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Immunology

Purine metabolism in adenosine deaminase deficiency*

(immunodeficiency/pyrimidines/adenine nucleotides/adenine)

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ABSTRACT **Purine and pyrimidine metabolites were measured in erythrocytes, plasma, and urine of a 5-month-old infant with adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) deficiency. Adenosine and adenine were measured using newly devised ion exchange separation techniques and a sensitive fluorescence assay. Plasma adenosine levels were increased, whereas adenosine was normal in erythrocytes and not detectable in urine. Increased amounts of adenine were found in erythrocytes and urine as well as in the plasma. Erythrocyte adenosine 5′-monophosphate and adenosine diphosphate concentrations were normal, but adenosine triphosphate content was greatly elevated. Because of the possibility of pyrimidine starvation, pyrimidine nucleotides (pyrimidine coenzymes) in erythrocytes and orotic acid in urine were measured. Pyrimidine nucleotide concentrations were normal, while orotic acid was not detected. These studies suggest that the immune deficiency associated with adenosine deaminase deficiency may be related to increased amounts of adenine, adenosine, or adenine nucleotides.**

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), an enzyme that is widespread in animal tissues, plays a key role in (*a*) the normal catabolic pathway for adenine nucleotides, and (*b*) the production of hypoxanthine for use in the purine salvage pathway (Fig. 1). Intense interest has centered on this enzyme since Giblett *et al.* (1) described the association of its deficiency with an autosomal recessive immunodeficiency. The disease is characterized by serious infections, severe thymus-dependent (T) lymphocyte and variable bone marrow-dependent (B) lymphocyte deficiencies, and characteristic skeletal abnormalities. This disease is fatal unless the immune system of affected patients is reconstituted. While the clinical and genetic aspects of this disorder have been well described (2–6), the relationship between the enzyme deficiency and the immunodeficiency is not understood. Although recent evidence suggests a causal relationship (7), elucidation must ultimately depend upon the measurement of key metabolites and enzyme activities in the affected purine and pyrimidine pathways.

The identification and quantitation of adenine and adenosine in blood has been difficult because the amounts of these substances are low and adenine nucleotides are relatively high within erythrocytes. The recent development of a sensitive fluorescence assay for adenine-containing compounds has enhanced the detection of these compounds in red cells. In the present study, various ion exchange procedures were used for preliminary separations, and adenine- containing compounds were identified and quantitated using either ultraviolet absorption or fluorescence spectra. With these techniques, we have studied changes in purine and pyrimidine pathways in an adenosine deaminase deficient individual.

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Ado, adenosine; Ade, adenine.

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MATERIALS AND METHODS

Subject. A 5-month-old boy born on December 5, 1974 with severe combined immunodeficiency was investigated. Physical examination revealed enlarged costochondral junctions and sparse lymphoid tissue (5). The serum IgG (normal values in parentheses) was 31 mg/dl (263–713); IgM, 8 mg/dl (34–138); and IgA was less than 3 mg/dl (13–71). C1q was not detectable by double immunodiffusion. The blood lymphocyte count was 200–400/mm^3 with 2–4% sheep erythrocyte (E)-rosettes, 12% sheep erythrocyte-antibody-complement (EAC)-rosettes, 10% IgG bearing cells, and no detectable IgA or IgM bearing cells. [^{3}H]Thymidine incorporation into blood lymphocytes stimulated by phytohemagglutinin and concanavalin A was not significantly greater than in unstimulated lymphocytes. Intradermal delayed hypersensitivity tests to common microbial antigens, including *Candida albicans*, were negative. Adenosine deaminase activity in red cells, as measured by Kalckar's method (8), was less than 1% of normal. At the time of these studies, the child was free of clinical infection, but he exhibited severe growth failure.

