Pergamon

PII: S1357-2725(96)00080-5

Int. J. Biochem. Cell Biol. Vol. 28, No. 12, pp. 1405–1414, 1996

1357-2725/96 $15.00 + 0.00

Metabolism of Adenosine and Deoxyadenosine by Human Erythrocytes and CCRF-CEM Leukemia Cells

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Human lymphocytes lacking adenosine deaminase die and T-cell leukemias are killed by deoxycoformycin (dCf), an inhibitor of adenosine deaminase, due to impaired metabolism of dAdo. The initial metabolism of exogenous adenosine (Ado) and deoxyadenosine (dAdo) has been compared in human erythrocytes and CCRF-CEM leukemia cells and the data obtained have been simulated using kinetic constants obtained *in vitro* for the enzymes involved. Cells were mixed with ^{3}H-labelled Ado or dAdo, samples were taken at 3 sec intervals and progress curves for the ^{3}H-labelled metabolites formed were determined by quantitative two-dimensional thin layer chromatography. Erythrocytes rapidly take up Ado and the predominant metabolite after 60 sec is hypoxanthine (Hyp), while for dAdo, deoxyinosine (dIno) predominates. By contrast, leukemia cells convert Ado predominantly to AMP, while dAdo is converted first to Hyp and then to AMP. The presence of dCf had little effect upon Ado metabolism but induced accumulation of dAdo. Erythrocytes rapidly degrade Ado and dAdo to Hyp, although the phosphorolysis of dIno is relatively slow. Human CCRF-CEM leukemia cells convert most of the Ado or dAdo to AMP after 60 sec. For dAdo, the sequence of reactions would be dAdo→dIno→Hyp→IMP→sAMP→AMP. dCf does not significantly affect the conversion of Ado→AMP, but dCf blocks AMP accumulation from dAdo, consistent with the reaction sequence shown above. A computer model has been developed for the metabolism of Ado and dAdo, but some of the kinetic constants determined *in vitro* for this model do not pertain to intact cells. Copyright © 1996 Elsevier Science Ltd

Keywords: Adenosine deaminase Erythrocytes Leukemia Immunodeficiency Metabolism

Int. J. Biochem. Cell Biol. (1996) **28,** 1405–1414

INTRODUCTION

An inherited deficiency in adenosine (Ado) deaminase results in Severe Combined Immunodeficiency Syndrome associated with accumulation of dATP to millimolar concentrations in the erythrocytes of patients (Coleman *et al.*, 1978; Cohen *et al.*, 1978). Because hereditary Ado deaminase deficiency results in a selective toxicity for T- and B-lymphoid cells, inhibitors of Ado deaminase may be useful as chemotherapeutic agents for lymphoid malignancies. Coformycin (Cf), 2′-deoxycoformycin (dCf) and 9-(erythro-2-hydroxy-3-nonyl) adenine (EHNA) are potent inhibitors of adenosine deaminase from calf intestine with inhibition constants of: Cf, $K_i = 2.2 \times 10^{-10}$ M; dCf, $K_i = 2.5 \times 10^{-12}$ M and EHNA, $K_i = 1.7 \times 10^{-9}$ M (Frieden *et al.*, 1980). dCf,

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Abbreviations: Ade, adenine; Ado, adenosine; Ado deaminase, adenosine deaminase (EC 3.5.4.4) may deaminate Ado or deoxyadenosine (dAdo); AMP deaminase, adenylate deaminase (EC 3.5.4.6) may deaminate AMP or dAMP; Cf, coformycin; EHNA, 9-(erythro-2-hydroxy-3-nonyl)adenine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Hyp, hypoxanthine; Ino, inosine; sAMP, N-succinyl-AMP. The prefix "d" before an abbreviation denotes the 2′-deoxyribose form of a nucleoside.

Received 14 December 1995; accepted 8 July 1996.

the most potent inhibitor, has been tested clinically on patients with T-cell leukemia (Mitchell *et al.*, 1979; Yu *et al.*, 1979) and is effective against chronic lymphocytic leukemia (Grever *et al.*, 1985) and hairy cell leukemia (Kraut *et al.*, 1986). Whether a deficiency of Ado deaminase is inherent or induced artificially, the consequent cytotoxicity is attributed to accumulation of dAdo which is phosphorylated to dATP. A variety of mechanisms of cytotoxicity involving metabolites of dAdo have been proposed (Kredich and Hershfield, 1989), but the selective toxicity for T- and B-lymphocytes suggests that consequently unbalanced pools of deoxyribonucleotides may affect recombination events of the DNA of the precursors of such cells involved in generation of the diversity of T-cell receptors and immunoglobulins produced by B-cells.

The metabolism of Ado and dAdo is thus of importance to our understanding of an inborn error of metabolism and for the chemotherapy of leukemia. Barankiewicz and Cohen (1984) and Valentine *et al.* (1985) have proposed that Ado nucleotides are degraded via the sequence: ATP→ADP→AMP→IMP→Ino→Hyp while dAdo nucleotides are degraded: dATP→dADP→dAMP→dAdo→dIno→Hyp (Fig. 1). Thus, Ado deaminase may be required for degradation of dAdo and a deficiency of Ado deaminase would lead to the reported accumulations of dAdo, dAMP, dADP and dATP. Data presented in this paper further define the pathways for Ado and dAdo metabolism in erythrocytes and leukemia cells. The *de novo* pathways for biosynthesis of purine and pyrimidine nucleotides are not functional in erythrocytes but are active in leukemia cells which are replicating DNA. The kinetic constants for the individual enzymes of the metabolic network for Ado and dAdo have been determined *in vitro* and used to simulate short-term progress curves for intermediates quantified from cell extracts.

