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Research Article

Deficiency of erythrocytic and lymphocytic adenosine deaminase (ADA) occurs in some patients with severe combined immunodeficiency disease (SCID). SCID with ADA deficiency is inherited as an autosomal recessive trait. ADA is markedly reduced or undetectable in affected patients (homozygotes), and approximately one-half normal levels are found in individuals heterozygous for ADA deficiency. The metabolism of purine nucleosides was studied in erythrocytes from normal individuals, four ADA-deficiency patients, and two heterozygous individuals. ADA deficiency in intake erythrocytes was confirmed by a very sensitive ammonia-liberation technique. Erythrocytic ADA activity in three heterozygous individuals (0.07,0.08, and 0.14 mumolar units/ml of packed cells) was between that of the four normal controls (0.20-0.37 mumol/ml) and the ADA-deficient patients (no activity). In vitro, adenosine was incorporated principally into IMP in the heterozygous and normal individuals but into the adenosine nucleotides in the ADa-deficient patients. Coformycin (3-beta-D-ribofuranosyl-6,7,8-trihydroimidazo[4,5-4] [1,3] diazepin-8 (R)-ol), a potent inhibitor of ADA, made possible incorporation of adenosine nucleotides in the ADA-deficient patients...

Purine Nucleoside Metabolism in the Erythrocytes of Patients with Adenosine Deaminase Deficiency and Severe Combined Immunodeficiency

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ABSTRACT Deficiency of erythrocytic and lymphocytic adenosine deaminase (ADA) occurs in some patients with severe combined immunodeficiency disease (SCID). SCID with ADA deficiency is inherited as an autosomal recessive trait. ADA is markedly reduced or undetectable in affected patients (homozygotes), and approximately one-half normal levels are found in individuals heterozygous for ADA deficiency.

The metabolism of purine nucleosides was studied in erythrocytes from normal individuals, four ADA-deficient patients, and two heterozygous individuals. ADA deficiency in intact erythrocytes was confirmed by a very sensitive ammonia-liberation technique. Erythrocytic ADA activity in three heterozygous individuals (0.07, 0.08, and 0.14 μmolar units/ml of packed cells) was between that of the four normal controls (0.20–0.37 μmol/ml) and the ADA-deficient patients (no activity). In vitro, adenosine was incorporated principally into IMP in the heterozygous and normal individuals but into the adenosine nucleotides in the ADA-deficient patients. Coformycin (3-β-D-ribofuranosyl-6,7,8-trihydroimidazo-[4,5-*d*][1,3]diazepin-8(*R*)-ol), a potent inhibitor of ADA, made possible incorporation of adenosine into the adenosine nucleotides in normal and heterozygous erythrocytes. Coformycin did not alter the pattern of nucleotide incorporation in the ADA-deficient cells. These results indicate that coformycin causes normal and heterozygous cells to behave like ADA-deficient cells. Similarly, *p*-nitrobenzylthioguanosine(2-amino-6-([4-nitrobenzyl]thio)-9-β-D-ribofuranosylpurine) (NBTGR), an inhibitor of nucleoside transport, drastically reduced the incorporation of adenosine into the inosine nucleotides and enhanced the incorporation into adenosine nucleotides in the normal erythrocytes. NBGTR had no effect upon ADA-deficient cells. NBTGR inhibited the liberation of ammonia from adenosine in intact normal cells. In two patients previously treated with bone marrow or fetal liver transplantation, incubation of erythrocytes with inosine resulted in accumulation of ITP, whereas in the erythrocytes from the heterozygotes, the normal individuals and the two other ADA-deficient patients, IMP accumulated. Reexamination of one of the transplanted patients 10 and 15 mo later revealed his erythrocytic inosine incorporation into ITP to be decreased from that observed at the initial examination. Guanosine was incorporated into guanosine nucleotides in all erythrocytes studied, suggesting that the nucleoside transport system and the enzymes purine nucleoside phosphorylase, hypoxanthine-guanine phosphoribosyltransferase, and 5-phosphoribosyl-1-pyrophosphate synthetase are functional in the erythrocytes of ADA-deficient patients. Formycin A (7-amino-3-β-D-ribofuranosylpyrazolo-[4,3-*d*]pyrimidine), an adenosine analogue, was converted to formycin A-mono-, di-, and triphosphate nucleotides in ADA-deficient patients'

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erythrocytes to a much greater extent than occurred in normal cells. This confirms the ability of formycin A to act as an excellent substrate for both human erythrocytic ADA and adenosine kinase.

These results are consistent with the hypothesis that the disposition of adenosine in human erythrocytes is the result of the relative activities and Michaelis constants of the enzymes ADA and adenosine kinase. Various possible biochemical mechanisms by which ADA deficiency causes cytotoxicity are discussed.

INTRODUCTION

Recently an inherited deficiency of the enzyme adenosine deaminase (ADA)[1] has been described (1–6). Deficiency of ADA is inherited as an autosomal recessive trait, and patients with this disorder also have a severe combined immunodeficiency disease (SCID) characterized by deficits both in thymus-derived and bone marrow-derived cell-mediated immunity. In addition, most of these children have striking skeletal abnormalities as well as malfunction of the thymus gland (4). Approximately one-third of the children with SCID studied have erythrocytes and lymphocytes that are deficient in ADA (4, 5). This finding is of particular interest because it is the first enzymic defect to be associated with an immune deficiency disease. Children with this condition usually succumb to infection early in life. However, several children have survived for prolonged periods with aseptic environmental conditions, and at least two children have become immunocompetent after bone marrow transplantation (6).

It has been previously suggested that the disposition of adenosine by human erythrocytes is, in part, determined by the relative activities and Michaelis constants of the enzymes ADA and adenosine kinase (7–10). Adenosine as well as adenosine analogues that are good substrates for ADA were not incorporated into the erythrocytic nucleotide pools. On the other hand, adenosine analogues that are inhibitors or that lack substrate activity with ADA but are good substrates for adenosine kinase in many cases are incorporated into the nucleotide pools (7–9). Several of the latter compounds formed notably high concentrations of the respective analogue triphosphate nucleotides (7, 9). When adenosine was incubated with normal erythrocytes, ammonia and large concentrations of IMP accumulated, probably as the result of the sequential reactions of the enzymes ADA, purine nucleoside phosphorylase, and hypoxanthine-guanine phosphoribosyl transferase (7–9, 11, 12). On the other hand, when human erythrocytes were treated with the potent ADA inhibitor, coformycin (8, 9, 12–14), adenosine and several adenosine analogues that are good substrates for ADA readily entered the nucleotide pools (9, 12). Thus, it is clear that ADA can play a major role in the control of intracellular adenine nucleotide metabolism.

