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DEOXYADENOSINE TRIPHOSPHATE ACCUMULATION IN ERYTHROCYTES OF DEOXYCOFORMYCIN-TREATED MICE*

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Abstract—The accumulation of deoxyadenosine triphosphate (dATP) in erythrocytes of mice treated with the adenosine deaminase inhibitor deoxycoformycin was studied in an attempt to establish and evaluate a model system for the study of at least some biochemical aspects of hereditary adenosine deaminase deficiency. Mouse erythrocytes *in vitro* readily phosphorylated deoxyadenosine to dATP, and this nucleotide was relatively stable once formed. dATP accumulated *in vivo* in mice treated with deoxycoformycin both as a function of dose from 0.25 to 10 mg/kg, and with time after administration. Major sources of the deoxyadenosine used for dATP formation *in vivo* appear to be normoblast nuclei produced during erythropoiesis, and dying cells; minor sources would appear to include dietary DNA, overproduction of deoxyribonucleotides, and DNA repair.

Patients with inherited deficiency of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.5) generally suffer from severe combined immunodeficiency disease and die from infections at an early age (reviews: Refs. 1–3). Recent biochemical studies have detected relatively high concentrations of deoxyadenosine triphosphate (dATP) in erythrocytes of such patients [4–8], though this nucleotide is virtually absent from erythrocytes of normal individuals; dATP concentrations may also be elevated in lymphocytes and bone marrow [3], though this has not always been observed [9]. As erythrocytes do not synthesize deoxyribonucleotides from ribonucleotides [10], it is presumed that dATP is synthesized from deoxyadenosine when this nucleoside is not catabolized by deamination. Deoxyadenosine levels are greatly elevated in the urine [6, 11–13] and plasma [8] of adenosine deaminase deficient patients.

Potent inhibitors of adenosine deaminase, such as deoxycoformycin [14], are tools both for the study of inherited adenosine deaminase deficiency and of their own lymphotoxic effects [15–21]. As with inherited adenosine deaminase deficiency, elevated dATP concentrations in blood cells have been reported following deoxycoformycin treatment [16, 21, 22].

Whether the lymphoid toxicity observed in these situations is due to the effects of free deoxyadenosine or to those of dATP is not yet clear (review: Ref. 23). However, it would appear that deoxyadenosine is produced in situations of adenosine deaminase deficiency, and that some deoxyadenosine is excreted in the urine while some is phosphorylated to dATP; probably deoxyadenosine is also produced in normal individuals, but this is not detected because it is rapidly catabolized by adenosine deaminase. The question that is posed here is, what are the sources of deoxyadenosine in the body? Chan [24] has shown recently that one source may be phagocytized cells, as deoxyadenosine was excreted by mouse peritoneal macrophages *in vitro* in following phagocytosis of nucleated cells; however, studies of this question have not yet been reported using model whole-animal systems.

The present study was an attempt, first to establish and evaluate a model system for the study of at least some biochemical aspected of adenosine deaminase deficiency, namely mice treated with deoxycoformycin. The accumulation of dATP in whole blood of deoxycoformycin-treated mice has been reported previously by Nelson *et al.* [25]. Using this system, then, experiments have been carried out to estimate the importance of various potential sources of deoxyadenosine, using dATP accumulation in erythrocytes as the criterion of deoxyadenosine production.

MATERIALS AND METHODS

Materials. [8-^{3}H]Deoxyadenosine, 24 Ci/mmole, was obtained from Moravek Biochemicals, Los Angeles, CA, [8-^{3}H]polydeoxyadenylate, 46.4 μCi/μmole phosphorus, was purchased from Miles Laboratories, Elkhart, IN, and [8-^{14}C]adenine, 50 mCi/mmole, was obtained from the Schwarz/Mann Co., Orangeburg, NY.

Deoxycoformycin was a gift of Dr. G. A. LePage of this Unit and methotrexate was a product of Lederle Laboratories, Pearl River, NY. Other drugs and biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO.

Blood collection. Mice were anesthesized using Penthrane (Abbott Laboratories, North Chicago,

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IL) and bled from the throat. Blood was collected with a pasteur pipet rinsed with heparin in saline, and was transferred to a heparin-rinsed glass centrifuge tube. After centrifugation the supernatant fraction was removed and the cells were washed once with Krebs–Ringer phosphate medium. Erythrocytes were centrifuged for 5 min at top speed in a clinical centrifuge to measure packed cell volume.