MATERIALS AND METHODS

Materials

RPMI 1640 growth medium, bicarbonate-free, with 20 mM K.Hepes as buffer, and gentamicin were purchased from Flow Laboratories (Sydney, Australia), fetal calf serum was from the Commonwealth Serum Laboratories (Parkville, Australia). The dCF was a generous gift from Dr V. L. Narayanan of the National Cancer Institute (Bethesda, U.S.A.) and EHNA was kindly provided by Wellcome (Cabarita, Australia). [G-^{3}H]-Hypoxanthine (obtained as a solid, 3.80 Ci/mmol), [8-^{3}H]adenine (43.5 μM, 23.0 Ci/mmol), [2-^{3}H]adenosine (37.0 μM, 27.0 Ci/mmol), [G-^{3}H]deoxyadenosine (34.5 μM, 29.0 Ci/mmol), [8-^{14}C]inosine (877 μM, 57.0 Ci/mol), [2-^{3}H]AMP (43.5 μM, 23.0 Ci/mmol) and [2,8-^{3}H]dAMP (62.5 μM, 16.0 Ci/mmol) were purchased from Amersham International plc (Amersham, U.K.). [8-^{3}H] Deoxyinosine (52.5 μM, 29.0 Ci/mmol) was obtained by incubation of the [8-^{3}H]deoxyadenosine with Ado deaminase type VI (Sigma Chemical Company, St Louis, U.S.A.; 0.021 units) in 50 mM K.Hepes (240 μl, pH 7.4) at 25°C for 1 hr. The reaction was stopped with 0.4 M $HClO_4$, and after neutralization, the [8-^{3}H]deoxyinosine formed was purified by HPLC using a Brownlee RP-18 column (Applied Biosystems, San Jose, U.S.A.; 0.42 × 22.0 cm) eluted with 10 mM ammonium formate (pH 5.0). The [8-^{3}H]deoxyinosine had a retention time of 23–26 min and was collected and lypophilized several times from water to remove the ammonium formate.

Growth of human CCRF-CEM leukemia cells

Cultures of leukemia cells were inoculated at a density of 4×10^4 cells/ml in RPMI 1640 medium containing 10% (v/v) fetal calf serum, gentamicin (50 μg/ml) and sodium bicarbonate (100 μM); the final pH was 7.3. The serum was heated at 56°C for 30 min prior to use and was free of Ado deaminase activity. Cells grew with a doubling-time of 24 hr. Human erythrocytes were separated from freshly drawn blood by centrifugation, washed three times in phosphate-buffered saline and resuspended at a density of 4.8×10^9 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. Erythrocytes prepared by this procedure contained less than 0.008% lymphocytes. All subsequent procedures were as described above for leukemia cells.

Incubation of cell suspensions and preparation of cell extracts

Human erythrocytes were separated from freshly-drawn blood (20 ml) by centrifugation (8000 ***g***, 3 min), washed three times in phosphate-buffered saline (20 ml) then once in Hanks' balanced salts solution (20 ml) and resuspended at a density of 4.8×10^9 cells/ml in Hanks' balanced salts solution. Any remaining lymphocytes were removed from the top of the cell pellet at each of these washing steps. As described elsewhere (Szabados and Christopherson, 1995), dibutylphthalate (800 μl, density 1.04 g/ml) was layered onto 200 μl of 0.8 M $HClO_4$ in 25% (v/v) glycerol in 1.5 ml Eppendorf tubes in the rotor of a centrifuge. Aliquots (200 μl) of cell suspensions at 37°C were rapidly mixed with [^{3}H]Ado or [^{3}H]dAdo (550 μM, 500 Ci/mol, 20 μl) and layered onto the dibutylphthalate layer of sequential tubes using a Hamilton dual syringe