Chemicals and Solutions. Analytic grade ion exchange resins (200/400 mesh) were obtained from Bio-Rad Laboratories. Chloroacetaldehyde was prepared as follows: 100 ml of chloroacetaldehyde, dimethylacetal (Aldrich Chemical Co.), was refluxed for 30 min with 30 ml of 1.5 M H_2SO_4, and the chloroacetaldehyde was collected by distillation. Concentrations were determined by conversion to the bisulfite derivative, with excess bisulfite determined iodometrically. This provided chloroacetaldehyde, which had minimal absorbance at 280 and 305 nm and minimal fluorescence when excited at those wave lengths. Sodium acetate buffers, used as eluants for cation exchange analysis, were prepared from solutions of sodium acetate (anhydrous, reagent grade) and acetic acid (analytical reagent). Since the cation was the eluting ion, listed sodium acetate buffer concentrations refer to the Na^+ concentration. Buffer pH values refer to pH at 22°.

Preparation of Protein-Free Supernates. Heparinized venous blood was centrifuged within 15 min of collection. Plasma was removed carefully from the erythrocytes, which were resuspended in a corresponding amount of 0.154 M NaCl. The erythrocyte suspension was deproteinized with trichloroacetic acid (9). After recentrifugation to remove traces of cells, plasma was deproteinized in the manner described for erythrocytes. The protein-free supernates were stored frozen until analyzed.

Analytical Procedures. Anion exchange chromatography of erythrocyte nucleotides and phosphate esters was carried out using a multiple column technique as described previously (10, 11). For fluorescence assay of adenine compounds, the procedure of Avigad and Damle (12) was used with minor modifications. Chloroacetaldehyde condenses with adenine com-

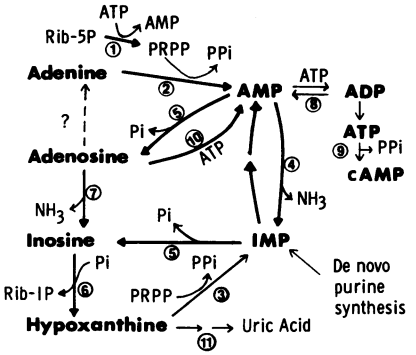

FIG. 1. Abbreviated scheme of adenine nucleotide metabolism. Enzymes catalyzing the various reactions are (*1*) phosphoribosylpyrophosphate synthetase, (*2*) adenine phosphoribosyltransferase, (*3*) hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyl transferase, EC 2.4.2.8), (*4*) adenylate deaminase, (*5*) 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), (*6*) nucleoside phosphorylase, (*7*) adenosine deaminase, (*8*) adenylate kinase, (*9*) adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], (*10*) adenosine kinase, and (*11*) xanthine oxidase.

pounds to form the highly fluorescent 1,N^6-etheno derivative (13). 2,8-Dioxyadenine also reacts readily, but the product has a different excitation spectrum. Pyrimidines with a 6-amino group (e.g., cytosine) also react, but maximal fluorescence in this case is at a lower wavelength. In our procedure, a concentration of chloroacetaldehyde of 0.067 M in the heated sample was used with a reaction time of 40 min at 80°. Either a phosphate or acetate buffer (0.05 M) was used for pH control. Adenine or adenosine standards were analyzed simultaneously with each determination. An unheated sample containing all components was used as a blank. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer with an 416-992 Xenon lamp and an R136 photomultiplier tube, using slit arrangement number three. In most cases, fluorescence spectra were obtained to assure identification of ethenoadenine compounds. Since the excitation spectrum of 1,N^6-ethenoadenine has a rather flat peak, an excitation wave length of either 280 or 310 nm was used for routine assays. The wavelength giving the lowest blank fluorescence was usually chosen, with fluorescence being measured at 425 nm. This assay will detect adenine-containing compounds at approximately 1/20th the concentration required to provide a measurable absorbance at 260 nm. With higher concentrations of adenine-containing compounds, absorbance measurements were used. 2,8-Dioxyadenine was determined by fluorescence assay after a preliminary separation on a short anion exchange column. The assay was carried out as described for adenine, with excitation at 310 nm and fluorescence measurement at 425 nm. Uric acid in urine was determined by spectrophotometry after anion exchange separation (14). This procedure was modified to include an orotic acid determination by elution of the orotic acid with 0.10 M HCl after removal of uric acid from the anion exchange column. The limit of sensitivity is about 50 μmol/liter of urine, so this assay does not detect orotic acid in urine of normal subjects.

