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SPECIFICITY OF ADENOSINE DEAMINASE INHIBITORS*

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Abstract—The specificity of the potent adenosine deaminase inhibitors deoxycoformycin (covidarabine), coformycin and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), has been assessed in Ehrlich ascites tumor cells *in vitro* and in cultured mouse lymphoma L5178Y cells. EHNA is both less potent an inhibitor of adenosine deaminase than deoxycoformycin, and is less specific. High concentrations of deoxycoformycin and EHNA inhibit all pathways of purine ribonucleotide synthesis, and inhibit the conversion of inosinate to adenine and guanine nucleotides. These drugs also inhibit purified adenylate deaminase, but inhibition of this enzyme in intact cells can only be detected at high rates of deamination of adenylate. Deoxycoformycin potentiates the toxicity of adenine against cultured cells.

Several highly potent inhibitors of adenosine deaminase (EC 3.5.4.4) have been identified in the past few years: coformycin [1–4], deoxycoformycin (also called covidarabine) [5], and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) [6]. The structures of these compounds are shown in Fig. 1.

As coformycin [7–14], deoxycoformycin [15–20] and EHNA [21–24] are coming into increasing use in studies of the metabolism of intact cells, and are being used in enzyme assays [25–27], it is important to determine if they are in fact specific inhibitors of adenosine deaminase, or if they also inhibit other aspects of purine metabolism as well. In this study we have determined the effects of deoxycoformycin and EHNA, and in some cases coformycin, on a number of aspects of purine metabolism in Ehrlich ascites tumor cells incubated *in vitro* and in cultured mouse lymphoma L5178Y cells.

MATERIALS AND METHODS

Coformycin, deoxycoformycin and EHNA were gifts of Drs H. Umezawa and M. Hori (Institute of Microbiol Chemistry, Tokyo), G. A. LePage (University of Alberta Cancer Research Unit), and G. B. Elion (Burroughs Wellcome and Co., Research Triangle Park, NC), respectively. Each was dissolved in distilled water for use.

Sources of most other materials [28, 29], methods of preparation and incubation of Ehrlich ascites tumor cells [28] and culture of lymphoma L5178Y cells [30], and procedures for the separation and measurement of radioactivity in purine bases, ribonucleosides and ribonucleotides [31, 32] and for the incorporation of radioactive glycine into phosphoribosyl formylglycineamide in the presence of azaserine [33], have been reported previously. Rabbit muscle adenylate deaminase (EC 3.5.4.6) was obtained from the Sigma Chemical Co. The AMP deaminase was assayed spectrophotometrically at 20° in 0.10 M sodium citrate at pH 6.5.

RESULTS

The aim of this study was to determine whether deoxycoformycin, coformycin and EHNA affected aspects of purine metabolism in intact mammalian cells in addition to their inhibitory effects on adenosine deaminase. As a basis for assessing the signifi-

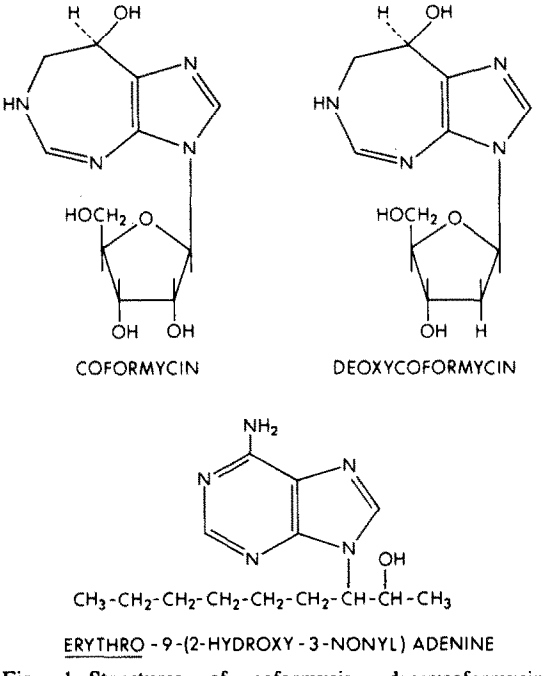

Fig. 1. Structures of coformycin, deoxycoformycin (covidarabine), and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA).