Several recent technical advances have greatly facilitated the study of nucleoside and nucleotide metabolism. Especially useful is the technique of high-pressure liquid chromatography (HPLC) for the study of nucleotide pools in cells (7, 15, 16). Also of value is the availability of a variety of purine nucleoside analogs and specific inhibitors, such as coformycin, a tight-binding inhibitor of ADA (13, 14), and *p*-nitrobenzylthioguanosine (NBTGR), a potent inhibitor of the facilitated nucleoside transport system of human erythrocytes (17). The present report describes studies in which several of these new biochemical tools have been applied to an examination of purine nucleotide metabolism in erythrocytes of four SCID-ADA-deficient patients as well as two heterozygous individuals.

METHODS

Adenosine, inosine, and guanosine were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. [8-^{14}C]Adenosine (51 mCi/mmol) and [8-^{14}C]inosine (35 mCi/mmol) were obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., and [8-^{14}C]guanosine was a product of New England Nuclear, Boston, Mass. A mixture of penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml) was obtained from Grand Island Biological Co., Grand Island, N. Y. Formycin A (7-amino-3-β-D-ribofuranosylpyrazolo-[4,3-*d*]pyrimidine) and coformycin(3-β-D-ribofuranosyl-6,7,8-trihydroimidazo[4,5-*d*][1,3]diazepin-8(*R*)-ol) were gifts of Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan, and NBTGR (2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine) was provided by Dr. Harry B. Wood, Jr., of the Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute.

Heparinized or citrated blood used in the present study was obtained from the donors listed below. In each case, the blood specimens were placed on ice immediately after phlebotomy and were hand-carried to the laboratory for study. All experiments were initiated as soon as practical after drawing the blood, i.e., within a period of 2–20 h. In each experiment, control blood specimens from normal subjects were included and were handled in a similar manner. It should be noted that in unpublished experiments in this laboratory, nucleotide profiles obtained by HPLC were performed daily on the same refrigerated normal blood sample over a 5-day period. No significant differences in these nucleotide profiles were seen. Also in other experiments the ADA activity was measured by the ammonia liberation procedure described here, with aliquots of the same specimen of refrigerated normal erythrocytes on several occasions over a 2-wk period. No significant difference in the activity of the enzyme was detected.

Patient 1 (A. W.) is a 5-yr-old male. The diagnosis of SCID was reinforced by the occurrence of this disorder in a previous sibling. He was treated successfully by transplantation of bone marrow from an older sister. Although

[1] *Abbreviations used in this paper:* ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4); HPLC, high-pressure liquid chromatography; NBTGR, *p*-nitrobenzylthioguanosine; SCID, severe combined immunodeficiency disease.

this child had a successful bone marrow transplant about 4 yr ago and now has normal thymus-derived and bone marrow-derived cell function, his erythrocytes still display the ADA deficiency. Details of the history, diagnosis, and treatment were reported previously (6).

Patient 2 (M. R.), who was a male infant diagnosed at birth as having SCID by the absence of lymphocytes in cord blood, had a family history of SCID. Successful engraftment of fetal liver, transplanted at 3 mo of age, resulted in the gradual development of functional thymus-derived and bone marrow-derived lymphocytes with ADA activity. Red cell chimerism was transient, and his erythrocytes lacked ADA activity at the time of study. 1 yr post-transplantation he developed an ultimately fatal nephrotic syndrome associated with immune complex nephritis. A detailed report of this case is in press (17*a*).

Patient 3 (C. W.) is a male who was 5-mo-old at the time of study (Feb. 1975). ADA deficiency was diagnosed prenatally by amniocentesis (18), and SCID was subsequently confirmed by immunologic studies.

Patient 4 (I. B.) is a female whose diagnosis of SCID was suggested by profound lymphopenia and absent thymus shadow on X-ray study (6). Familial history and immunological studies confirming the diagnosis have been reported by Parkman et al. (6).

Heterozygotic individuals. Characteristics of all three heterozygotic individuals used in the present study have been described by Parkman et al. (6). G. W. and M. W. are the brother and mother, respectively, of A. W. (patient 1). The third heterozygotic individual (M. B.) is the mother of I. B. (patient 4). With respect to M. W., only ADA activity studies were performed. No nucleoside incorporation studies were done with this heterozygotic individual's erythrocytes.

Preparation of cells. Plasma-free erythrocytes were washed 2–3 times with equal volumes of 0.9% NaCl solution and then suspended in the incubation medium (potassium phosphate buffer, 50 mM, pH 7.5; $MgSO_4$, 2 mM; NaCl, 75 mM; and glucose, 10 mM). These cell suspensions were used for studies of ADA activity and nucleoside incorporation.

Assay of ADA activity in intact erythrocytes. The activity of ADA in intact cells was calculated from the rate of liberation of ammonia during the deamination of adenosine. The ammonia was determined by slight modifications of the microdiffusion method of Seligson and Seligson (19) and the colorimetric procedure of Chaney and Marbach (20) as described below. Erythrocytic suspensions (2% in the above medium) were incubated with 1 mM adenosine. 1-ml samples were withdrawn at various time intervals after incubation in a shaking water bath at 30°C and were added to flasks containing 1 ml of saturated K_2CO_3 solution and equipped with glass rods having flared ground tips and coated with 1 M citric acid. The flasks were placed on a rotator for 30 min and the ammonia was allowed to diffuse and be trapped on the citric acid-coated rods (19). The ammonia collected on the glass rods was washed into test tubes with 5-ml aliquots of the phenol-nitroprusside solution (20), and the color was developed by the addition of 5 ml of the sodium hydroxide-sodium hypochlorite solution as described by Chaney and Marbach (20).

