per se*, impede substrate binding to ADA, since the adenosine analog 4-amino-1(β-D-ribofuranosyl) pyrazolo[3,4-*d*]pyrimidine is an active substrate with ADA from calf intestine [26] and human erythrocytes [27]. On the other hand, with the latter compound, if a carboxamide group is substituted on the carbon atom in purine position 7 (forming an analog similar to sangivamycin) [28], activity with ADA is abolished [27]. The fact that methylation of N-1 of formycin (corresponding to purine position 7) abolishes substrate activity with ADA is additional evidence that a bulky group on purine position 7 impedes binding of adenosine analogs to the active site of the enzyme, probably due to steric effects.

The above observations demonstrate that N-1-methylation of formycin provides a derivative that can form intracellular nucleotides similarly to formycin in combination with a potent ADA inhibitor, i.e. coformycin or 2′-deoxycoformycin [13]. Consistent with the results reported here with erythrocytic ADA (Table 1) is a recent report which indicates that 2-methylformycin shows substrate activity with the calf intestinal enzyme [29]. Of particular relevance is a report on the effects of 1-methyl- and 2-methylformycins on the vaccinia, herpes simplex, and vesicular stomatitis viruses in primary rabbit kidney (PRK) cell cultures [29]. It was found that 2-methylformycin has relatively strong activity against vaccinia virus (approaching the potency of 5-iodo-2′-deoxyuridine). On the other hand, 1-methylformycin was inactive against all three viruses. The results presented above, which indicate that 1-methylformycin is superior to 2-methylformycin in the rates of synthesis and the total quantities of analog nucleotides formed in human erythrocytes, appear to be inconsistent with the result of these antiviral studies. One might have expected that an adenosine analog that reacts most readily with the mammalian nucleoside and nucleotide kinases necessary to form polyphosphate nucleotides in cells should display the greatest antiviral activity.

Formycin and its 1-methyl-, 2-methyl- and N^7-methyl-derivatives all showed similar cytotoxicity to L1210 cells, whereas formycin B and the other *N*-methyl derivatives were comparatively inactive (Table 2). In contrast, Giziewicz *et al.* [29] reported that formycin and formycin B both showed cytotoxicity, whereas the 1-methyl- and 2-methylformycins had no toxic effects on the PRK cells used for evaluation of antiviral activity. The discrepancies between the results reported here and those of Giziewicz *et al.* [29] may be due to cell line differences or to the different conditions of assay.

The compounds that show pronounced cytotoxicity to L1210 cells are also capable of forming nucleotides in human erythrocytes, if deamination is prevented either by the molecular structure of the analog or by an ADA inhibitor such as 2′-deoxycoformycin. However, the extent of nucleotide formation from N^7-methylformycin is much less than from formycin, 1-methyl- or 2-methylformycin. This apparent discrepancy may be due to *bona fide* differences in the metabolism of N^7-

methylformycin by human erythrocytes and L1210 cells, and should be investigated further. The two most potent cytotoxic agents, 1-methyl- and 2-methylformycin, formed significant quantities of analog nucleotides in L1210 cells.

The cytotoxicity of formycin and 2-methylformycin to L1210 cells in culture is unexpected in view of the rapid deamination of these compounds by human erythrocytic ADA reported here, and the high level of ADA previously reported in L1210 cells [30]. However, the ID_{50} values for L1210 growth inhibition by these compounds are about three orders of magnitude smaller than their K_m values for human erythrocytic ADA, and therefore deamination may not be significant at the nucleoside concentrations needed to inhibit cell growth. On the other hand, deamination by erythrocytes must still be considered a pharmacologically important reaction when these compounds are administered to intact animals. In fact, the potent ADA inhibitor, coformycin, has been shown to enhance the activity of formycin against Ehrlich ascites carcinoma cells in mice [31]. Therefore, the possibility that ADA inhibitors might enhance the antiviral and/or antitumor activity of 2-methylformycin should be studied further.

The observations presented above raise many intriguing questions. For example, can the antiviral or cytotoxic activity of 2-methylformycin be increased by concurrent treatment with a potent ADA inhibitor as indicated by the greater formation of analog nucleotides as seen in Figs. 4 and 6? Is 1-methylformycin ineffective against vaccinia virus because of inactivity of its nucleotides with enzymes such as ribonucleotide reductase or viral DNA polymerase? How do formycin and its active *N*-methyl derivatives cause cytotoxicity? Obviously, compounds of this class deserve further study.

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