Adenine and Adenosine in Urine and Red Cells. Use of a cation exchange resin (H^+form) with HCl elution to separate adenine and adenosine is illustrated in Fig. 2A. Adenine in urine of the adenosine deaminase deficient child was readily detected by this technique (Fig. 2B). For identification, ultraviolet absorption spectra of adenine in HCl and NaOH were used, and the adenine was converted to 1,N^6-ethenoadenine and identified by fluorescence spectra. Urine from normal adults and

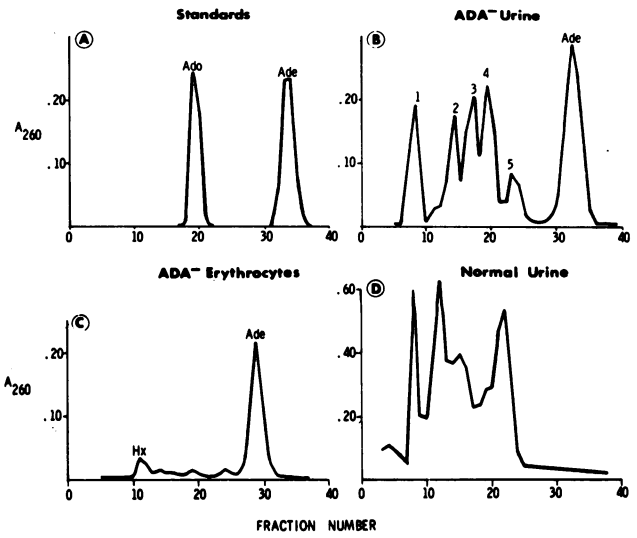

FIG. 2. Cation exchange separation of adenosine (Ado) and adenine (Ade) using HCl as an eluant. Four Dowex AG50-X4 columns (0.5 × 20 cm, H^+ form) were eluted simultaneously using gradient elution with 1.5 M HCl added dropwise to a closed mixing reservoir containing 300 ml of water (75 ml/column). Fraction volumes were 3.5–4.0 ml. Elution profile A shows the separation of known samples of Ado and Ade. Three milliliters of the patient's urine were used for elution profile B, and 4.5 ml of normal urine (0.10 hr excretion volume) were used for elution profile D. For elution profile C, the protein-free supernate corresponding to 0.50 ml of the patient's erythrocytes was used. In each case, samples were passed through short Dowex AG1-X4 columns (1 × 5 cm, formate form) to remove anionic components prior to cation exchange chromatography. Elution profiles A and B were run simultaneously. Since elution profiles C and D were run separately, the peak positions did not match exactly those noted for profiles A and B. A_{260}, absorbance at 260 nm. ADA, adenosine deaminase.

children showed no significant adenine in elution profiles† (Fig. 2D). Although peak 4 (Fig. 2B) corresponds to the adenosine position, fluorescence assay showed that adenosine was not present in these fractions. When this separative technique was applied to the protein-free supernate from the patient's red blood cells (Fig. 2C), the adenine peak was also noted.

Adenine Separation by Elution from Cation Exchange Resins with Sodium Acetate Buffer. Because the reaction of adenine with chloroacetaldehyde to form ethenoadenine requires a pH of 4–6, the sensitivity of adenine assays was increased greatly by eluting adenine from the column in this pH range. Since blank fluorescence is lower if the concentration of the eluting buffer is low (0.10 M or less), a procedure was devised using 0.077 M sodium acetate buffer to elute adenine from a cation exchange resin column at a pH of 5.2.

In this procedure, adenine was separated from tryptophan and adenosine, and was detected with great sensitivity (Fig. 3A and B). This assay was particularly useful for plasma adenine determinations. The identity of the isolated adenine was confirmed by determining fluorescence spectra of the ethenoadenine produced during the assay of eluate fractions (Fig. 4).