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cance of such side effects, it was first necessary to determine the lowest concentration of each compound that would virtually completely inhibit adenosine deaminase activity in Ehrlich ascites tumor cells incubated *in vitro* under the particular conditions used in this investigation. Previous studies [7] had shown that the concentration of coformycin that was required for this purpose was 3.5 μM (1 μg/ml), although in slices of mouse liver, kidney, brain and heart, 35 μM was required [9].

Figure 2 shows the relationship between concentration of deoxycoformycin, coformycin and EHNA and the deamination of adenosine by intact tumor cells. Deoxycoformycin was slightly more potent than coformycin, and both were appreciably more effective than EHNA. Concentrations of between 1.8 and 3.6 μM deoxycoformycin and coformycin gave 98–99 per cent inhibition, while 3.6 μM EHNA gave 96 per cent inhibition. For purposes of the following experiments, 3.6 μM of each of the three inhibitors was considered to be effective for inhibiting adenosine deaminase activity.

In order to assess the specificity of deoxycoformycin and EHNA in intact tumor cells, the effects of a range of concentrations of each compound on each of the following reactions or processes was determined: (a) conversion of radioactive adenosine to nucleotides, (b) conversion of radioactive adenine, guanine and hypoxanthine to nucleotides, (c) conversion of radioactive glycine to phosphoribosyl formylglycineamide in the presence of azaserine (this is a measure of the first few reactions in the pathway of purine biosynthesis *de novo*), (d) conversion of inosinate to adenine and guanine nucleotides, (e) breakdown of adenine nucleotides (an indicator of effects on energy metabolism), and (f) the deamination of adenylate. Because of limited availability of coformycin, it was not used in all of the experiments described. Concentrations of deoxycoformycin used in these studies ranged from 3.6 to 185 μM, and the concentrations of EHNA used ranged from 3.6 to 76 μM.

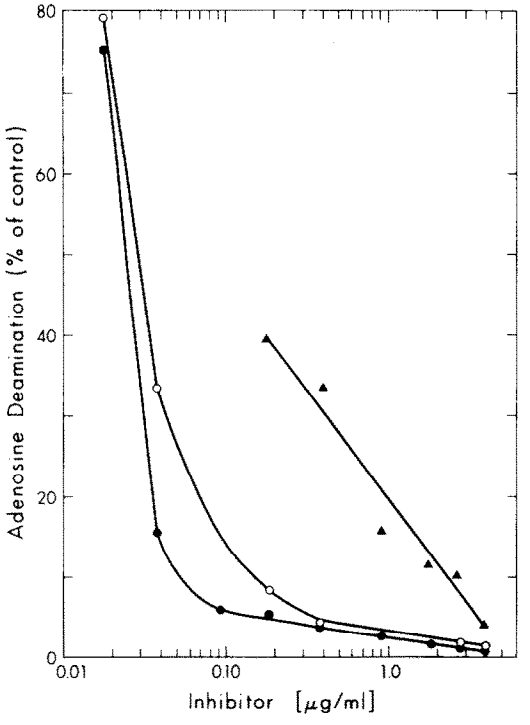

Fig. 2. Inhibition of deamination of adenosine. Ehrlich ascites tumor cells (0.1 ml of a 2%, v/v suspension) in Fischer's medium containing 25 mM phosphate and no sodium bicarboxate were incubated at 37° with shaking, in an air atmosphere, with various concentrations of coformycin (○), deoxycoformycin (●), or EHNA (▲). After 20 min, [^{14}C]adenosine (28 mCi/m-mole, final concentration, 200 μM) was added and the incubation was continued for 30 min. Deamination of adenosine was calculated as radioactivity in inosine plus hypoxanthine. Results presented are averages of duplicate determinations, and are representative of data obtained in two separate experiments.