Incubation in vitro. Erythrocytes were suspended (4% v/v) and incubated in calcium-free Krebs–Ringer medium containing 25 mM phosphate buffer, pH 7.4, and 5.5 mM glucose; incubations were carried out at 37° in a shaking water bath, using an air atmosphere. When deoxycoformycin (3.7 μM) was used, it was added for 30 min and cells were then transferred to fresh medium prior to addition of deoxyadenosine.

dATP analysis. After incubation *in vitro*, 1 or 2 ml of cell suspensions was centrifuged, the supernatant fractions were removed, and dATP was extracted with 100 or 200 μl of 0.4 M perchloric acid. These extracts were neutralized either using a 4 M KOH–$KHCO_3$ or with alamine–freon [26].

Blood collected directly from mice was in some cases diluted to 4% (v/v) incubation medium, and extracts were made as described above. Usually, 100 μl of blood was collected into a heparinized micro-sampling pipet (Corning) and then mixed with 0.4 ml of saline. After centrifugation and removal of the supernatant fraction, the erythrocytes were extracted with 100 μl of 0.4 M perchloric acid; the extract was neutralized using 100 μl of alamine–freon.

Nucleotides were separated by high-pressure liquid chromatography (HPLC) on a Varian–Aerograph LCS-1000 chromatograph using a 4.6 × 250 mm Whatman Partisil-10SAX column. Elution was carried out using 0.25 M KH_2PO_4, pH 4.5, and 0.5 M KCl, at a flow rate of 1.25 ml/min. Peak areas were measured using an Autolab Minigrator (Spectra-Physics) and were compared to standards of authentic compounds analyzed under the same conditions.

Very low levels of dATP had to be analyzed by a modification of the DNA polymerase procedure [27].

Adenosine deaminase assay. Twenty microlitres of tissue homogenate was incubated in 60 μl Tris–HCl buffer, pH 7.4, and 20 μl of 2.5 mM [^{3}H]deoxyadenosine, for 15 min at 37°. Then 5 μl of 4 M perchloric acid was added, the extract was neutralized with KOH, and hypoxanthine, deoxyinosine and deoxyadenosine were separated by thin layer chromatography (TLC) on cellulose plates using 1-butanol–propionic acid–water (5:3:2). The reaction was linear with time and with protein concentration, and the rate of deamination of deoxyadenosine was calculated from the radioactivity in deoxyinosine plus hypoxanthine.

To assay for adenosine deaminase activity in duodenum, a 4-cm section of small intestine immediately beyond the stomach was removed, the contents were squeezed out, and the lumen was rinsed with water. The section was cut open and the mucosa was scraped off. The collected mucosa was homogenized in 1 ml of cold 50 mM Tris–HCl buffer, pH 7.4, and diluted 100-fold in the same buffer for assay.

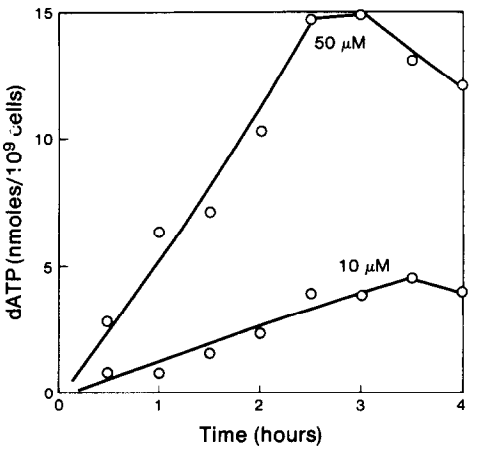

Fig. 1. dATP accumulation in mouse erythrocytes incubated with deoxyadenosine. Mouse erythrocytes (4% by volume) were incubated, with shaking in an air atmosphere at 37°, in Krebs–Ringer medium containing 25 mM phosphate buffer, pH 7.4, 5.5 mM glucose and 3.7 μM deoxycoformycin for 30 min. The cells were then sedimented by centrifugation and suspended in fresh medium without deoxycoformycin. Incubation was then continued with 10 μM deoxyadenosine (lower curve) and 50 μM deoxyadenosine (upper curve). Values reported are averages of duplicate measurements and are representative of results obtained in three experiments.