Adenosine Determinations by Using Two Successive Cation Exchange Column Separations. Since adenosine was not separated from tryptophan by the previous procedure using acetate buffer as eluant, a combination of the separative procedures shown in Figs. 2 and 3 was required for adenosine assay

† Previous studies have shown that adenine excretion in normal adults is approximately 1 mg/day (15), an amount too low to detect by this assay.

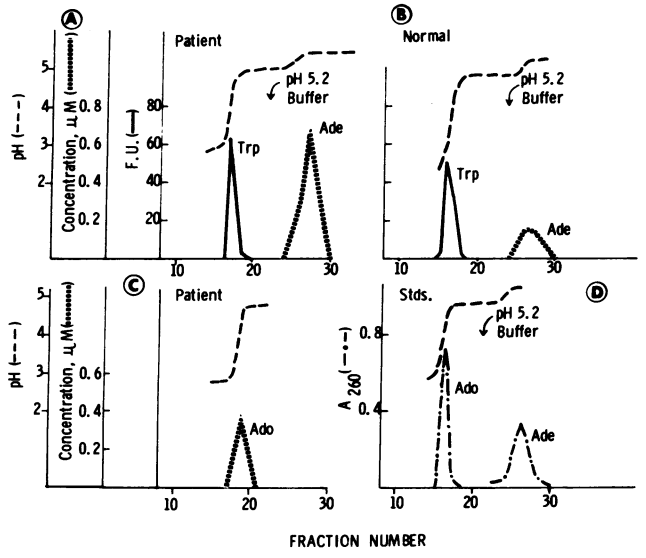

FIG. 3. Cation exchange separations using sodium acetate buffers as eluants. Four Dowex AG50-X4 columns (0.5 × 20 cm) (H^+ form) were eluted simultaneously using 0.077 M sodium acetate buffer at pH 4.7. After tryptophan (Trp) was eluted, the pH 4.7 buffer was replaced with a pH 5.2 buffer with the same Na· concentration. Fraction volumes were 3.0–3.3 ml. The determination of Ade in plasma of an adenosine deaminase deficient patient (A) and in normal plasma (B) are shown. The solutions used for elution profiles A and B were equivalent to 3.3 and 3.1 ml of plasma, respectively. The elution profile from the second column in the two-column procedure for separation of adenosine is shown in C. The solution used for this elution profile was equivalent to 1.1 ml of the patient's plasma. Ado had been separated previously from Trp and Ade on the first column (see *text* for details). Ade and Ado concentrations in A, B, and C were determined by the fluorometric assay. Separation of known samples of Ado and Ade are shown in elution profile D.

in plasma and red cells. Adenosine and tryptophan were first separated on a cation exchange column using HCl as eluant. Tryptophan appeared in the elution profile at about fraction 50 (Fig. 2A). Spectrophotometric assay of fractions from one column containing an adenosine standard served to identify the position of adenosine in the HCl elution profile for the three unknown samples. Adenosine-containing fractions‡ (about 18 ml) were diluted 5-fold to reduce the HCl concentration to 0.15–0.20 M and were applied to a cation exchange column (H^+form). Adenosine was then displaced from these columns with 0.077 M, pH 4.7 sodium acetate buffer. With tryptophan absent, adenosine was readily identified in eluant fractions by the fluorescence assay (Figs. 3C and 4).

RESULTS

Purine and pyrimidine compounds in body fluids in adenosine deaminase deficiency

The data are summarized in Table 1. In the subject with adenosine deaminase deficiency, adenine was slightly elevated in plasma and greatly elevated in red cells and urine. Adenine excretion was about 5% of uric acid excretion, while the value for normal individuals is about 0.2% (15). Plasma adenosine was also elevated. Control subjects for plasma and red cell studies included the patient's parents (obligate heterozygous carriers of the enzyme deficiency), two normal adults, and two infants of approximately the same age as the patient. One infant had a severe combined immunodeficiency with normal adenosine deaminase activity; the other had no immune defect but did have a mild reticulocytosis.