Incorporation of nucleosides into nucleotide pools. Erythrocytic suspensions (15–25% in the above medium containing penicillin, 10 U/ml, and streptomycin, 10 μg/ml) were incubated with or without coformycin (1 μg/ml) or NBTGR (10 μM) in a shaking water bath at 30°C for 20 min. Then one of the following nucleosides was added: adenosine, 0.5 mM (0.98 mCi/mmol); inosine, 0.27 mM (1.8 mCi/mmol); guanosine, 0.12 mM (8.3 mCi/mmol), or formycin A, 1.0 mM. Aliquots (200 μl) were withdrawn after incubation at 30°C for 0, 1, 2, and 4 h after addition of the nucleoside and were added to 100 μl of cold 12% perchloric acid. The samples were stirred on a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.), centrifuged, and the resulting supernatant solutions were neutralized with KOH with phenol red as the pH indicator. Precipitates of $KClO_4$ were removed by centrifugation and the neutralized supernatant fluids were stored frozen at −20°C for further study.

Nucleotides were resolved either by thin-layer chromatography or by HPLC. The method of Crabtree and Henderson (21) was used to separate the nucleotides on PEI-cellulose thin-layer plates (J. T. Baker Chemical Co., Phillipsburg, N. J.). This method separates cleanly the adenine-, hypoxanthine-, and guanine-containing nucleotides. In studies with isotopically labeled compounds, unlabeled, known nucleotides were added as carriers to facilitate the identification of the labeled compounds. After development of the chromatograms, the individual nucleotide spots were visualized by UV light at 2537 Å, cut out, and the radioactivity was counted in an Omnifluor (98% PPO and 2% *p-bis-*(*o*-methylstyryl)benzene, New England Nuclear)-toluene (4 g/liter) scintillation fluid in a liquid scintillation counter (Packard Tri Carb, Packard Instrument Co., Inc., Downers Grove, Ill.). HPLC was performed on a Varian LCS 1000 analyzer (Varian Aerograph, Palo Alto, Calif.) (15), and the concentrations of the nucleotides were calculated by planimetery using known nucleotides as standards. The recent availability of a multiwave-length detection system (model SF 770, Schoeffel Instrument Corp., Westwood, N. J.) adaptable to HPLC has made practical the ready detection and estimation of the concentrations of intracellular analogue nucleotides that have absorbancy maxima different from those of the natural nucleotides. For example, nucleotides of formycin A were detected and estimated at 295 nm, a wave length at which the natural nucleotides do not absorb (See Fig. 8).

In some instances, incorporation of inosine into IMP, IDP, and ITP was followed by HPLC with a Waters Associates liquid chromatograph equipped with Bondapak AX columns (2.3 mm ID × 122 cm, Waters Associates, Inc., Milford, Mass.). The flow rate was 2 ml/min, and a linear gradient from 5 mM $NH_4H_2PO_4$, pH 3.0, to 125 mM $NH_4H_2PO_4$ plus 250 mM KCl was formed in 15 min. This system permits the separation of inosine ribonucleotides from adenosine ribonucleotides, but does not separate adenosine nucleotides from guanosine nucleotides. However, in control erythrocytic extracts, the guanosine nucleotide pool is considerably lower than that of the adenosine compounds (16).

RESULTS

ADA activity in intact erythrocytes of SCID-ADA-deficient patients

Fig. 1 presents measurements of ADA activity in intact erythrocytes from six normals, three ADA-deficient patients (A. W., C. W., and I. B.), and three heterozygous individuals. The normal intact erythrocytes liberated ammonia from adenosine at a mean rate of 0.29 μmol/min/ml of packed cells (values ranged from 0.2 to 0.4 μmol/min/ml), which is in good agree-

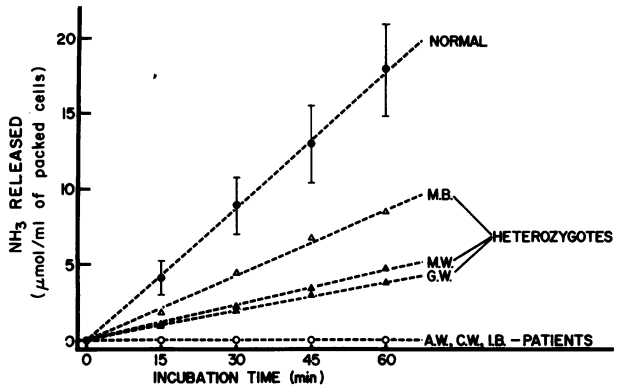

FIGURE 1 ADA activity in intact erythrocytes. Erythrocytic suspensions (2% in a medium consisting of potassium phosphate buffer, 50 mM, pH 7.5; $MgSO_4$, 2 mM; NaCl, 75 mM; and glucose, 10 mM) were incubated with 1 mM adenosine at 30°C in a shaking water bath. At various times after addition of adenosine, 1-ml aliquots were removed and the ammonia liberated by the deamination of adenosine was determined as described in Methods. "Normal" line is the mean of six samples. Ranges from the mean are indicated by vertical bars.

ment with the values reported earlier for normal hemolysates (8, 9, 11). No detectable liberation of ammonia from adenosine was observed with the ADA-deficient cells. This confirms the reports of Parkman et al. (6) and Hirschhorn et al. (18), that the erythrocytes of these patients lack the enzyme ADA. The activity of ADA in the intact erythrocytes from the heterozygous individuals ranged from 0.07 to 0.14 μmol/min/ml of packed erythrocytes. Interestingly, the heterozygous individuals (G. W. and M. W.) of the same family showed similar rates of ammonia liberation. These values are substantially below the range of ADA activity usually observed by this method with normal erythrocytes (see above). These results are consistent with earlier reports which have examined ADA activities in heterozygous individuals (6, 18, 22).

Metabolism of purine nucleosides in the erythrocytes of ADA-deficient SCID patients

Adenosine metabolism. The nucleotide levels of erythrocytes from six normal, two heterozygous, and four ADA-deficient individuals are shown in Table I. With the exception of one blood sample from M. R., ATP concentrations from all samples fell within the range normally found in human erythrocytes. In most cases the ratios of ATP/ADP were similar in the erythrocytes of all three types of donors. When first examined, the erythrocytes of A. W. had an ATP/ADP ratio of 17:1, but on reexamination 10 and 15 mo later, the ATP/ADP ratio was about 4:1 and 9:1, respectively, which is close to the normal range. Although in most samples examined the adenine nucleotides fell within the normal range, individual deviations from this range were seen. Therefore, it will be interesting to examine additional patients as they become available and to do serial adenine nucleotide determinations on individual patients. As discussed below, it seems possible that fluctuations in adenosine nucleotide concentrations may occur more readily with ADA-deficient than with normal cells.