RESULTS

The first aim of this study was to determine if the deoxycoformycin-treated mouse was a satisfactory model for at least some aspects of inherited adenosine deaminase deficiency, particularly the accumulation of dATP in erythrocytes *in vivo*.

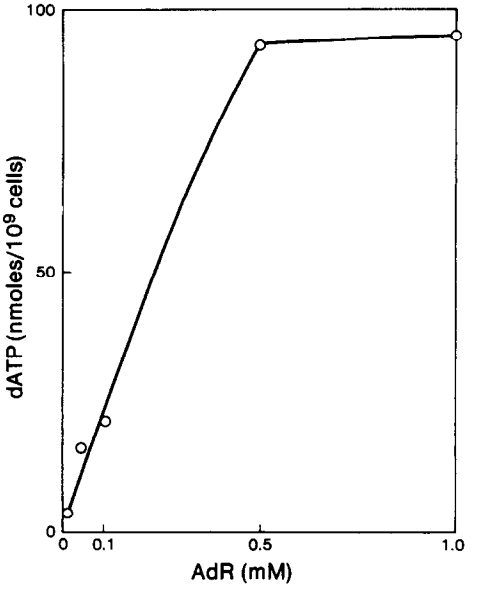

Fig. 2. Dependence of dATP accumulation on deoxyadenosine (AdR) concentration. Mouse erythrocytes were first treated with deoxycoformycin as described in the legend of Fig. 1 and then incubated for 4 hr with a range of concentrations of deoxyadenosine. Values reported are averages of duplicate measurements and are representative of results obtained in two experiments.

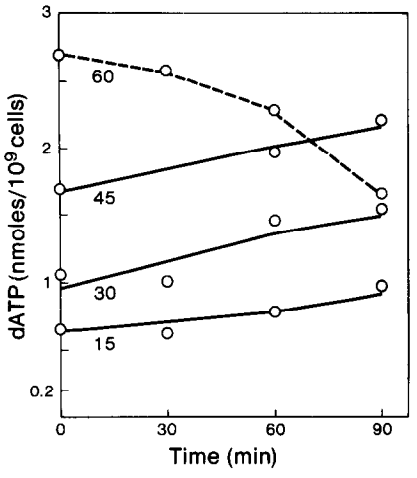

Fig. 3. Stability of dATP in mouse erythrocytes. Mouse erythrocytes were treated with deoxycoformycin as described in the legend of Fig. 1 and then were incubated with 500 μM deoxycoformycin for 15, 30, 45 and 90 min. The cells were then centrifuged, resuspended in medium without deoxycoformycin, and incubated for up to 90 min. Values reported are averages of duplicate analyses and are representative of results obtained in two experiments.

Experiments were therefore conducted to determine if mouse erythrocytes were permeable to deoxyadenosine and phosphorylated it to dATP *in vitro* at appreciable rates, and if such dATP was relatively stable after its formation. Preliminary studies showed that 3.7 μM (1 μg/ml) deoxycoformycin completely inhibited the deamination of adenosine and deoxyadenosine by mouse erythrocytes.

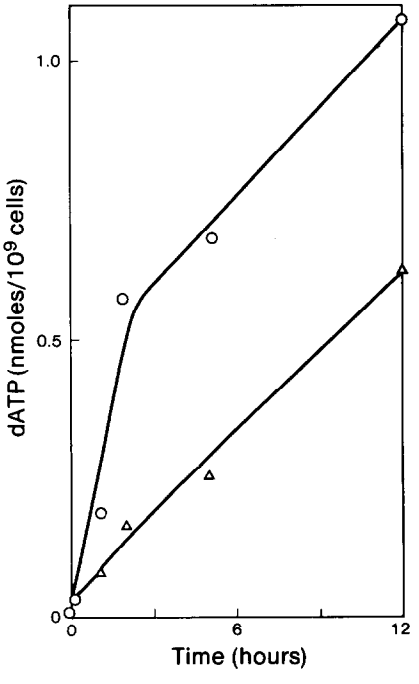

Fig. 4. dATP accumulation in mouse erythrocytes *in vivo*. Mice were injected intraperitoneally with 1 (△) or 10 (○) mg/kg deoxycoformycin. Values are averages of duplicate analyses from two mice and are representative of results from three experiments.