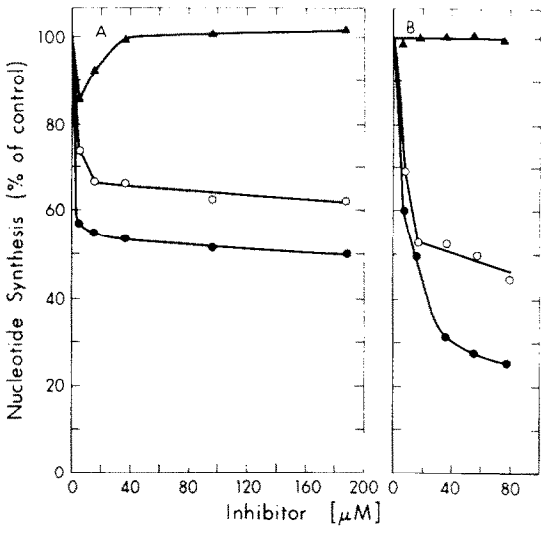

Fig. 3. Effects of adenosine deaminase inhibitors on conversion of adenosine to nucleotides. Cells were incubated generally as described in Fig. 2, with either deoxycoformycin (A) or EHNA (B). Cells were either incubated with inhibitor for 20 min, followed by addition of [^{14}C]adenosine and further incubation for 30 min (●); or they were incubated for 20 min without inhibitor, followed by simultaneous addition of inhibitor and [^{14}C]adenosine and further incubation for 30 min (○); or they were incubated with inhibitor for 20 min, after which inhibitor was removed by centrifugation and resuspension twice in fresh medium, [^{14}C]adenosine was then added and incubation was continued for 30 min (▲). Total radioactivity in acid-soluble nucleotides was determined. Results presented are averages of duplicate determinations, and are representative of data obtained in two experiments.

Experiments were first conducted to determine if deoxycoformycin or EHNA inhibited the conversion of radioactive adenosine to nucleotides. As such inhibition could be produced either by inhibition of adenosine transport or by inhibition of the phosphorylation of adenosine, or both, these experiments were conducted in three different ways. First, cells were incubated with drug (and glucose) for 20 min prior to addition of radioactive adenosine. Second, drugs and radioactive adenosine were added simultaneously, following 20 min incubation with glucose alone. Third, cells were incubated with drug (and glucose) for 20 min, and extracellular free drug was removed by centrifugation and resuspending the cells twice in fresh medium containing no drug. Radioac-

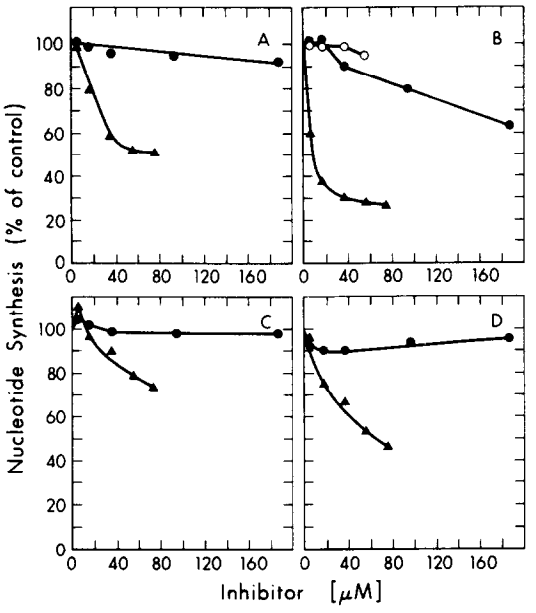

Fig. 4. Effects of adenosine deaminase inhibitors on nucleotide synthesis from purine bases and on purine biosynthesis *de novo*. Tumor cells were incubated as described in Fig. 2 with deoxycoformycin (●), coformycin (○), or EHNA (▲). After 20 min, [^{14}C]adenine (A), [^{14}C]hypoxanthine (B), or [^{14}C]guanine (C), were added to final concentrations of 100 μM and incubations were continued for 30 min. Total radioactivity in acid-soluble nucleotides was determined. Alternatively, cells were incubated for 20 min in calcium-free Krebs–Ringer medium containing 25 mM phosphate, 5.5 mM glucose, adenosine deaminase inhibitors and 10 μM azaserine, after which 2 μM glutamine and 1 mM [^{14}]glycine were added and incubation was continued for 30 min (D). Radioactivity in phosphoribosyl formylglycineomide was determined. Results presented are averages of duplicate determinations, and are representative of results obtained in two to three experiments.