The nucleotide profiles obtained by HPLC were similar in the normal and the ADA-deficient patients (Fig. 2). Figs. 2 and 3 illustrate the response to incubation with adenosine of erythrocytes from one patient (A. W.). Similar findings were obtained with erythrocytes from all four patients. After incubation of erythrocytes with [8-^{14}C]adenosine (0.5 mM) at 30°C for 4 h substantial differences were observed both in the nucleotide profiles and in the concentrations of nucleotides between normal and ADA-deficient cells. Fig. 2 illustrates that with normal erythrocytes, in agreement with earlier findings (7, 9), a large new peak appears with a retention time consistent with that of the monophosphate nucleotide, IMP. The identity of this peak as IMP was confirmed by collecting effluent from the HPLC and resolving the contents of this peak by thin-layer chromatography according to the method of Crabtree and Henderson (21). On the other hand, in the ADA-deficient cells, this monophosphate nucleotide peak was not present. In these cells the ADP and ATP peaks increased in size. These observations are supported by

TABLE I
Adenine Nucleotide Concentrations in Human Erythrocytes from Normal Donors, SCID-ADA-Deficient Patients, and Heterozygous Individuals

	ADP	ATP
	μmol/ml packed erythrocytes	
Normal	0.18±0.07*	1.06±0.30*
SCID-ADA-deficient patients		
No. 1 (A. W.)‡	0.08	1.34
(A. W.)§	0.24	0.91
(A. W.)‖	0.10	0.89
No. 2 (M. R.)¶	0.13	1.28
(M. R.)**	0.52	1.99
No. 3 (C. W.)	0.23	1.30
No. 4 (I. B.)	0.10	0.88
Heterozygotes		
G. W.	0.27	1.14
M. B.	0.09	0.78

* Values from six normal donors (mean±SD).
‡ Blood drawn on 7 July 1974.
§ Blood drawn on 12 May 1975.
‖ Blood drawn on 13 October 1975.
¶ Blood drawn on 22 December 1973.
** Blood drawn on 19 January 1974.

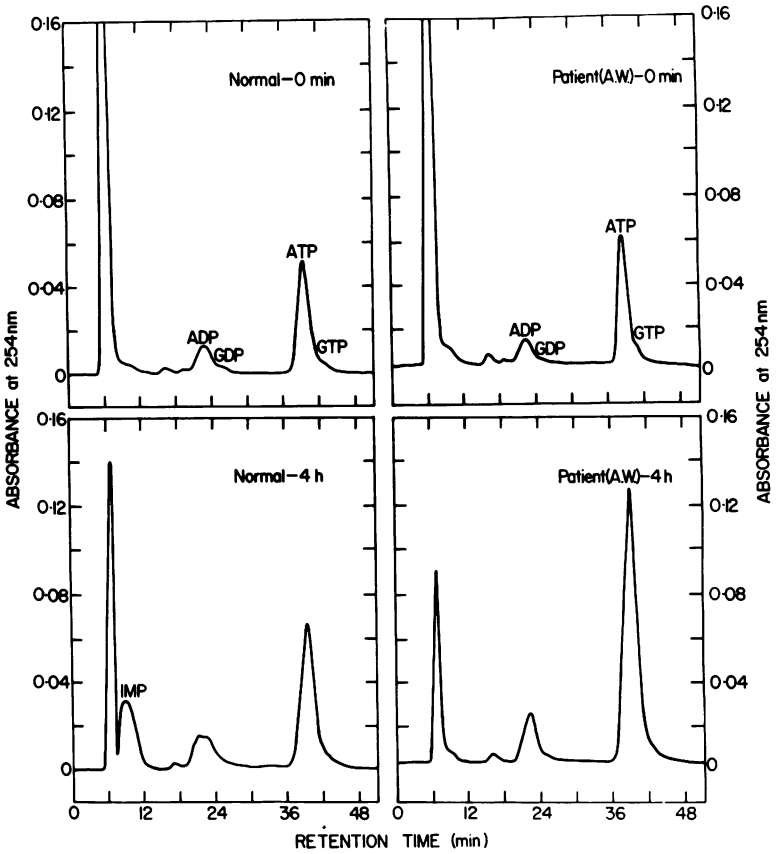

FIGURE 2 Erythrocytic nucleotide profiles obtained by HPLC. Erythrocytic suspensions (15–25% in the medium described in the legend to Fig. 1 supplemented with penicillin, 10 U/ml and streptomycin, 10 μg/ml) were incubated with 0.5 mM adenosine. Aliquots (200 μl) were removed at 0 and 4 h after addition of adenosine and added to 100 μl of cold 12% perchloric acid. After thorough mixing the samples were centrifuged and the supernatant solutions were neutralized with KOH. After removal of precipitated $KClO_4$ by centrifugation, 20 μl of the neutralized extracts were chromatographed on a Varian LCS-1000 liquid chromatograph equipped with a Reeve-Angel AS-Pellionex SAX (3-m × 1-mm) column. A linear elution gradient was used which consisted of 0.002 M potassium phosphate, pH 4.5, as the low concentrate buffer and 0.5 M potassium phosphate, pH 4.5, in 1.0 M KCl as the high concentrate buffer. The starting volume was 40 ml and the flow rates were 14 and 7 ml/h for the column and gradient, respectively. Although the profile of only one SCID-ADA-deficient patient (A. W.) is shown here, the profiles of erythrocytes from all other SCID-ADA-deficient patients examined were similar. The nucleotide profile of erythrocytes from two heterozygous individuals (G. W. and M. B.) were similar to those of erythrocytes from normal individuals.

data shown in Fig. 3, which illustrates the incorporation of [8-^{14}C]adenosine into the various nucleotides. In normal and heterozygous cells, large amounts of adenosine were converted to IMP (and ITP) rather than to adenine nucleotides. In ADA-deficient cells, however, the isotope was incorporated principally into the adenine nucleotides (ADP and ATP) with negligible incorporation into the inosine nucleotides. In this experiment, after 4 h of incubation, the ATP levels in the patients' cells increased from about 1.1 to about 2.6 μmol/ml, i.e., an increase of approximately 135%. In contrast, the increases in ATP levels in the heterozygous and normal cells were 29 and 15%, respectively. In ADA-deficient erythrocytes from all four patients studied, the ATP levels increased about 130–150% after 4 h of incubation with 0.5 mM adenosine.