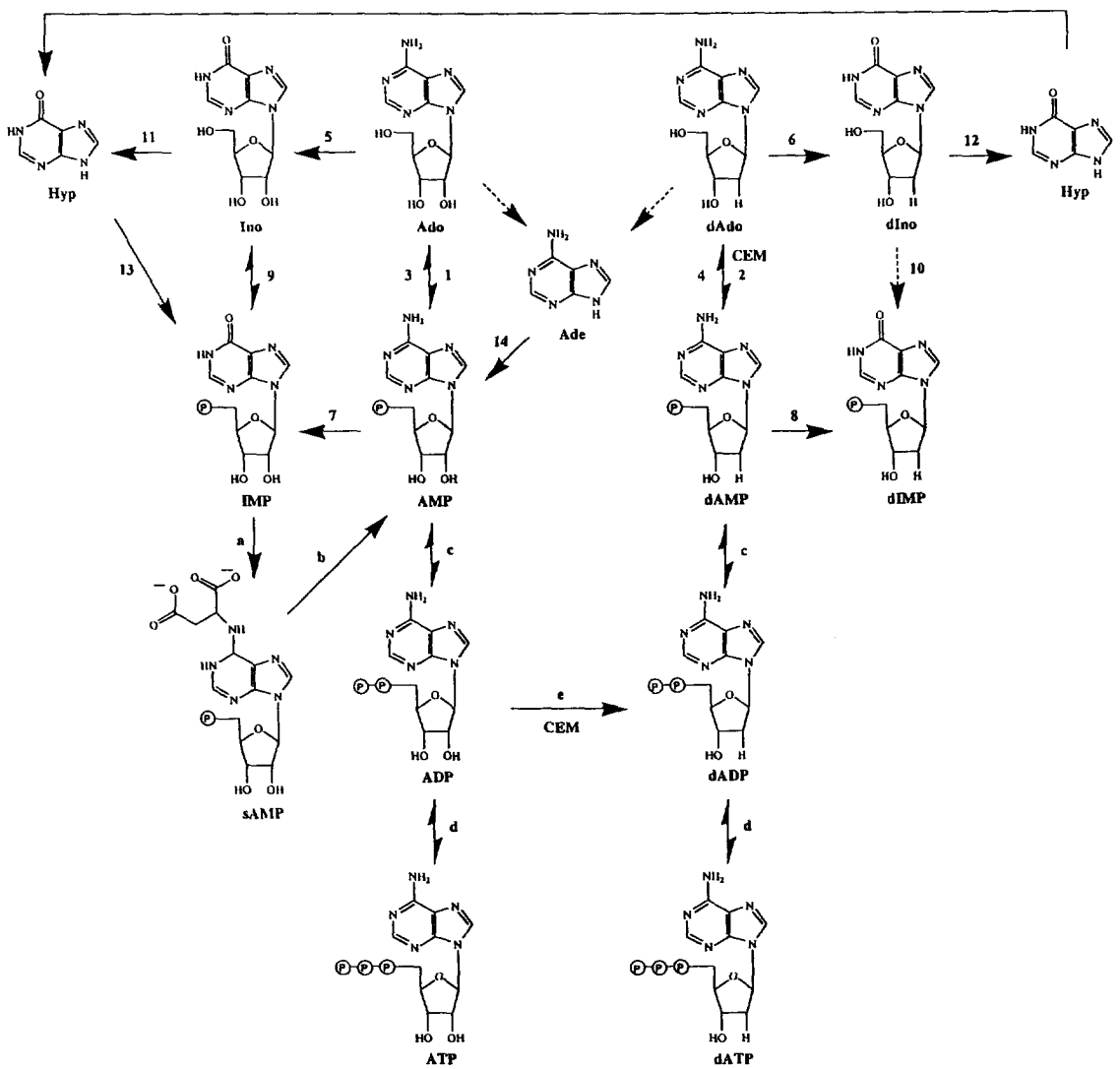


Fig. 1. Pathways for metabolism of adenine nucleotides. The enzymes catalyzing these reactions are: 1, 2, adenosine kinase (EC 2.7.1.20); 3, 4, 5′-nucleotidase (EC 3.1.3.5); 5, 6, adenosine deaminase (EC 3.5.4.4); 7, 8, AMP deaminase (EC 3.5.4.6); 9, 10, inosine kinase (EC 2.7.1.3); 11, 12, purine nucleoside phosphorylase (EC 2.4.2.1); 13, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); 14, adenine phosphoribosyltransferase (EC 2.4.2.7); a, adenylosuccinate synthetase (EC 6.3.4.4); b, adenylosuccinate lyase (EC 4.3.2.2); d, nucleoside diphosphate kinase (EC 2.7.4.6); e, ribonucleotide reductase (EC 1.17.4.1). Arrows marked "CEM" indicate a reaction found only in leukemia cells, broken arrows indicate an enzyme activity which was very low or absent.

apparatus fitted with repeating dispensers (Szabados and Christopherson, 1995). One second after delivery of the last mixture of cells plus nucleoside, the centrifuge was started (8500 ***g***, 1 min) and the cells sedimented through the dibutylphthalate into the perchloric acid giving a total elapsed time of 3 sec for the final sample. After standing on ice for 15 min, the tube above the $HClO_4$ was washed with dibutylphthalate (800 μl) which was then removed. A sample (180 μl) of the acidic cell extract was neutralized by vortex-mixing for 1 min with an equal volume of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Sherman and Fyfe, 1989). The two phases were separated by centrifugation (8500 ***g***, 5 min) and the upper aqueous layer was removed and stored at $-20°C$ for subsequent analysis.

Human CCRF-CEM leukemia cells in late exponential phase (6×10^5 cells/ml) were harvested by centrifugation, washed in phosphate-buffered saline and resuspended at a density of 4.0×10^7 cells/ml. Dibutylphthalate/mineral oil (86:14, v/v, density 1.01 g/ml, 700 μl) was layered onto 100 μl of 0.8 M $HClO_4$ in 25% (v/v) glycerol in Eppendorf tubes. The cell suspension was preincubated at 37°C for 30 min in the absence or presence of 5 μM dCf and at 3 sec intervals aliquots of cells (200 μl) and [^{3}H]Ado or [^{3}H]dAdo (550 μM, 100 Ci/mol, 20 μl) were mixed and layered onto dibutylphthalate/mineral oil as for erythrocytes. Uptake and metabolism of [^{3}H]Ado and [^{3}H]dAdo by cells was stopped by centrifugation and cell extracts were prepared as described above.

Determination of cellular volumes

Suspensions (1.0 ml) of erythrocytes or leukemia cells in Hanks' balanced salts solution at 10 densities between 5 and 100×10^7 cells/ml or 2 and 40×10^7 cells/ml, respectively, were mixed with ^3H_2O (100 μl, 1.0 μCi) and [^{14}C]inulin (100 μl, 1.0 μCi). Aliquots (240 μl) of these mixtures were layered on top of dibutylphthalate/mineral oil (700 μl) underlayed with $HClO_4$ (100 μl, 0.8 M) in an Eppendorf tube. The cells were centrifuged (8500 ***g***, 30 sec) through the oil into the acid and aliquots (50 μl) were taken of the aqueous upper and lower layers for scintillation counting in 5 ml of cocktail (5.5 g PPO/l toluene:Triton X – 100, 2:1, v/v). Counts from ^3H_2O and [^{14}C]inulin were plotted vs number of cells and the slopes to these plots were determined by linear regression. With the assumption that [^{14}C]inulin can occupy only the extracellular space, the intracellular spaces for human erythrocytes and CCRF-CEM leukemia cells were calculated as the difference between the slopes of the two plots.

Chromatography of ^{3}H-labelled nucleosides and bases

Cell extracts (20 μl) were analysed by two-dimensional thin layer chromatography on poly(ethyleneimine)–cellulose (Machery-Nagel, Duren, Germany; Szabados and Christopherson, 1995). Glycerol, in neutralized extracts of erythrocytes and leukemia cells, was removed after application of the sample to a poly(ethyleneimine)–cellulose chromatogram by immersion of the chromatogram in anhydrous isopropanol containing 1.2 g Tris/l. The developing solvent was 200 mM LiCl saturated with H_3BO_3 (pH 3.5):ethanol, 1:1 (v/v) for the first dimension and 1.2 M ammonium sulfate (pH 3.5) for the second dimension. The positions of purine nucleosides or the nucleotides (see below) were determined by visualization of appropriate marker compounds under UV light (Szabados and Christopherson, 1995). The separated spots were excised and quantified by scintillation counting.