tive adenosine was added at this point and nucleotide synthesis was measured as usual.

Figure 3 shows that both deoxycoformycin and EHNA inhibited conversion of adenosine to nucleotides when both drug and adenosine were present together in the medium. Greater inhibition was achieved when cells were incubated for 20 min with drug before addition of radioactive adenosine than if drug and adenosine were added together. In these experiments EHNA was somewhat more potent than deoxycoformycin, and its effects were more strongly dose dependent than were those of deoxycoformycin.

However, when deoxycoformycin and EHNA were removed by changing the medium prior to addition of radioactive adenosine, no inhibition of nucleotide synthesis was observed. This would suggest that these drugs do not inhibit adenosine kinase, (EC 2.7.1.20) but instead are inhibitors of adenosine entry into the cells.

Figure 4 shows the effects of a range of concentrations of deoxycoformycin and EHNA on nucleotide synthesis from radioactive adenine (Fig. 4A), hypoxanthine (Fig. 4B), and guanine (Fig. 4C), and on the early steps of the pathway of purine biosynthesis *de novo*, as measured by the incorporation of radioactive glycine into phosphoribosyl formylglycineamide in the presence of azaserine (Fig. 4D). Figure 4C also shows the effects of a smaller range of concentrations of coformycin on nucleotide synthesis from radioactive hypoxanthine.

Figs 4A, 4C and 4D show that deoxycoformycin had little or no effect on nucleotide synthesis from adenine or guanine or on purine biosynthesis *de novo*. In each case, however, EHNA produced considerable inhibition at the higher concentrations.

Figure 4B shows that EHNA produced greater inhibition of nucleotide synthesis from radioactive hypoxanthine than it did when the other precursors were used. In addition, high concentrations of deoxycoformycin also inhibited this process, in contrast to its lack of effect on other pathways of nucleotide formation. Coformycin appeared to affect nucleotide synthesis from hypoxanthine less than deoxycoformycin, but it was not tested at very high concentrations.

One possible basis for a differential effect of deoxycoformycin and EHNA on nucleotide synthesis from hypoxanthine would be inhibition of the conversion of inosinate to either adenine nucleotide or guanine nucleotides, or both. It has previously been established [8, 12, 31, 34] that inhibition of these processes does not lead to accumulation of nucleoside monophosphate, but instead that such nucleotides are dephosphorylated to nucleosides. In order to test this possibility, the ratio of radioactivity in adenine nucleotides to that in guanine nucleotides was calculated from data obtained in the experiment of Fig. 4C. As shown in Fig. 5, this ratio was moderately decreased in cells incubated with deoxycoformycin, whereas EHNA caused a very substantial decrease. These results suggest that both compounds inhibit the conversion of inosinate to adenylate. In fact, 76 μM EHNA inhibited incorporation of radioactive hypoxanthine into adenine nucleotides by 80 per cent, whereas that into guanine nucleotides was inhibited only 32 per cent. Similarly, 185 μM deoxycoformycin inhibited incorporation of radioactive hypoxanthine into adenine nucleotides by 47 per cent, whereas that into guanine nucleotides was inhibited only 19 per cent.

One sensitive indicator of the effects of drugs on energy metabolism is increased formation of radioactive inosine and hypoxanthine in cells incubated with radioactive adenine, as well as a decrease in the ratio of radioactivity in ATP to that in ADP in the same experiment [12]. Neither deoxycoformycin nor EHNA affected these processes.