Coformycin, a tight-binding inhibitor of ADA, has been shown capable of inhibiting this enzyme in intact erythrocytes and of significantly modifying the patterns of adenosine and adenosine analogue metabolism in these cells (9, 12). The effect of coformycin on the adenosine metabolism of ADA-deficient erythrocytes was also examined (Fig. 4). As expected, pretreatment of these cells with coformycin (1 μg/ml reaction mixture) did not affect either the nucleotide profiles or the amount of adenosine incorporated into the nucleotide

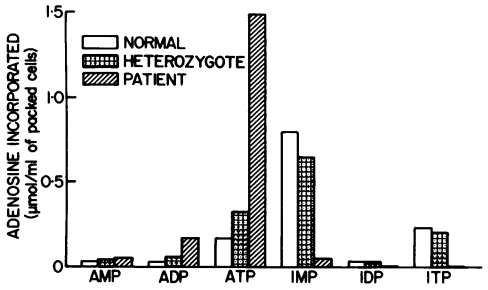

FIGURE 3 Incorporation of adenosine into nucleotides by intact erythrocytes. Erythrocytes were incubated and extracts were prepared as described in Fig. 2 except that labeled [8-^{14}C]adenosine (0.5 mM, 0.98 mCi/mmol) was used. Aliquots (25 μl) of the 4-h samples were chromatographed on PEI-cellulose thin-layer sheets and developed with a stepwise gradient of sodium formate solutions according to the method of Crabtree and Henderson (21). Concentrations of nucleotides refer only to the incorporation of the precursor and do not represent total erythrocytic concentrations. Results are presented for one of the SCID-ADA-deficient patients (A. W.), his heterozygous sibling (G. W.), and a normal individual.

pools. However, in normal and heterozygous cells, coformycin abolished the formation of inosine nucleotides and markedly enhanced the incorporation of adenosine into the adenine nucleotides. In fact, treatment of normal cells with coformycin caused these cells to metabolize adenosine in a manner very similar to that of erythrocytes from the ADA-deficient patients.

It has been shown recently that NBTGR, a potent inhibitor of the facilitated nucleoside transport system of erythrocytes (17), greatly inhibits the deamination of adenosine and enhances adenosine incorporation into the adenine nucleotides in intact normal erythrocytes (11). To examine whether NBTGR affects the adenosine metabolism of ADA-deficient erythrocytes, cells from A. W. and normal cells were pretreated with 10 μM NBTGR, after which they were incubated with [8-^{14}C]adenosine. As shown in Fig. 5, the NBTGR did not alter the incorporation of adenosine into the nucleotides of patients' cells whereas in normal cells the incorporation of adenosine into adenine nucleotide pools was enhanced and the formation of IMP was decreased. The latter observation is consistent with earlier reports which proposed that ADA is physically associated with the cell membrane (23) at a site close to the nucleoside transporter of human erythrocytes (11).

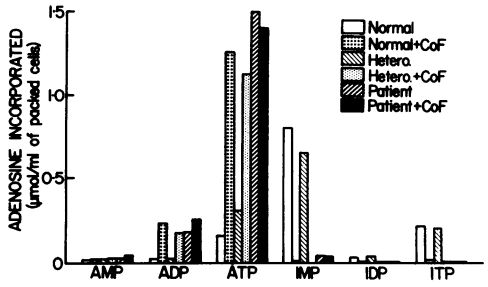

FIGURE 4 Effects of coformycin on incorporation of adenosine into nucleotides by intact erythrocytes. Cells were incubated with and without coformycin (1 μg/ml of reaction mixture) for 20 min before addition of [8-^{14}C]adenosine. Conditions for incubation, extraction, and analysis are described in Figs. 2 and 3. Concentrations of nucleotides refer only to incorporation of precursor. Results from only one of the SCID-ADA-deficient patients (A. W.) are represented here. However, similar findings were obtained with the other two patients studied. "Hetero" refers to heterozygote G. W. and "CoF" refers to coformycin.

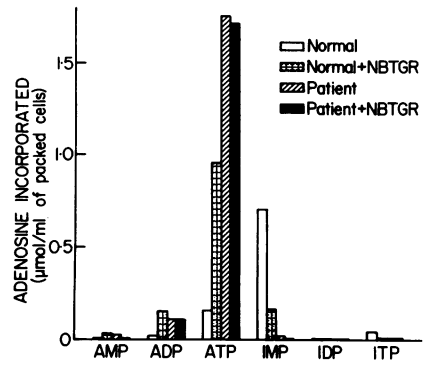

FIGURE 5 Effects of NBTGR on the incorporation of adenosine into nucleotides by intact erythrocytes. Cell were incubated with and without NBTGR (10 μM) for 20 min before addition of [8-^{14}C]adenosine. Conditions for incubation, extraction, and analysis are presented in Figs. 2 and 3. Concentrations of nucleotides refer only to incorporation of precursor. The patient studied was A. W.

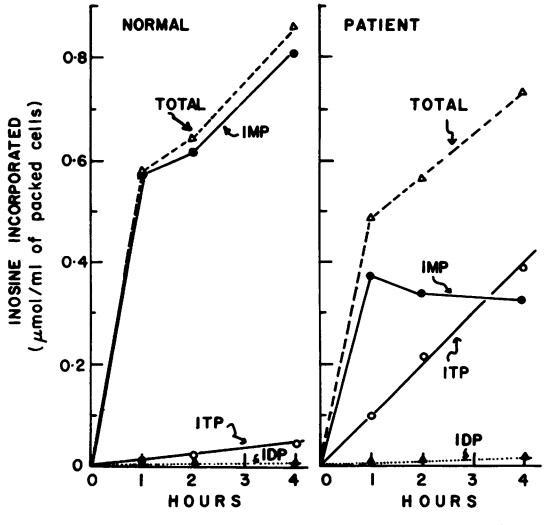

FIGURE 6 Time-course of incorporation of inosine into nucleotides by intact erythrocytes. Incubation, extraction, and analysis conditions are as described in Figs. 2 and 3 except that [8-^{14}C]inosine (0.27 mM, 1.8 mCi/mmol) was used in place of [8-^{14}C]adenosine. "Total" refers to the sum of IMP+IDP+ITP. "Patient" was A. W.