Chromatography of ^{3}H-labelled nucleotides

Cell extracts (20 μl) were analysed on poly(ethyleneimine)–cellulose (Szabados and Christopherson, 1995), after removal of glycerol as described above, by development in the first dimension with 0.75 M LiCl, 1.0 M acetic acid for 5 cm, followed by 1.25 M LiCl, 1.0 M acetic acid for a further 13 cm. The chromatograms were desalted by immersion in anhydrous methanol containing 1.2 g Tris/l for 10 min and then developed in the second dimension with 2.5 M ammonium acetate, 3.6% (w/v) H_3BO_3 (pH 7.0) for 5 cm, followed by 3.5 M ammonium acetate, 5.0% (w/v) H_3BO_3 (pH 7.0) for a further 13 cm.

Preparation of cell-free extracts for enzyme assays

Human CCRF-CEM leukemia cells (50 ml) were harvested by centrifugation (500 ***g***, 10 min) in late exponential phase (8×10^5 cells/ml), washed in Hanks' balanced salts solution and resuspended in enzyme buffer [1 ml of 30% (v/v) glycerol, 1 mM dithiothreitol and 20 mM K.Hepes (pH 7.4)]. Erythrocytes from freshly-drawn blood (10 ml) were washed three times in phosphate-buffered saline and then once in

enzyme buffer before being resuspended in enzyme buffer (1 ml). Both human CCRF-CEM leukemia cells and erythrocytes were disrupted by sonication with a Branson Sonifier Cell Disruptor Model B15 (Branson Sonic Power Company, Danbury, U.S.A.) three times (50 W, 30 sec), the lysate was centrifuged (20 000 ***g***, 30 min) and the supernatant was dialyzed against enzyme buffer and stored as aliquots at − 20°C. Protein concentrations for typical cell-free extracts were: leukemia cells, 10.4 mg/ml; and erythrocytes, 121 mg/ml.

Enzyme assays

The kinetic parameters of enzymes in cell-free extracts from erythrocytes and human CCRF-CEM leukemia cells were determined at 37°C in assay mixtures containing in a total volume of 25 μl:50 mM K.Hepes (pH 7.4), 5% (v/v) glycerol and 10 different substrate concentrations. The second substrate, if required, was at a saturating concentration: P-Rib-PP (1.0 mM) and $MgCl_2$ (1.0 mM) were required for the phosphoribosyltransferases, inorganic phosphate (1.0 mM) for purine nucleoside phosphorylase and MgATP (5.0 mM) for the kinases. After preincubating the assay mixture for 5 min at 37°C, the reaction was started by addition of enzyme. For assay of AMP deaminase, Ado and dAdo kinases, the cell-free extract was preincubated with 5 μM EHNA for 5 min at 37°C to inhibit Ado deaminase. Samples (7 μl) were withdrawn and applied to poly(ethyleneimine)–cellulose chromatograms at three appropriate times. The radiolabelled substrate and product(s) were separated by ascending chromatography using one of two developing solvents. Ammonium sulfate (1.2 M, pH 3.5) gave the following R_f values: Ade, 0.25; Ado and dAdo, 0.38; Hyp and IMP, 0.54; Ino and dIno, 0.69; AMP, ADP and ATP, 0.84. Butanol:acetic acid:water (5:3:2, v/v/v) gave the following R_f values: IMP, AMP, ADP and ATP, 0; Ino, 0.39; dIno, 0.50; Hyp, 0.53; Ado, 0.65; dAdo, 0.70. For most assays, 1.2 M ammonium sulfate was used as the developing solvent. For 5′-nucleotidase assays, butanol:acetic acid:water was the developing solvent. For AMP deaminase and Ado kinase assays, 3.5 μl samples were applied to two poly(ethyleneimine)–cellulose chromatograms, one of which was developed with 1.2 M ammonium sulfate and the other with butanol:acetic acid:water. Radiolabelled substrate and product(s) were located on chromatograms by co-chromatography with marker compounds with UV absorbance. Appropriate spots were excised and quantified by scintillation counting. Initial reaction velocities were determined by linear regression of data for product formed vs time. Reaction velocities at different substrate concentrations were fitted by non-linear regression to the Michaelis–Menten equation using the BASIC program DNRP53 (Duggleby, 1984).

Metabolic simulations

The short-term uptake and metabolism of dAdo and Ado by erythrocytes or leukemia cells were simulated to obtain progress curves for the concentration of each metabolite as a function of time. These simulations were done with a custom-written BASIC computer program that used a routine adapted from the CRICF Fortran program (Chandler *et al.*, 1972) based on a 3rd/4th-order Runge–Kutta–Fehlberg algorithm (Press *et al.*, 1986) with automatic step-size control.

The metabolism of dAdo by erythrocytes will be used to illustrate the procedure. The experimental data show that the only metabolites detected in the cells were dAdo, dIno and Hyp, indicating the sequence:

$$dAdo_o \xrightarrow{R00} dAdo_i \xrightarrow{R06} dIno \underset{R12b}{\overset{R12}{\rightleftarrows}} Hyp \qquad (1)$$

where $dAdo_o$ is extracellular, $dAdo_i$ is intracellular and R00 is the rate of uptake. The changes in metabolite concentrations were calculated using the following equations:

$D[Ado]_i = R00 - R06;$

$D[Ino] = R06 + R12b - R12;$

$D[Hyp] = R12 - R12b.$

Reaction rates are designated by R with the reaction numbers in Fig. 1 and Table 1. The total uptake (i.e. $[dAdo]_i + [dIno] + [Hyp]$) was calculated and fitted by non-linear regression, using the program DNRP53 (Duggleby, 1984), to a double-exponential curve consisting of a fast initial component and a second slower

component that approaches a combined asymptote equal to the external dAdo concentration of 50 μM.

$$\text{Total} = C_1[1 - \exp(-k_1 t)] + [50 - C_1][1 - \exp(-k_2 t)]. \quad (2a)$$

This model describes uptake by two different facilitated diffusion systems working in parallel, with no efflux, where the rate of uptake is proportional to the extracellular Ado concentration.