Data on erythrocyte nucleotides in the patient and in control subjects are summarized in Table 2. Adenine nucleotides were greatly elevated in the patient's erythrocytes. The elevation was due to increased ATP, whereas ADP and AMP were similar to controls. We questioned whether this increase in ATP may have been due to a young population of erythroid cells, but the patient's reticulocyte count was less than 1%. Since red cell ATP concentrations of infants are very close to adult values (16), the latter were used as controls. Normal concentrations were noted for nicotinamide coenzymes (NAD and NADP), guanine-containing nucleotides (GDP and GTP), and pyrimidine nucleotide coenzymes, i.e., cytosine-containing nucleotides (cytidine and deoxycytidine diphosphate choline and cytidine and deoxycytidine diphosphate ethanolamine) (17) and uracil-containing nucleotides (uridine diphosphate glucose and uridine diphosphate *N*-acetylglucosamine) (18).

Several other compounds were determined in urine to evaluate possible alterations in purine catabolism or pyrimidine biosynthesis in adenosine deaminase deficiency. Uric acid excretion was normal (Table 1). Since adenine at elevated concentrations may be oxidized by xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2) (19), 2,8-dioxyadenine is found in urine after adenine administration. Although small amounts of 2,8-dioxyadenine (0.9 μmol/24 hr) were detected in the patient's urine, the amount was much below that associated with impaired renal function (20). Orotic acid was not detected in the urine of the patient.

DISCUSSION

The relationship between the absence of adenosine deaminase and severe combined immunodeficiency has remained unclear. Green and Chan (21) demonstrated that fibroblasts and lymphoid cell lines treated with exogenous adenosine at concentrations ranging from 10 to 100 μM were depleted by pyrimidine nucleotides. It was suggested that this was due to a buildup of ADP, a known inhibitor of phosphoribosyl-pyrophosphate synthetase (ATP:D-ribose-5-phosphate pyrophosphotransferase, EC 2.7.6.1) (22). Presumably, this would block pyrimidine synthesis *de novo* at the level of orotate due to lack of substrate phosphoribosyl-pyrophosphate synthetase. Purine synthesis *de novo* would also be reduced by the lack of this substrate. Significantly, uridine partially protected these two types of cells from pyrimidine depletion as well as cell death.

Wolberg *et al.* (23) offered an alternative explanation for the cellular immunodeficiency associated with adenosine deaminase deficiency. They showed that addition of adenosine to murine peritoneal exudate lymphocytes with or without *erythro*-9-(2-hydroxy-3-nonyl) adenine, a potent inhibitor of adenosine deaminase, resulted in inhibition of lymphocyte-mediated cytolysis and elevation of adenosine 3′:5′-cyclic monophosphate (cAMP). These changes were always greater with addition of *erythro*-9-(2-hydroxy-3-nonyl) adenine. Lymphocyte killer function was not ameliorated by uridine. Furthermore, no effect on pyrimidine pools could be demonstrated after 60 min of incubation with adenosine at the concentrations used. These experiments led the authors to suggest that the immunodeficiency in adenosine deaminase deficiency patients was due to increased intracellular levels of cAMP.

To our knowledge these possible explanations have not been previously tested in adenosine deaminase deficiency. The suggestion of Green and Chan (21) that the lymphocyte dys-

‡ This included one fraction on each side of the adenosine peak to assure that no adenosine from the unknown was lost due to slight differences in the elution pattern of the four columns.