Debatisse and Buttin have recently reported that in addition to inhibiting adenosine deaminase, coformycin also inhibited AMP deaminase in cell-free extracts of Chinese hamster fibroblasts [14]. They also observed that coformycin appeared to potentiate the toxicity of adenine in these cells. These observations prompted us to examine the inhibition of partially purified rabbit muscle adenylate deaminase by deoxycoformycin and EHNA. The partially purified adenylate deaminase was chosen rather than a crude cell-free extract to minimize the possibility that any inhibition observed may be caused by a phosphorylated derivative. Plots of velocity against AMP concentration gave the expected sigmoid curves with an apparent Michaelis constant of approximately 0.10 mM. Similar plots in the presence of either in-

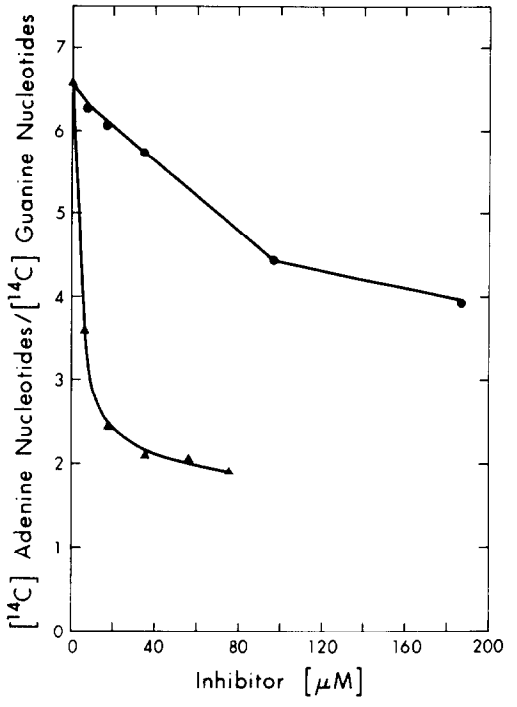

Fig. 5. Effects of adenosine deaminase inhibitors on conversion of inosinate to adenine nucleotides and guanine nucleotides. Cells were incubated as described in Fig. 2 for 20 min with deoxycoformycin (●) or EHNA (▲), after which [^{14}C]hypoxanthine was added to a final concentration of 100 μM, and incubation was continued for 30 min. Radioactivity in radioactive adenylate, ADP, ATP, guanylate, GDP and GTP was determined. Results presented are averages of duplicate determinations, and are representative of data obtained in two experiments.

hibitor showed a decrease in reaction velocity. The studies were not extensive enough to determine whether the observed slight increase in the apparent Michaelis constant was significant. Figure 6 shows the relative velocity of the adenylate deaminase reaction at various inhibitor concentrations. These results are similar to the reported effects of coformycin on the unpurified adenylate deaminase from Chinese hamster fibroblasts.

It is difficult to measure possible effects of drugs on adenylate deaminase activity in intact Ehrlich ascites tumor cells, as the rate of this process in cells incubated with radioactive adenine or adenosine is very low [19, 28]. However, the rate of this process is greatly accelerated in cells treated with 2-deoxyglucose or 2,4-dinitrophenol, and the extent of deamination of adenylate varies both with time and with concentration of the inhibitor of energy metabolism [8, 12]. In a previous study [12] of ATP catabolism in cells incubated with deoxyglucose but in the absence of coformycin, the relationship between deamination of adenylate and formation of adenylate from ATP was shown to be linear over quite a wide range. Though not published at the time, similar studies had also been conducted in the presence of 3.5 μM coformycin, and these data appear to be relevant to the question of inhibition of adenylate deaminase in intact cells.