Inosine metabolism. Usually, the concentrations of inosine nucleotides in both normal and ADA-deficient erythrocytes are too low for ready detection by HPLC, i.e., below 10 nmol/ml packed cells. However, a large amount of IMP accumulates in normal but not in ADA-deficient erythrocytes when these cells are incubated with adenosine (See Fig. 3). To examine whether ADA-de..cient cells have the capacity to metabolize inosine and guanosine, the incorporation of these compounds into nucleotide pools was studied.

Time-courses of the incorporation of inosine into the nucleotides of normal and ADA-deficient erythrocytes (A. W.) are presented in Fig. 6. Cells were incubated at 30°C with [8-^{14}C]inosine (0.27 mM) for various times up to 4 h. Aliquots were extracted, the nucleotides were separated by thin-layer chromatography, and the radioactivity in the various purine nucleotides was determined. As shown in Fig. 6, after 4 h of incubation with inosine, IMP accumulated in the normal erythrocytes to a concentration of about 0.8 μmol/ml of packed cells, and ITP to a concentration of about 0.05 μmol/ml of packed cells, i.e., an ITP/IMP ratio of about 0.06. The accumulation of IDP was negligible at all time periods examined. On four occasions we have performed similar studies with normal erythrocytes and have found ITP/IMP ratios between 0.06 and 0.4 with an average of about 0.2. The question of the normal variation in this parameter has not yet been subjected to detailed study by this laboratory, but results to date suggest that significant variability exists. We have had the opportunity to perform similar inosine incorporation experiments with the erythrocytes of patient A. W. on three separate occasions over a 15-mo period, and Fig. 6 illustrates the first of these experiments. On these three occasions, ITP/IMP ratios of about 1.2. 0.4, and 0.5 were obtained. With the erythrocytes of a second patient, M. R., after 17 h of incubation with inosine a ratio of ITP/IMP of about 4.0 was seen. In contrast, with the erythrocytes of the two other ADA-deficient patients (C. W. and I. B.), after 4 h of incubation with inosine, ITP/IMP ratios of about 0.02 and 0.07 were observed. In addition, in similar experiments, the ITP/IMP ratios of the erythrocytes of two of the heterozygous subjects studied similarly were about 0.08 and 0.2. It is not yet possible to evaluate the significance of the apparently rapid synthesis of ITP seen with the erythrocytes of two of the patients (A. W. and M. R.). Possible reasons for these differences in inosine metabolism are discussed below.

The ATP concentrations remained unchanged in both normal and patients' cells during the course of incubation with inosine, suggesting that the formation of inosine nucleotides was not at the expense of cellular ATP under conditions used here. Although the rates of glycolysis were not determined in the various erythrocytes in this study, it has been shown that during the incorporation of large amounts of 2-fluoroadenosine into erythrocytic nucleotide pools, the rate of glycolysis increased about threefold, whereas the ATP and 2,3-diphosphoglycerate concentrations were unaffected (7).

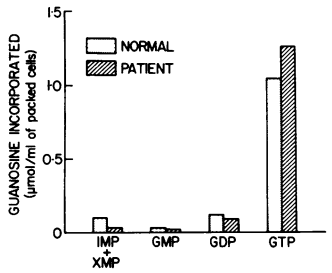

FIGURE 7 Incorporation of guanosine into nucleotides by intact erythrocytes. Conditions for incubation, extraction, and analysis are as described in Figs. 2 and 3 except that [8-^{14}C]guanosine (0.12 mM, 8.3 mCi/mmol) was used in place of [8-^{14}C]adenosine. "Patient" refers to A. W.

Guanosine metabolism. Incubation of both normal and ADA-deficient erythrocytes with [8-^{14}C]guanosine (0.16 mM) resulted in the rapid incorporation of label into the nucleotide pools. The total amounts of guanosine incorporated were similar and represented virtually total conversion of the added guanosine to nucleotides after 4 h of incubation. At the end of this incubation time most of the [8-^{14}C]guanosine had accumulated as GTP in both types of cells (Fig. 7).

The results of this experiment and that of inosine incorporation noted above indicate that the purine nucleoside transport process, purine nucleoside phosphorylase, hypoxanthine-guanine phosphoribosyl transferase, and 5-phosphoribosyl-1-pyrophosphate synthetase, all function normally in the erythrocytes of the ADA-deficient patients studied. Fig. 7 also shows that a small amount of label appears as IMP + XMP. Although the significance of this observation is not known at the present time, a trace contamination of the [8-^{14}C]guanosine by hypoxanthine, xanthine, or their nucleotides cannot be ruled out. It should be noted that ATP levels remained unchanged during these guanosine incorporation experiments (data not shown).

Metabolism of formycin A. Fig. 8 shows the incorporation of the C-nucleoside analogue of adenosine, formycin A, into the nucleotide pools of ADA-deficient erythrocytes (from A. W.). Cells were incubated with 1 mM formycin A at 30°C and nucleotide profiles were obtained at various time intervals. As shown in Fig. 8, the profile at 0 time does not have a peak in the nucleotide region that absorbs at 295 nm (the λ_{max} for formycin A). Incubation of normal cells for 4 h produced small peaks in the di- and triphosphate regions. By contrast, in ADA-deficient erythrocytes, throughout the 4-h incubation period, substantial quantities of di-