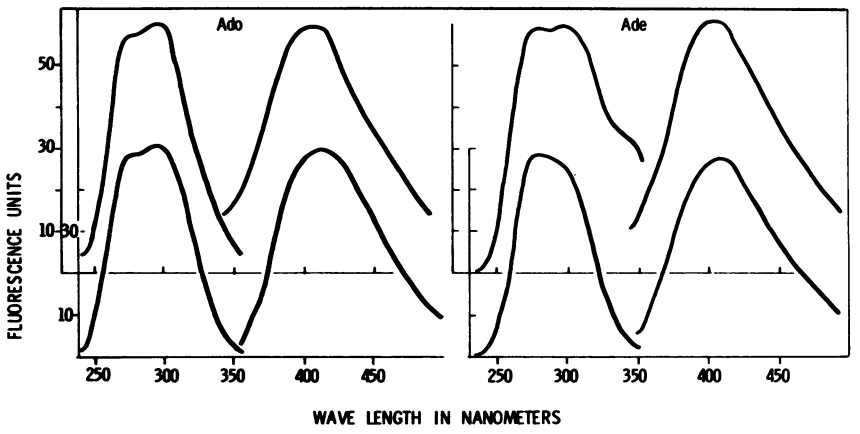

FIG. 4. Fluorescence spectra of reaction products with chloroacetaldehyde of Ado and Ade. In each case, the lower curves show the fluorescence of known compounds (Ado and Ade) and the upper curves, the corresponding compounds isolated by cation exchange chromatography from the patient's plasma. Fluorescence units are arbitrary since peak heights have been adjusted to make the curves more readily comparable. In each case, the blank fluorescence was subtracted prior to plotting. As actually determined, maximal fluorescence for the samples isolated from the patient's plasma was Ado, 65, and Ade, 52 (meter multiplier setting of 0.01). The emission spectra (the curve at the right in Ado and Ade) were determined with an excitation wavelength of 280 nm while scanning with the emission monochromator. The excitation spectra (the curve at the left in Ado and Ade) were determined by measuring fluorescence at 410 nm while scanning with the excitation monochromator. Other instrument settings are described in *Materials and Methods*. The pH of the samples during fluorescence measurement was Ado, 4.7, and Ade, 5.2.

function is due to pyrimidine starvation was not supported by our findings of normal pyrimidine nucleotides in adenosine deaminase deficient red cells. Although the nucleotides that we measured are not direct precursors of DNA, but nucleotide coenzymes (Table 2), the synthesis of these compounds is dependent on an intact pathway for pyrimidine biosynthesis *de novo* in erythrocyte precursors. In addition, orotic aciduria, as predicted by the hypothesis of pyrimidine starvation, was not found in our patient.

We then examined the possibility that adenosine might inhibit lymphocyte function in adenosine deaminase deficiency by formation of excess adenine nucleotides. In our patient, adenosine was elevated in plasma (Table 1) to a level (about 2 μM) that approximates the level of exogenous adenosine necessary to produce a deficit in lymphocyte killer function in the presence of *erythro*-9-(2-hydroxy-3-nonyl)adenine (23). Red cell ATP levels were increased markedly. The cause of the increased ATP in adenosine deaminase deficient red cells appears to be related to the block in adenine nucleotide catabolism. Since adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) (Fig. 1) is the primary enzyme acting on adenosine in these deficient cells, most of the adenosine would be converted back to AMP (Fig. 1). The K_m for human erythrocyte adenosine kinase (about 1 μM) (24) is low enough to maintain a low intracellular concentration of adenosine (Table 1). The action of adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) and a normally functioning glycolytic pathway might increase ATP levels without significantly changing AMP or ADP levels. Although AMP can be deaminated to IMP by AMP aminohydrolase, EC 3.5.4.6 (Fig. 1), it appears that the alternate catabolic pathway through adenosine might be more important, in at least some tissues.

Table 1. Concentration of adenine, adenosine, and related compounds in body fluids in adenosine deaminase deficiency

	Patient	Controls
Plasma		
—Adenine	1.7; 1.1; 1.3	0.64 ± 0.15*
—Adenosine	3.6; 1.6; 0.7	0.31 ± 0.29†
Erythrocytes		
—Adenine	86; 110; 91	13 ± 7‡
—Adenosine	1.3	0.9, 1.5
Urine		
—Adenine	82; 88; 83	Not detectable

Plasma and urine values are expressed as μM; erythrocyte values are expressed as μmol/liter of cells. The three urine samples were collected on April 10, May 9, and May 13, 1975, respectively. The 24-hr excretion of adenine in these samples was 7.0, 11.5, and 8.6 μmol; and of uric acid, 162, 253, and 127 μmol. The three blood samples were drawn on April 6, May 14, and July 21, 1975, respectively.