Figures 1 and 2 show that mouse erythrocytes incubated *in vitro* readily converted deoxyadenosine to dATP. Accumulation of dATP increased progressively for *ca.* 3.5 hr and was detectable following incubation with as little as 10 μM deoxyadenosine. In these experiments cells were incubated for 30 min with deoxycoformycin, and the inhibitor was then removed prior to addition of the deoxyadenosine. This permitted the deoxycoformycin to enter the cells and effect complete inhibition of adenosine deaminase prior to addition of substrate. Removal of drug also prevented any competition with deoxyadenosine for transport or phosphorylation [28].

Once formed, dATP was relatively stable in mouse erythrocytes. Figure 3 shows the results of an experiment in which cells were incubated with deoxyadenosine for varying periods, in order to achieve a range of intracellular concentrations of dATP, and then were transferred to deoxyadenosine-free medium and incubated for a further 90 min. Only at the highest initial dATP concentration was there any apparent catabolism of dATP; at the three lower initial dATP concentrations it appeared to be completely stable during subsequent incubation.

These experiments demonstrated that mouse erythrocytes had the capacity to synthesize and accumulate dATP when supplied with deoxyadenosine, at least under the *in vitro* conditions used. Experiments published previously showed that dATP was accumulated in mouse erythrocytes when exogenous deoxyadenosine was administered to deoxycoformycin-treated mice [21]. Further experiments were next carried out to determine if deoxyadenosine was produced endogenously and accumulated as dATP in mouse erythrocytes *in vivo* when adenosine deaminase was inhibited by deoxycoformycin. Previous studies showed that 1 mg/kg deoxycoformycin produced complete or almost complete inhibition of adenosine deaminase activity for a 24-hr period in a number of mouse tissues [29, 30]; consequently, this dose plus a higher dose (10 mg/kg) were used in these experiments.

Figure 4 shows that in fact dATP concentrations in mouse erythrocytes were elevated, even a few hours following injection of deoxycoformycin, and continued to increase over the 12-hr period studied. The rate of dATP accumulation and final dATP concentration achieved were appreciably greater at 10, than at 1 mg/kg deoxycoformycin.

In view of this marked dose effect and because deoxycoformycin has been used *in vivo* at doses even lower than 1 mg/kg, a dose–response curve was determined for dATP accumulation in erythrocytes, using a range of deoxycoformycin concentrations from 0.25 to 10 mg/kg. These results are shown in Fig. 5, and a considerable dose dependence within this range is demonstrated. It is not certain that 10 mg/kg gives maximum dATP accumulation, but the effects of still higher doses were not studied.

The basis for the increased effectiveness of 10 mg/kg deoxycoformycin was then investigated. In comparing the extent of inhibition of adenosine deaminase in tissues of deoxycoformycin-treated mice, it was noted that there was incomplete inhibition (24 hr after treatment) only in the small intes-

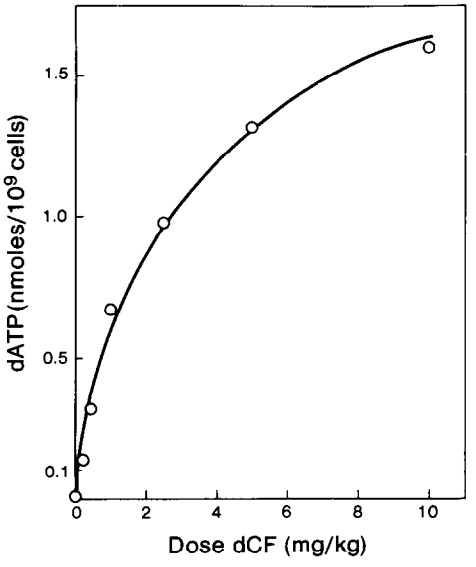

Fig. 5. Effect of deoxycoformycin (dCF) dose on dATP accumulation in erythrocytes. Mice were injected intraperitoneally with a range of concentrations of deoxycoformycin, and erythrocyte dATP concentrations were measured after 24 hr. In this experiment only the DNA polymerase assay for dATP was used. Values are averages of duplicate analyses from each of two mice, and are representative of results from two experiments.