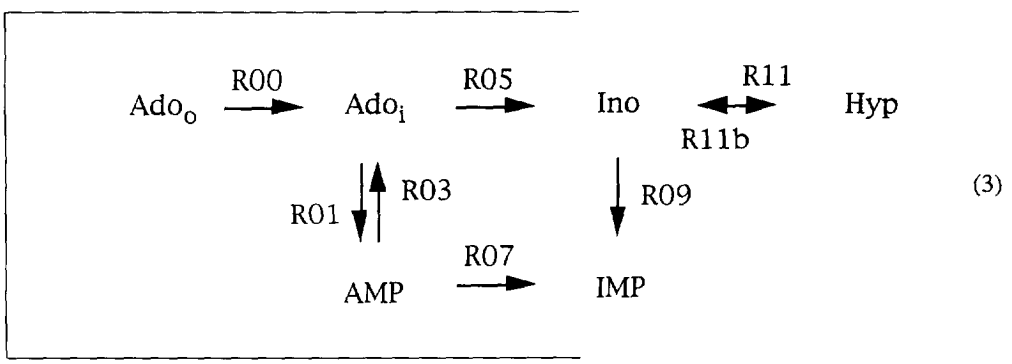
The uptake kinetics were then modelled using the parameter values obtained from this analysis.

$$\text{R00} = v_1\exp(-k_1 t) + v_2\exp(-k_2 t) \quad (2b)$$

where R00 is total velocity for uptake, $v_1 = C_1k_1$ is the fast uptake component, k_1 is a first-order rate constant, $v_2 = [50 - C_1]k_2$ is the slow uptake component and k_2 is a first-order rate constant.

The Ado deaminase and purine nucleoside phosphorylase reactions were modelled as simple Michaelis–Menten enzymes using a maximum velocity and Michaelis constant for each enzyme, as measured in cell extracts (see Results). dRib-1-P is one of the substrates for the reverse reaction catalyzed by purine nucleoside phosphorylase (dRib-1-P + Hyp→dIno + P_i). Since the concentration of dRib-1-P was not available, the reversibility of the purine nucleoside phosphorylase reaction was modelled using selected values of V_{max}/K_m for the reverse reaction until an acceptable simulation of the data was found. In addition, in this example, it was found to be necessary to normalize the simulation by assuming a value of V_{max} for adenosine deaminase that was 2.5-fold higher than that observed in cell extracts; enzymic activity could have been lost during extraction and storage prior to assay.

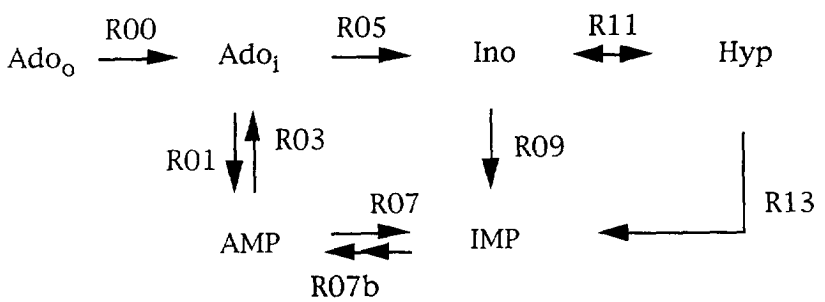
A similar procedure was adopted for the metabolism of Ado by erythrocytes, and for leukemia cells with Ado or dAdo as precursor. Most of these cases involved more complex metabolic networks and normalization factors for some V_{max} values and a partitioning factor (P) for compartmentation of dAdo were introduced. The metabolic networks for each of the remaining experiments are shown below together with the equations used to simulate progress curves for metabolite concentrations. Velocity equations for the reactions (R01–R13) are listed in the legends to Figs 2 and 3.For Ado metabolism in erythrocytes:



(3)

$D[Ado]_i = R00 + R03 - R01 - R05;$
$D[Ino] = R05 + R11b - R11 - R09;$
$D[Hyp] = R11 - R11b;$
$D[AMP] = R01 - R03 - R07.$

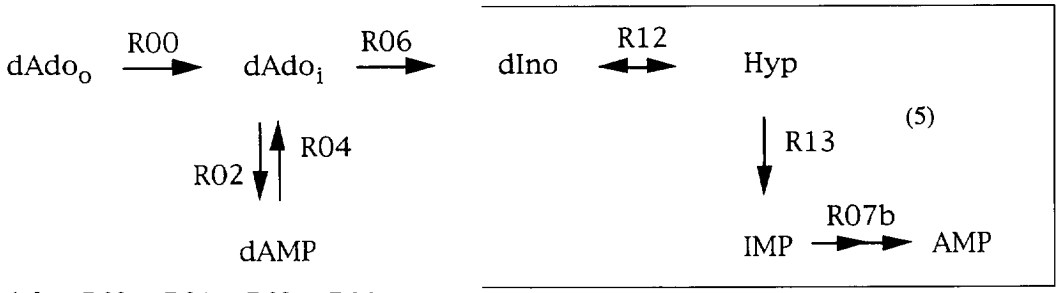
For Ado metabolism in leukemia cells:



(4)

$D[Ado]_i = R00 + R03 - R01 - R05;$
$D[Ino] = R05 - R11 - R09;$
$D[Hyp] = R11 - R13;$
$D[AMP] = R01 + R07b - R03 - R07;$
$D[IMP] = R07 + R09 + R13 - R07b.$

For dAdo metabolism in leukemia cells:



$D[dAdo]_i = R00 + R04 - R02 - R06;$
$D[dIno] = R06 - R12;$ $D[Hyp] = R12 - R13;$
$D[IMP] = R13 - R07b;$ $D[AMP] = R07b;$
$D[dAMP] = R02 - R04.$ The reaction R07b actually represents two reactions (IMP→sAMP→AMP) and is effectively the reverse of R07.