* Mean values ± SD for five subjects.

† Mean values ± SD for four subjects.

‡ Mean values ± SD for 18 subjects.

Table 2. Nucleotide and phosphate ester levels in adenosine deaminase deficient erythrocytes

	Patient		Patient's father	Normal values*
AMP	24	21	23	24 ± 7
ADP	204	203	168	201 ± 29
ATP	2380	2640	1520	1390 ± 170
NAD	49	48	50	67 ± 21
NADP	48	45	45	46 ± 8
CDPX†	18	20	33	36 ± 12
UDPX‡	50	35	32	41 ± 12
GDP	10	7	12	18 ± 8
GTP area	119	112	100	108 ± 35
2,3-Diphospho-glycerate	6410	6130	4220	5210 ± 580
Total adenine nucleotides	2610	2860	1710	1620 ± 180

Values are expressed as μmol/liter of cells. The two sets of values for the patient are from samples drawn on April 8 and July 21, 1975, respectively.

* Mean values ± SD for 26 normal adults.

† CDPX, cytidine and deoxycytidine diphosphate choline and cytidine and deoxycytidine diphosphate ethanolamine.

‡ UDPX, uridine diphosphate glucose and uridine diphosphate *N*-acetyl-glucosamine.

The finding of increased levels of adenine in our patient was unexpected. We questioned whether adenosine was the direct source of the increased adenine. Nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) cleaves inosine and guanosine readily, but has not been reported to act upon adenosine (25, 26). Zimmerman *et al.* (27), in a study of the reverse reaction (adenine + ribose 1-phosphate → adenosine + inorganic phosphate), noted a slight activity of nucleoside phosphorylase, but the V_{max} was much lower and the K_m much higher than for hypoxanthine. However, since nucleoside phosphorylase is an exceedingly active enzyme, it is conceivable that it might act upon adenosine to produce adenine. Snyder and Henderson (28) recently demonstrated an enzyme in mammalian tissues that cleaves deoxyadenosine to form adenine. Their study did not indicate whether the cleavage was phosphorylytic or hydrolytic. Presumably, this enzyme also acts on adenosine to form adenine, although the experimental design of their study did not permit them to test this. Although adenine might be formed from AMP and inorganic pyrophosphate by the action of adenine phosphoribosyltransferase (AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7), the equilibrium for this reaction strongly favors AMP synthesis. Furthermore, normal levels of AMP in red cells or our patient make the reversal of this reaction unlikely. Indeed, elevated adenine levels imply a decrease in activity of adenine phosphoribosyltransferase. The activity of this enzyme is thought to be inhibited by the concentrations of 2,3-diphosphoglycerate and ATP (29). In that regard, our patient's red cells contained only slightly elevated amounts of 2,3-diphosphoglycerate, but greatly elevated amounts of ATP.

The abnormalities in erythrocyte nucleotides in our patient may provide a clue to the metabolic disturbance that causes the lymphocyte dysfunction in adenosine deaminase deficiency. It should be pointed out that in this study, we did not measure the nucleotides in the cells in question, the lymphocytes. However, our preliminary studies have shown elevated concentrations of ATP in the patient's lymphocytes (F. C. Schmalstieg, J. A. Nelson, G. C. Mills, T. M. Monahan, A. S. Goldman, and R. M. Goldblum, unpublished work). Therefore, lymphocyte function might be corrected by lowering the levels of adenine-containing compounds. It is possible that this could be accomplished by transferring normal human cells rich in adenosine deaminase, which have prolonged survival and would not elicit a graft versus host reaction. Normal human erythrocytes fit these criteria. In conjunction with further studies of purine pathways, we are presently examining the effect of human erythrocyte transfusions in patients with adenosine deaminase deficiency.

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