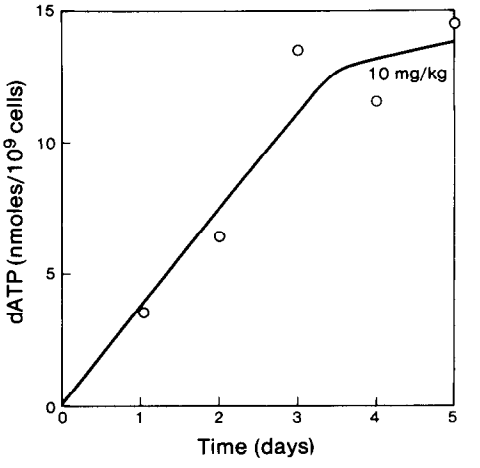

Fig. 6. Effect of prolonged deoxycoformycin treatment on dATP accumulation in mouse erythrocytes *in vivo*. Mice were injected intraperitoneally with 10 mg/kg deoxycoformycin every 24 hr. Values are averages of duplicate analyses from two mice and are representative of results from two experiments.

tine. Table 1 shows the efects of a wide range of deoxycoformycin doses on adenosine deaminase activity in the mucosa of the duodenum. (Adenosine deaminase activity was much higher in duodenal mucosa than in ileum, and in all experiments mucosa was obtained from the top 4 cm of duodenum.) Table 1 shows that adenosine deaminase activity in duodenal mucosa was relatively resistant to inhibition by intraperitoneally administered deoxycoformycin; 1 mg/kg had little effect, and even 10 mg/kg only produced *ca.* 50% inhibition. On the basis of this result and the data of Figs. 4 and 5, most subsequent studies used 10 mg/kg deoxycoformycin.

Figure 6 shows the results of an experiment in which mice were treated with 10 mg/kg deoxycoformycin daily for 4 days and erythrocyte dATP concentrations were measured 23–24 hr following each treatment. It may be seen that erythrocyte dATP concentration continued to increase sharply during the first 3 days and more slowly for the remainder of the period studied.

Table 1. Effect of deoxycoformycin on adenosine deaminase activity in duodenal mucosa*

Deoxycoformycin dose (mg/kg)	Adenosine deaminase activity (% of control)
0.25	100
0.5	100
1.0	98
5.0	85
10	48

* Mice were injected intraperitoneally with various doses of deoxycoformycin. After 24 hr, animals were killed and mucosa was scraped from the top 4 cm of duodenum and assayed for residual adenosine deaminase activity. Control value: 1.08 nmoles per min per μg protein. Values are averages of six measurements in three experiments.

Though this point was not explored in any detail, concentrations of dATP were also found to be elevated in tissues other than erythrocytes, 24 hr following treatment with 10 mg/kg deoxycoformycin. Thus, in duplicate mice in a single experiment, dATP concentrations in liver increased from 6.25 to 61 pmoles per mg protein following treatment, in kidney from 14 to 66, and in skeletal muscle, from 1.56 to 85.

Sources of deoxyadenosine in vivo. The experiments just reported demonstrated that the deoxycoformycin-treated mouse is a satisfactory model system in which to study the synthesis and accumulation of dATP by erythrocytes *in vivo* as well as *in vitro*. It was therefore possible to address the question of the sources of the endogenous deoxyadenosine that is used for dATP synthesis when adenosine deaminase is inhibited or genetically absent. As erythrocytes contain no DNA, and cannot convert ribonucleotides to deoxyribonucleotides, the deoxyadenosine has to come from sources outside the erythrocyte. Possible sources include (a) the diet, (b) excessive reduction of adenine ribonucleotides to deoxyribonucleotides in other tissues, followed by dephosphorylation to deoxyadenosine, (c) DNA repair, (d) DNA of dead cells, and (e) DNA of nuclei extruded from normoblasts in the course of erythropoiesis. The relative importance of these was therefore studied.