RESULTS AND DISCUSSION

Determination of kinetic parameters for enzymes in vitro

Enzymes catalyzing conversions between purine nucleoside 5′-monophosphates, nucleosides and bases (Fig. 1) were assayed in dialyzed, cell-free extracts of human leukemia cells and erythrocytes and the kinetic data obtained were used to determine K_m and V_{max} values (Table 1). The enzyme assays *in vitro* were carried out at a physiological pH of 7.4 using a minimal number of assay components. Chromatography of standard substrate and product(s) as UV markers enabled accurate excision of radiolabelled compounds. The accuracy of the individual assays is indicated by the standard deviations of values obtained for K_m and V_{max} (Table 1). Ado and dAdo phosphorylase activities (Ado→Ade) were not detected; Ino and dIno kinase activities (Ino→IMP) were just detectable and only the first-order rate constants are listed. Similarly, there was little adenylate deaminase activity when dAMP was the substrate, but significant activity with dAdo as substrate (dAdo→dIno). Thus, as reported by Barankiewicz and Cohen (1984), dAdo compounds would be deaminated only via Ado deaminase. Barankiewicz and Cohen proposed that Ado compounds are deaminated via AMP deaminase but the first-order rate constant for Ado→Ino when compared with that for AMP→IMP is 47-fold higher for leukemia cells and 86-fold higher in erythrocytes (Table 1). Thus, when Ado and AMP are low in cells, Ado→Ino would be the preferred route of deamination and there could be dual deamination via Ado and AMP deaminases [equation (12), Fig. 1] when Ado and AMP concentrations were high.

The kinetic constants of Table 1 are for enzymes found in the soluble fraction of cells, but enzymes such as Ado deaminase are also found associated with membranes (Van der Weyden and Kelley, 1976). To gain an overall picture of the activities of the enzymes of adenine nucleotide metabolism found in intact cells, significant enzymes were also assayed in the particulate fraction (Table 2). As the protein content of the particulate fraction represents only 0.0239% and 3.19% of the total protein in erythrocytes and CCRF-CEM leukemia cells, respectively, the enzymic activities measured in the particulate fractions are not significant when compared with the cell-free extracts.

Initial metabolism of Ado and dAdo by cells

The uptake of nucleosides by cells and their subsequent intracellular metabolism are rapid processes. To determine the initial events in the metabolism of exogenous [^{3}H]Ado and [^{3}H]dAdo by leukemia cells and erythrocytes, cell suspensions were incubated at 37°C for up to 60 sec before stopping uptake and metabolism by centrifuging the cells through oil into perchloric acid. The stability of [^{3}H]Ado and [^{3}H]dAdo under acidic conditions was determined by incubating [^{3}H]Ado and [^{3}H]dAdo in 0.4 M $HClO_4$ at 0°C for up to 23 hr. After allowance for ^{3}H-labelled impurities present initially in these solutions there was no decrease in radioactivity in [^{3}H]Ado or [^{3}H]dAdo over the 23 hr of incubation. The two-dimensional chromatographic system separates Ade from Hyp and all the nucleosides.

Table 1. Kinetic constants for enzymes of adenine nucleotide metabolism in soluble, cell-free extracts

Reaction		CCRF-CEM leukemia cells			Erythrocytes		
		V_{max}(pmol min^{-1} μg^{-1})	K_m (μM)	V_{max}/K_m(min)$^{-1}$	V_{max}(pmol min$^{-1}\mu$g^{-1})	K_m(μM)	V_{max}/K_m(min)$^{-1}$
1	Ado→AMP	0.605 ± 0.023	0.923 ± 0.143	115	0.0885 ± 0.0066	1.67 ± 0.468	17.9
2	dAdo→dAMP	0.648 ± 0.063	4.86 ± 1.14	22.7	0.00674 ± 0.00039	29.0 ± 3.65	0.0783
3	AMP→Ado	25.3 ± 8.0	2860 ± 1570	1.50	1.44 ± 0.08	339 ± 62	1.43
4	dAMP→dAdo	6.01 ± 2.32	740 ± 510	1.38	0.153 ± 0.015	428 ± 114	0.120
5	Ado→Ino	94.7 ± 6.8	142 ± 36	113	1.77 ± 0.08	115 ± 19.8	5.19
6	dAdo→dIno	23.3 ± 1.5	84.5 ± 22.5	46.9	0.315 ± 0.013	51.0 ± 10.2	2.08
7	AMP→IMP	26.4 ± 4.27	1860 ± 631	2.41	0.263 ± 0.077	1480 ± 110	0.0599
8	dAMP→dIMP	2.87 ± 0.323	3840 ± 680	0.127	—	—	0.00788*
9	Ino→IMP	—	—	0.278*	—	—	0.00226*
10	dIno→dIMP	—	—	0.0674*	—	—	0.0354*
11	Ino→Hyp	16.9 ± 0.7	157 ± 16	18.3	1.20 ± 0.06	15.7 ± 3.7	25.8
12	dIno→Hyp	0.924 ± 0.055	126 ± 20	1.25	2.63 ± 0.15	11.7 ± 3.5	75.8
13	Hyp→IMP	2.49 ± 0.11	9.76 ± 2.93	43.4	2.72 ± 0.07	32.2 ± 4.9	28.5
14	Ade→AMP	0.253 ± 0.007	6.95 ± 1.09	6.18	0.122 ± 0.007	5.19 ± 0.99	7.93
	Ado→Ade	N.D.			N.D.		
	dAdo→Ade	N.D.			N.D.		

Extracts of human CCRF-CEM leukemia cells and erythrocytes were prepared, enzyme assays were performed and kinetic data were analysed as described in Materials and Methods. First-order rate constants (V_{max}/K_m) were calculated assuming a cytosolic protein concentration of 170 mg/ml for leukemia cells and 337 mg/ml for erythrocytes. Reaction numbers correspond to those in Fig. 1. N.D.,not detectable.