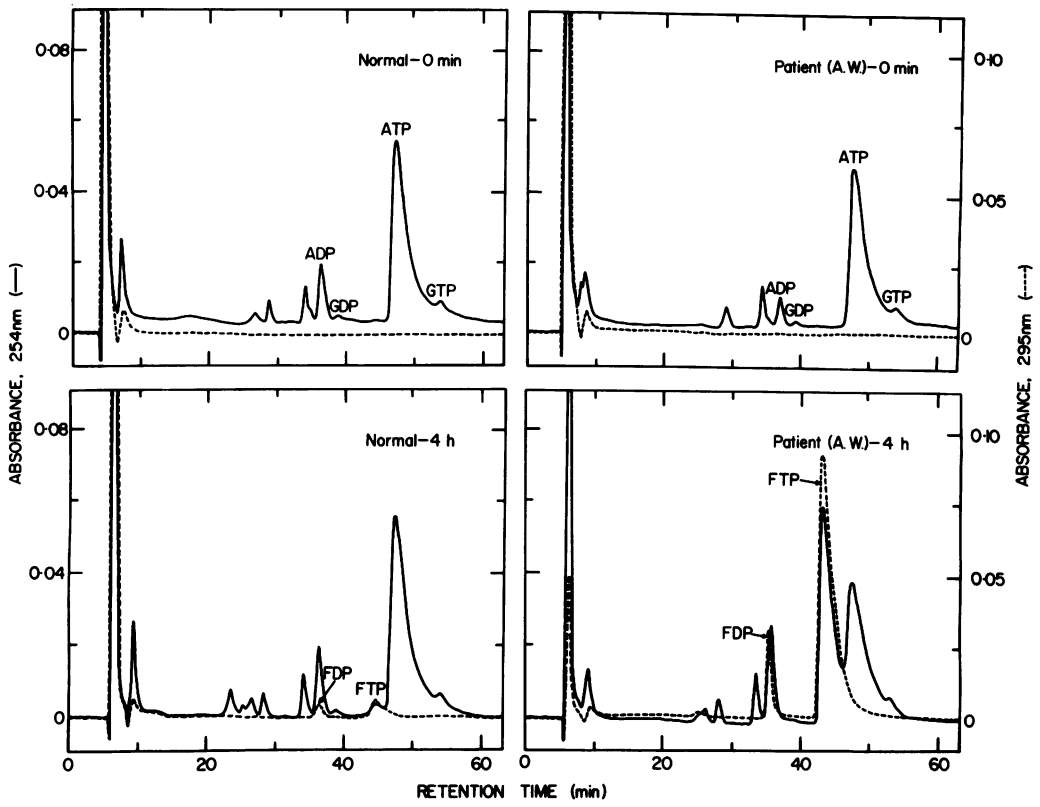

FIGURE 8 HPLC profiles of erythrocytes incubated with formycin A. Conditions of incubation, extraction, and chromatography are described in Fig. 2 except that formycin A (1 mM) was used in place of adenosine. Column effluents were monitored at both 254 nm and 295 nm (the λ_{max} for formycin A). "FDP" and "FTP" refer to the di- and triphosphate nucleotides of formycin A.

and triphosphate nucleotides of formycin A were formed. The amount of analogue monophosphate nucleotide that accumulated was insignificant during this period. The total concentrations of formycin nucleotides reached about 4.5 μmol/ml of erythrocytes (about three times the normal ATP concentration) after 4 h of incubation which represents almost complete conversion of the added formycin A to analogue nucleotides. As with guanosine and 2-fluoroadenosine, the ATP levels remained unchanged during the incubation period. Similar results were obtained with the erythrocytes of two other SCID-ADA-deficient patients (C. W. and I. B.) studied.

DISCUSSION

The above results with erythrocytes from SCID-ADA-deficient patients as well as normal cells treated with coformycin, a tight-binding inhibitor of erythrocytic ADA, are consistent with the hypothesis presented previously that the disposition of adenosine in human erythrocytes is the result of the relative activities and Michaelis constants of the enzymes, ADA and adenosine kinase (7–9, 12). When normal erythrocytes were incubated with adenosine, principally the monophosphate nucleotide, IMP, accumulated. Furthermore, only small quantities of analogue nucleotides were formed from formycin A, a C-nucleoside analogue of adenosine that is an excellent substrate for ADA (8, 12). In contrast, with SCID-ADA-deficient erythrocytes or normal cells treated with coformycin, marked increases in the adenine nucleotides were produced on incubation with adenosine (Fig. 4), and when formycin A was used as precursor, quantities of analogue nucleotides were produced that exceeded the normal adenine nucleotides by about two- to threefold (Fig. 8).

Mature human erythrocytes cannot synthesize purine nucleotides *de novo* but actively salvage purine bases and purine nucleosides to form nucleotides. Natural purine bases, as well as several base analogues, can react with 5-phosphoribosyl-1-pyrophosphate and the appropriate phosphoribosyl transferase to form directly the 5'-monophosphate nucleotides. Nucleosides of hypoxanthine and guanine as well as many related analogues can be phosphorolyzed by purine nucleoside phosphorylase to form the purine base and ribose-1-phosphate. The existence of inosine or guanosine kinases has not been demonstrated in human erythrocytes—rather the formation of nucleotides from inosine and guanosine or their analogues is readily explained by

the sequential actions of the enzymes, purine nucleoside phosphorylase and hypoxanthine-guanine phosphoribosyl transferase. On the other hand, adenosine, which is a very poor substrate for purine nucleoside phosphorylase (24), can be converted directly to AMP by reaction with adenosine kinase. This enzyme is also capable of reacting with many adenosine-like analogues. Since the activity of ADA is about eightfold higher than that of adenosine kinase, normal human erythrocytes, in the presence of high concentrations of adenosine, principally deaminate adenosine to inosine with the subsequent formation and accumulation of IMP by the reactions noted above (7, 9). Thus, the results presented above with SCID-ADA-deficient erythrocytes and with coformycin-treated normal erythrocytes effectively demonstrate the key role played by ADA in the disposition of adenosine in the human erythrocyte.

Despite considerable investigation and speculation since the discovery of the first ADA-deficient patient, a satisfactory explanation of the relationship between ADA deficiency and the deficit in thymus-derived and bone marrow-derived cell function is not yet available. It seems likely, however, that the inability of ADA-deficient lymphocytes, or perhaps lymphatic stem cells, to deaminate and detoxify adenosine is somehow involved. Adenosine has been reported to cause interference with pyrimidine biosynthesis in lymphoid cell lines (25, 26) and with RNA, DNA, and protein synthesis in phytohemagglutinin-stimulated peripheral lymphocytes (27). Incubation with adenosine produced an increase in cyclic AMP levels in lymphocytes and an inhibition of lymphocyte-mediated cytolysis (28). As reported elsewhere (29), the lymphocytes of an SCID-ADA-deficient individual (patient C. W., this paper) lacked responsiveness to the mitogens phytohemagglutinin and concanavalin A. Significantly, addition of calf intestinal or human erythrocytic ADA to cultures of this patient's lymphocytes restored their responsiveness to mitogen stimulation, suggesting a causal relationship between the ADA deficiency and the cellular immune defects.