Dietary DNA was felt to be an unlikely source of much deoxyadenosine that could be used for erythrocyte dATP synthesis, because of the high adenosine deaminase activity of intestine, because the vegetarian diet of the mouse has a relatively low DNA content, and because previous studies [31] had shown that dietary nucleic purines are used by the mouse only to a very small extent. To test this point

directly, however, mice were treated either with a single intraperitoneal injection of 10 mg/kg deoxycoformycin, or with an initial injection of 5 mg/kg drug followed 7 hr later by an identical treatment, or by first administering 5 mg/kg deoxycoformycin orally followed by an intraperitoneal injection (5 mg/kg) 7 hr later; virtually the same results were obtained in each case. The mice were administered 5 μCi of tritiated polydeoxyadenoylate (38 μg), and radioactivity in erythrocyte dATP was measured after 24 hr; in some experiments radioactivity in urine and feces was also determined.

In control mice not treated with deoxycoformycin, 0.12% of the administered radioactivity was found in erythrocyte dATP, and when adenosine deaminase was inhibited by the deoxycoformycin treatment, this percentage increased to 0.30 and 0.45 in two separate experiments. It is tentatively concluded that, although dietary DNA may be one source of erythrocyte dATP in the absence of adenosine deaminase activity, this probably is not the sole or major source. The fact that most of the administered radioactivity was excreted in the urine and very little in feces indicates that the polydeoxyadenylate that was fed was, in fact, digested and absorbed.

Another source of deoxyadenosine is the catabolism of DNA of dead and dying cells. As one test of this possibility, mice were treated with several cytotoxic drugs to accelerate cell death, and the accumulation of dATP in erythrocytes was measured following such treatment. Table 2 shows that dATP accumulation was apparently increased after injection of cytosine arabinoside and hydroxyurea, though methotrexate had only a marginal effect under the conditions used. Similar studies were done using dexamethasone; results were erratic, but such treatment did lead to elevated dATP concentration in some animals. None of the drugs used caused an increase in dATP concentrations in mice not treated with deoxycoformycin. The possibility was also considered that the catabolism of the DNA in nuclei extruded from normoblasts during the final stages of erythropoiesis might be an important source of deoxyadenosine for erythrocyte dATP synthesis. This was tested by retarding erythropoiesis by hypertransfusing mice; erythrocytes from donor mice are injected intraperitoneally, and subsequently these enter the circulation, producing hematocrit values of 55–75% after 4–6 days [32]. The effect of deoxycoformycin treatment on erythrocyte dATP accumulation may then be compared in control and hypertransfused mice. Table 3 shows that as the hematocrit increased and, hence, as erythropoiesis slowed down, the accumulation of dATP in erythrocytes also decreased markedly.

Table 2. Effect of cytotoxic drugs on dATP accumulations*

Drug treatment	dATP (nmoles/10^9 cells)
None	Nil
Deoxycoformycin	1.73
Deoxycoformycin plus cytosine arabinoside	3.17
Deoxycoformycin plus methotrexate	2.20
Deoxycoformycin plus hydroxyurea	3.35

* Mice were treated with saline alone, with 10 mg/kg deoxycoformycin alone, or with 10 mg/kg deoxycoformycin plus 1000 mg/kg cytosine arabinoside, 15 mg/kg methotrexate, or 1.5 g/kg hydroxyurea. Treatment schedule was deoxycoformycin at t = zero, other drugs at 30 min, and removal of blood for analysis at 24 hr. Values are averages of duplicate measurements and are representative of results from three experiments.

Table 3. Effects of hypertransfusion on dATP accumulation in mouse erythrocytes *in vivo**

Condition	Hematocrit	dATP (nmoles/10^9 cells)
Control	36	2.9
Hypertransfused	67	0.70

* At 9:00 a.m. and 3:00 p.m. on day 1, mice were injected intraperitoneally with 0.75 ml of packed erythrocytes, and at 9:00 a.m. and 3:00 p.m. on day 2 this was repeated using 0.5 ml of packed erythrocytes. At 9:00 a.m. on day 7, 10 mg/kg of deoxycoformycin was injected, and at 9:00 a.m. on day 8 blood was taken for dATP measurement.