[a] V_{max} was not determined because substrate concentrations in the range of K_m could not be used.

The R_f value for Ade in the first dimension is the same as that of Hyp, which is between dIno/dAdo and Ino/Ado (Szabados and Christopherson, 1995). In the second dimension, the R_f value for Ade is the lowest of all compounds.

The uptake of Ado and dAdo by erythrocytes is initially faster than their subsequent metabolism; up to 10 sec, the intracellular concentrations of Ado and dAdo increase and are higher than other metabolites (Fig. 2). After 10 sec, Ado is deaminated via Ado→Ino→Hyp [equation (8)], with some concurrent phosphorylation of Ado→AMP (Fig. 2a). dAdo is also deaminated via Ado deaminase but without significant phosphorylation of dAdo→dAMP (Fig. 2b), as predicted from Table 1. Leukemia cells initially phosphorylate exogenous Ado→AMP with little deamination to Hyp (Fig. 3a). The concentration of AMP accumulated is not affected by 5 μM dCf (Fig. 3b). By contrast, exogenous dAdo, which is taken up more slowly, is deaminated to Hyp via dAdo→dIno→Hyp, which is then salvaged to AMP via Hyp→IMP→ sAMP→AMP (Fig. 3c), as shown by equation (7). dCf (5 μM) caused an accumulation of dAdo and decreases in dIno, Hyp, IMP and AMP (Fig. 3d), indicating partial inhibition of Ado deaminase (dAdo→dIno) by 5 μM dCf. No phosphorylation of dAdo (dAdo→dAMP) was detected over the first 60 sec. Figures 2 and 3 show progress curves for metabolism of Ado and dAdo by erythrocytes and leukemia cells. These data were reproducible between experiments with similar trends in the progress curves. The volume of the trapped extracellular space which is carried as a thin film around the cells as they are centrifuged through oil was 4 and 6% for human erythrocytes and CCRF-CEM leukemia cells, respectively, a small contribution which was not included in calculations.

Simulation of initial metabolism of Ado and dAdo

The progress curves through the experimental data of Figs 2 and 3 were generated using the metabolic networks defined in Experimental Procedures and velocity equations from the figure legends with parameter values from Table 1. To obtain simulations consistent with the metabolite concentrations from intact cells, several values for V_{max} and V_{max}/K_m were normalized from the values determined *in vitro* (Table 1) as indicated in the figure legends. For erythrocytes (Fig. 2), it was necessary to reduce V_{max01} for Ado→AMP, AMP→IMP was inactive, while an increase was required in V_{max06} for dAdo→dIno and values were assigned to V_{max11b} for Hyp→Ino and V_{max12b}/K_{m12b} for Hyp→dIno. For simulation of progress curves for metabolites in leukemia cells (Fig. 3), it was necessary to reduce V_{max07} (AMP→IMP) and V_{max13} (Hyp→IMP), while an increase was required in V_{max12} (dIno→Hyp) and a value assigned to V_{max07b}/K_{m07b} (IMP→ →AMP). For simulations of Ado or dAdo metabolism by leukemia cells in the presence of dCf (Fig. 3b,d), V_{max05} (Ado→Ino) was zero and V_{max06} (dAdo→dIno)

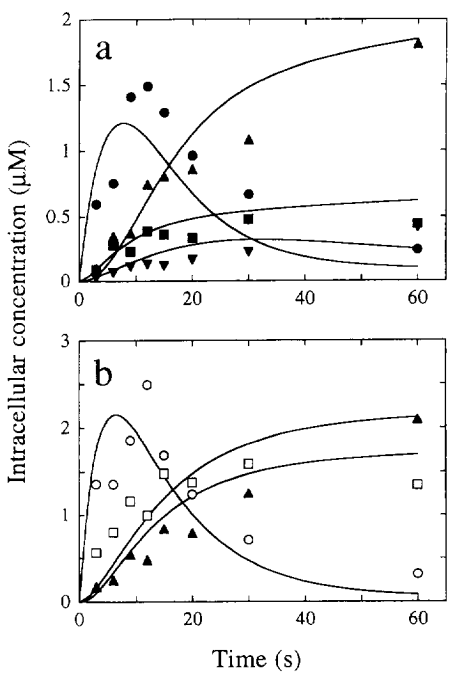


Fig. 2. Initial metabolism of Ado and dAdo by human erythrocytes. (a) + 50 μM [^{3}H]Ado; (b) + 50 μM [^{3}H]dAdo. Cell suspensions were incubated at 37°C, centrifuged through oil into perchloric acid at the indicated times and analysed by two-dimensional chromatography as described in the Experimental Procedures section. (●) Ado; (○) dAdo; (■) Ino; (□) dIno; (▲) Hyp; (▼) AMP; (△) IMP. The progress curves through the data points are simulations of the expected concentrations of the metabolites generated as described in Materials and Methods, using the following equations: (a) R01 = V_{max01} $[Ado]_i/(K_{m01} + [Ado]_i)$; R03 = $V_{max03}[AMP]/(K_{m03} + [AMP])$; R05 = V_{max05} $[Ado]_i/(K_{m05} + [Ado]_i)$; R07 = V_{max07} $[AMP]/(K_{m07} + [AMP])$; R09 = (V_{max09}/K_{m09}) [Ino]; R11 = V_{max11} $[Ino]/(K_{m11} + [Ino])$, R11b = (V_{max11b}/K_{m11b})[Hyp], with a normalization factor of 0.1 for V_{max01}, an assigned value of 0.135 for V_{max11b}/K_{m11b} and $V_{max07} = 0$; (b) R06 = V_{max06} $[dAdo]_i/(K_{m06} + [dAdo]_i)$; R12 = $V_{max12}[dIno]/(K_{m12} + [dIno])$; R12b = (V_{max12b}/K_{m12b}) [Hyp] with a normalization factor of 2.5 for V_{max06} and an assigned value of 80 for V_{max12b}/K_{m12b}.