These studies deal with purine metabolism in the erythrocytes of SCID-ADA-deficient patients, although the major abnormality in such patients appears to involve primarily lymphatic cells. ADA-deficiency in the erythrocyte does not appear to affect either the function or survival of such cells. Therefore, one must exercise caution in extrapolating directly to the lymphocyte conclusions derived from the study of erythrocytes. However, it should be noted that, although the purine metabolism of human erythrocytes is very limited, e.g., they lack the *de novo* purine biosynthetic pathway and do not synthesize nucleic acids, they represent an ideal cell for the study of many facets of purine salvage metabolism. Already several important differences between the purine salvage mechanisms of normal human erythrocytes and those of peripheral lymphocytes have been detected in this laboratory. For example, erythrocytes can synthesize very large quantities of GTP or of analogue nucleotides such as 2-fluoroATP, without causing significant alterations in the normal adenine nucleotide pools (7, 9). However, when peripheral lymphocytes are incubated with 2-fluoroadenosine, 2-fluoroadenine nucleotides are synthesized rapidly and largely replace the normal adenine nucleotides (9). Also, as noted elsewhere, the activity of ADA in peripheral lymphocytes is about 10-fold greater than that of human erythrocytes, which is further indication of an important role of this enzyme in the lymphocyte.

Perhaps the most significant observation made in these experiments with ADA-deficient erythrocytes (either from SCID-ADA-deficient patients or with normal erythrocytes treated with coformycin) is that these cells, on exposure to adenosine, undergo rapid surges in the adenine nucleotide concentrations with increases in the ATP levels of the order of 130 to 150% (see Fig. 4). By contrast, normal ADA-containing erythrocytes convert most of the adenosine to IMP with only minor increases (about 15%) in the ATP levels (see Fig. 3). Thus, one might speculate that the cells of SCID-ADA-deficient patients might also be subject to major surges in the adenine nucleotide levels after exposure to adenosine, as might occur after a purine-rich meal or after tissue breakdown. Thus, in addition to the proposed explanations of the cytotoxic action of adenosine noted above, other possibilities must be considered. For example, if effects such as those seen with erythrocytes also occur in lymphatic cells, they might have drastic effects on intracellular metabolism. It is well established that many key metabolic functions are sensitive to the adenylate energy charge (30) and to fluctuations in the adenine nucleotide levels of the cell, e.g., in many tissues the enzyme 5-phosphoribosyl-1-pyrophosphate synthetase is subject to strong allosteric regulation by these nucleotides (30, 31). Thus, if marked elevations in the ATP levels occur, there might be an inhibition of this enzyme with consequent deleterious effects on such vital reactions as pyrimidine nucleotide biosynthesis, as well as on the salvage mechanisms for purine and pyrimidine bases. Also, it has been known for about 20 yr that the key glycolytic enzyme phosphofructokinase in various tissues is strongly inhibited by ATP (32). It is now generally accepted that allosteric regulation of this enzyme by ATP, as well as by a number of other metabolites, plays a major role in the control of glycolysis in many tissues (for a recent thorough review see 33). It is intriguing to speculate that elevated intracellular adenine nucleotides might not have damaging effects in the resting peripheral lymphocyte. However, when such cells are subjected to great metabolic demands imposed by cellular division or transformation during the immune response or in re-

sponse to mitogens, blockade of glycolysis by high levels of ATP might have profound effects. For example, during these events there is a marked increase in intracellular membranous structures that require the availability of large amounts of fatty acids and glycerophosphate. Thus, blockade of glycolysis at the phosphofructokinase reaction might drastically impair membrane synthesis. It also seems likely that similar damaging effects might be produced on the process of maturation of lymphoid stem cells into mature lymphocytes. Thus, as mentioned previously ADA might offer a logical target enzyme for the design of immunosuppressive agents (8, 9).

At present we cannot explain the apparently increased rates of synthesis of ITP found in the erythrocytes of two of the four SCID-ADA-deficient patients studied above. No distinct IMP kinase activity has been demonstrated in human erythrocytes. However, IMP served as a weak substrate for several guanylate kinase isozymes purified from pooled human erythrocytes but had unfavorable kinetic characteristics compared with those of the natural substrate GMP (34, 35). The V_{max} for IMP was 1% or less than that of GMP, and the Michaelis constants (about 1×10^{-3} M) were about 50-fold greater than for GMP. Furthermore, normal human erythrocytes have an ITPase capable of the pyrophosphorolysis of ITP to IMP (36). The metabolic regulation and physiological role of this enzyme are still poorly understood. Additional investigations will be necessary to determine whether the apparently elevated rates of ITP accumulation are a common occurrence in SCID-ADA-deficient patients and to determine the mechanism, e.g., whether the elevated ITP levels are due to increased rates of synthesis or to an impairment of the activity of ITPase. The two patients who displayed elevated rates of ITP synthesis from inosine had been subjected to transplantation procedures with temporary and long-term restoration of immuncompetence. The erythrocytes from one patient (A. W.), were examined on three occasions over a period of 15 mo. On the second and third examinations, the rates of ITP accumulation had decreased markedly.

It will be both of interest and importance to examine other patients with SCID who display normal ADA activity. Recently, such a patient has been reported whose erythrocytes are deficient in purine nucleoside phosphorylase (37). It is tempting to speculate that defects in the facilitated nucleoside transport mechanism (17) or in the postulated binding of ADA to the cell membrane at a site near the nucleoside transporter (11) might be involved in other SCID patients who display normal ADA activity in hemolysates. It appears that the ammonia liberation procedure for the measurement of ADA activity in intact erythrocytes and hemolysates might be of particular value in the identification of such postulated defects.

It seems likely that the adenosine analogue, formycin A, will prove of special value in future studies of SCID cells with defects in adenosine metabolism. This analogue is deaminated about eight times more rapidly than adenosine by ADA (8, 9) and readily forms the 5'-monophosphate nucleotide by reaction with adenosine kinase. In ADA-competent cells, deamination predominates over phosphorylation and little analogue nucleotide is formed (see reference 7 and Fig. 8 of this paper). On the other hand, in ADA-deficient cells, very large amounts of formycin A nucleotides can accumulate (Fig. 8). Of great potential value is the fact that formycin A and its nucleotides fluoresce strongly at 340 nm upon excitation with ultraviolet light at 295 nm at pH 7 (38). Therefore, after incubation with formycin A, it should be possible to distinguish between normal cells and ADA-deficient cells (that contain large amounts of formycin A nucleotides). In addition to the use of fluorescence, it may be possible to distinguish between normal and ADA-deficient cells by radioautography after incubation with tritiated formycin A. Such a technique may prove of clinical value in following patients with mixed-cell populations as might occur after procedures such as blood transfusions or bone marrow transplantations.

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