Figure 7 shows plots of adenylate deamination as

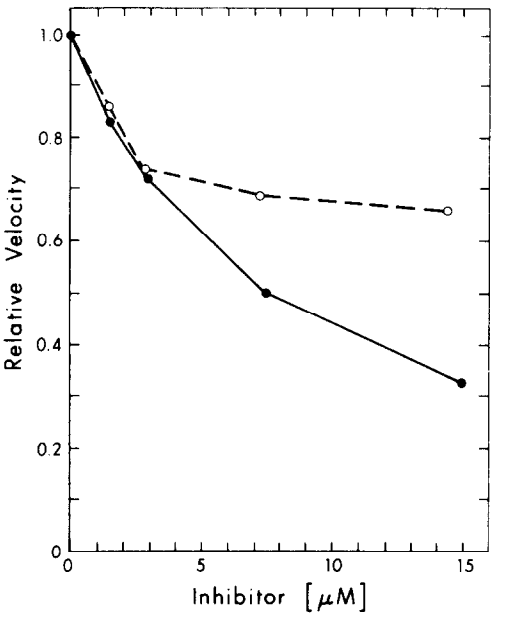

Fig. 6. Inhibition of rabbit muscle adenylate deaminase by various concentrations of deoxycoformycin (●) and EHNA (○). Reactions were started by addition of the enzyme.

a function of amount of adenylate formed by the breakdown of ATP under the influence of deoxyglucose, both in the presence and absence of coformycin. It can be seen that there is apparently less deamination of AMP in the presence of coformycin than in its absence. In a smaller experiment using dinitro-

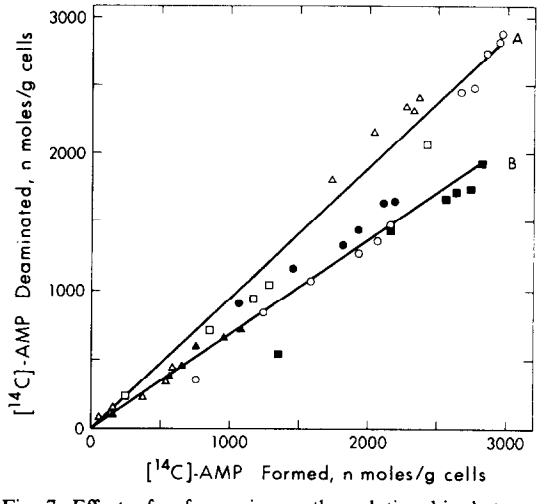

Fig. 7. Effect of coformycin on the relationship between [^{14}C]AMP deamination and [^{14}C]AMP formation. Cells were incubated for 30 min in Fischer's medium containing 25 mM phosphate and 100 μM [^{14}C]adenine. They were then centrifuged, washed, and resuspended in Fischer's medium containing the following concentrations of glucose and deoxyglucose: ■, 5.5 mM deoxyglucose; ○,●, 4.81 mM deoxyglucose + 0.69 mM glucose; △, ▲, 3.3 mM deoxyglucose + 2.2 mM glucose; ●, ▲, 400 μM aminoiodoribofuranosylpyrrolo-pyrimidine (an inhibitor of adenosine phosphorylation). A, without coformycin. B, with 3.5 μM coformycin. Data for B are taken from ref. 12.

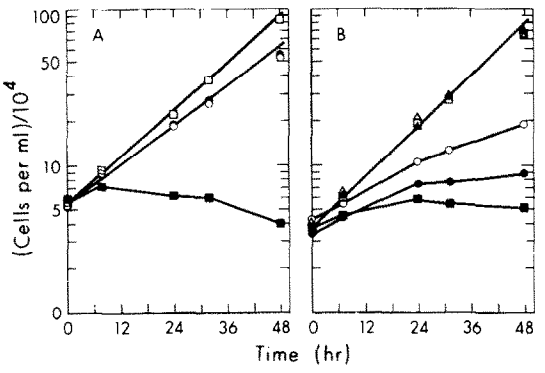

Fig. 8. Effects of deoxycoformycin on adenine metabolism in cultured mouse lymphoma L5178Y cells. L5178Y cells were subcultured 16 hr prior to the addition of reagents to start these experiments at time zero.