Finally, the possibility was tested that, in one or more tissues, adenine ribonucleotides were reduced to deoxyribonucleotides at rates greater than the utilization of dATP for DNA synthesis, and that the excess was converted to deoxyadenosine and, at least in part, was made available to erythrocytes for dATP synthesis. As deoxyribonucleotide synthesis generally is considered to be tightly regulated, this possibility seemed unlikely; however, to test it, mice were injected with radioactive adenine (100 μCi, 2 μmoles) in order to label the adenine ribonucleotide pools in tissues. The extent of accumulation of radioactivity in erythrocyte dATP was determined at 7 hr, 1, 2, and 3 days. The maximum labeling of dATP occurred at 7 hr, and amounted to only 0.03% of the injected radioactivity; radioactivity in dATP declined slowly thereafter. It cannot unambiguously be determined if the radioactivity in dATP originated solely by reduction of ADP to dATP that was in excess of requirements for DNA synthesis, or in the DNA of a very rapidly turning over cell fraction, or both.

DISCUSSION

Deoxycoformycin readily inhibits the adenosine deaminase of mouse erythrocytes, and the abilities of these cells to take up deoxyadenosine and phosphorylate it to dATP and to accumulate this metabolite both *in vitro* and *in vivo* make this system an appropriate model for the study of at least some aspects of inherited adenosine deaminase deficiency and of the effects of deoxycoformycin. It is of considerable interest that dATP accumulation *in vivo* continued to increase with deoxycoformycin doses up to at least 10 mg/kg, even though lower doses have often been used for biochemical and pharma-

cological purposes [16, 19, 20, 22, 29, 30, 33]. These results therefore are consistent with those of Nelson *et al.* [25], who administered deoxycoformycin daily at doses of 0.2 and 1.0 mg/kg, and found both dose- and time-dependent accumulation of dATP in whole blood (presumably mainly in the erythrocytes). The requirement for high doses of deoxycoformycin can be related (at least in part) to the insensitivity of duodenal adenosine deaminase to inhibition, even though lower doses of this drug can readily inhibit the enzyme of other tissues. In this connection it is of interest that Constine *et al.* [34] have reported that one of the three molecular species of adenosine deaminase from calf intestine is considerably less sensitive to deoxycoformycin than are the others (ID_{50} of 2×10^{-8} instead of 28×10^{-11} M); Tedde *et al.* [35] have also reported that intestinal adenosine deaminase was relatively resistant to inhibition to deoxycoformycin when this drug was infused for 5 days. Another possibility is that intestinal adenosine deaminase is initially inhibited to a considerable extent, but that normal activity is recovered exceptionally rapidly [33, 36].

The ready accumulation of dATP in mouse erythrocytes clearly indicates that appreciable quantities of deoxyadenosine are produced by mice under the conditions used. We hypothesize that such deoxyadenosine has several sources, some of which are physiological or normal processes (diet, normal cell turnover, normoblast nuclei). In addition, however, the toxic effects of deoxycoformycin treatment (presumably mediated by elevated deoxyadenosine or dATP concentrations) are another source of deoxyadenosine; this toxicity is exerted mainly on the thymus and peripheral lymphocytes under the conditions used (unpublished results). It is not possible to determine accurately the relative contribution of each source; however it seems reasonable to conclude that the nuclei destroyed as a byproduct of erythropoiesis constitute a more important physiological source than diet or normal cell turnover. Furthermore, deoxyadenosine produced very soon after deoxycoformycin administration is more likely to be produced from physiological sources while that resulting from the toxicity of deoxycoformycin itself may not be detectable until later times.

Inhibition of adenosine deaminase by deoxycoformycin has several distinct functions with respect to the production of lymphoid or other toxicity. Thus, inhibition in tissues which produce deoxyadenosine, such as bone marrow, has the function of allowing deoxyadenosine to accumulate and escape into the circulation. Other tissues (e.g. intestine and liver) probably neither produce deoxyadenosine nor are adversely affected by it, but have relatively high adenosine deaminase activities; in these, inhibition by deoxycoformycin serves to prevent catabolism and the lowering of its concentration in the circulation and body generally. Finally, in pharmacologically sensitive tissue, such as thymus, inhibition of adenosine deaminase permits the accumulation of toxic concentrations of deoxyadenosine or its metabolites. As demonstrated in the case of intestinal mucosa, adenosine deaminase in different tissues may vary in responsiveness to deoxycoformycin treatment.

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