(A). The growth of a L5178Y control culture (□) is shown together with that of cultures containing 1 μM methotrexate and 20 μM dThd (■); with 1 μM methotrexate, 20 μM dThd and 20 μM Ade (○); and with 1 μM methotrexate, 20 μM dThd, 20 μM Ade and 1 μM 2′-deoxycoformycin (●).

(B). The growth of a L5178Y control culture (□) is shown together with that of cultures containing 20 μM 2′-deoxycoformycin (△); with 20 μM Ade (▲); with 20 μM Ade and 5 μM 2′-deoxycoformycin (○); with 20 μM Ade and 10 μM 2′-deoxycoformycin (●); and with 20 μM Ade and 20 μM 2′-deoxycoformycin (■).

phenol (data not shown), there was also less deamination of adenylate in the presence of coformycin than in its absence.

In a further attempt to assess the significance of possible inhibition of adenylate deaminase for the metabolism of intact cells, several experiments were conducted using cultured mouse lymphoma L5178Y cells. Figure 8A shows results of experiments in which cells were treated with methotrexate to inhibit purine biosynthesis *de novo* as well as the synthesis of thymidylate; under these conditions cell growth requires addition of thymidine and a purine to the medium. Hypoxanthine is usually used in such experiments as it is readily converted to the adenine and guanine nucleotides that are required for growth. In order for adenine to be converted to guanine nucleotides in L5178Y cells, adenylate must be deaminated to inosinate by adenylate deaminase [19]. It can be seen that cells grow well in the presence of 20 μM adenine (plus 20 μM thymidine), though not quite as well as in the absence of methotrexate, or as in the presence of methotrexate plus hypoxanthine [30, 35]. The further addition of 1 μM deoxycoformycin did not alter the growth rate, indicating that at least under these conditions the activity of adenylate deaminase either was not inhibited at all or that it was not inhibited sufficiently to curtail guanine nucleotide synthesis from adenine.

That higher concentrations of deoxycoformycine do affect adenine metabolism, however, is shown in Fig. 8B. In this case L5178Y cells were grown in the absence of methotrexate, and it is seen that neither 20 μM adenine nor 20 μM deoxycoformycin affected cell growth. However, combinations of adenine and deoxycoformycin were toxic, and the growth inhibitory effects of such combinations increased with increasing concentration of deoxycoformycin. The precise basis of this toxicity is not yet defined.

DISCUSSION

These studies have shown that certain concentrations of coformycin, deoxycoformycin and EHNA inhibit several aspects of purine metabolism in addition to their "prime" target, adenosine deaminase. In general, EHNA seems to have more side effects, or be more potent in inhibiting other reactions, than deoxycoformycin. It is clear that the specificity of these adenosine deaminase inhibitors should be determined in each experimental system in which they are used, and that their possible side effects should be taken into account in interpreting results of experiments in which they are used, particularly in experiments using intact cells.

The extent to which results of the present work on the lack of specificity of deoxycoformycin, coformycin and EHNA can be extrapolated to other experimental systems remains to be determined. Two factors are important in this regard, the minimal concentration of drug that is required to inhibit adenosine deaminase, and the relative sensitivity to inhibition of other reactions of purine metabolism. Thus concentrations of deoxycoformycin required to potentiate the growth inhibitory activity of adenosine analogs have ranged from *ca.* 0.02 μM [18] to 3.7 μM [20] in different experimental systems, and concentrations of EHNA that have been used in different systems have ranged from 0.3 μM [23] to 7.9 μM [22]. The relationship between the two forementioned factors may be particularly important in clinical oncology as it is very likely that these adenosine deaminase inhibitors will be tested in conjunction with such adenosine analogues as arabinosyladenine for effectiveness against human tumors.

The observed variation in minimum effective concentrations of deoxycoformycin and EHNA may be due to variation in rates of drug uptake, variation in the concentration of adenosine deaminase (i.e. amount of enzyme per cell and cell density), and length of time cells have been exposed to drug prior to assay. In addition, little is known about the metabolism of these drugs.

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