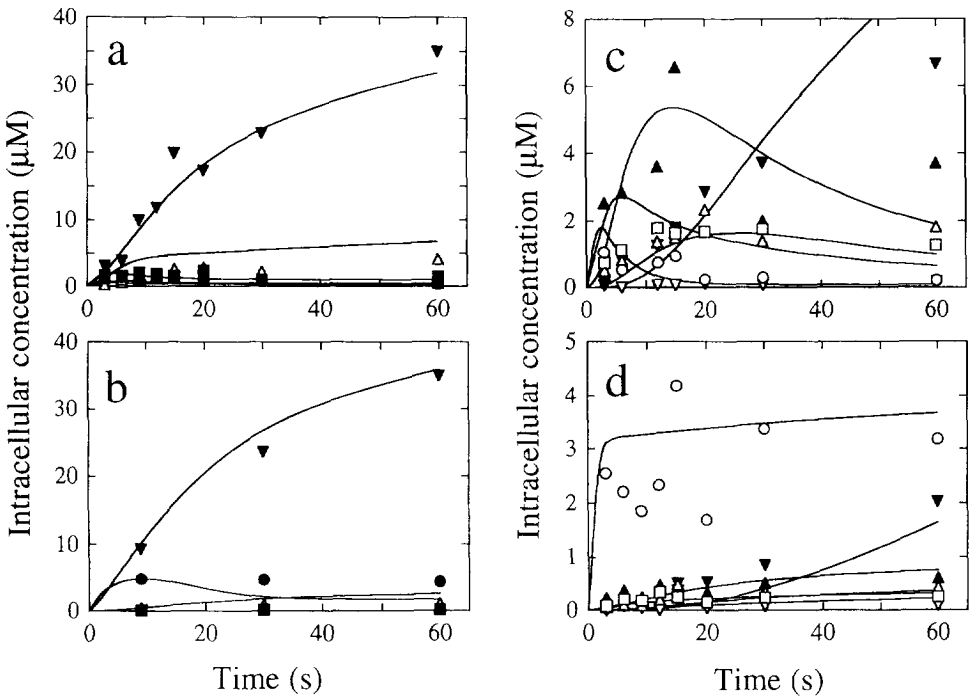


Fig. 3. Initial metabolism of Ado and dAdo by human CCRF-CEM leukemia cells. (a) + 50 μM [^{3}H]Ado; (b) + 50 μM [^{3}H]Ado + 5 μM dCf; (c) + 50 μM [^{3}H]dAdo; (d) + 50 μM [^{3}H]dAdo + 5 μM dCf. Cell suspensions were incubated at 37°C, centrifuged through oil into perchloric acid at the indicated times and analysed by two-dimensional chromatography as described in Experimental Procedures. (●) Ado; (○) dAdo; (■) Ino; (□) dIno; (▲) Hyp; (▼) AMP; (Δ) IMP; (∇) dAMP. The progress curves through the data points are simulations of the expected concentrations of the metabolites generated as described in Materials and Methods, using the following equations: (a) R01 = V_{max01} $[Ado]_i/(K_{m01} + [Ado]_i)$; R03 = V_{max03} $[AMP]/(K_{m03} + [AMP])$; R05 = V_{max05} $[Ado]_i/(K_{m05} + [Ado]_i)$; R07 = V_{max07} $[AMP]/(K_{m07} + [AMP])$; R09 = (V_{max09}/K_{m09}) [Ino]; R11 = V_{max11} $[Ino]/(K_{m11} + [Ino])$; R13 = V_{max13} $[Hyp]/(K_{m13} + [Hyp])$ with an assigned value of 0.27 for V_{max07b}/K_{m07b}; (b) R01 = V_{max01} $[Ado]_i/(K_{m01} + [Ado]_i)$; R03 = V_{max03} $[AMP]/(K_{m03} + [AMP])$; R05 = V_{max05} $[Ado]_i/(K_{m05} + [Ado]_i)$; R07 = V_{max07} $[AMP]/(K_{m07} + [AMP])$; R07b = $(V_{max07b}/K_{m07b})[AMP]$; R09 = (V_{max09}/K_{m09}) [Ino]; R11 = V_{max11} $[Ino]/(K_{m11} + [Ino])$; R13 = V_{max13} $[Hyp]/(K_{m13} + [Hyp])$ with a normalization factor of 0.5 for V_{max07} and an assigned value of 0.27 for V_{max07b}/K_{m07b} and $V_{max05} = 0$; (c) R02 = V_{max02} P $[dAdo]_i/(K_{m02} + P$ $[dAdo]_i)$; R04 = V_{max04} $[dAMP]/(K_{m04} + [dAMP])$; R06 = V_{max06} $[dAdo]_i/(K_{m06} + [dAdo]_i)$; R12 = V_{max12} $[dIno]/(K_{m12} + [dIno])$; R13 = V_{max13} $[Hyp]/(K_{m13} + [Hyp])$; R07b = (V_{max07b}/K_{m07b}) [IMP] with normalization factors of 20 for V_{max12}, 0.1 for V_{max13} and a partitioning factor (P) of 0.005 for dAdo; (d) R02 = V_{max02} P $[dAdo]_i/(K_{m02} + P$ $[dAdo]_i)$; R04 = V_{max04} $[dAMP]/(K_{m04} + [dAMP])$; R06 = V_{max06} $[dAdo]_i/(K_{m06} + [dAdo]_i)$; R12 = V_{max12} $[dIno]/(K_{m12} + [dIno])$; R13 = V_{max13} $[Hyp]/(K_{m13} + [Hyp])$; R07b = (V_{max07b}/K_{m07b}) [IMP] with normalization factors of 20 for V_{max12}, 0.1 for V_{max13}, 0.02 for V_{max06} and a partitioning factor (P) of 0.005 for dAdo.

Table 2. Enzymic activities of adenine nucleotide metabolism in particulate fractions

	Enzymic activity (pmol min^{-1} μg^{-1})	
	Erythrocytes	CCRF-CEM leukemia
AMP→Ado	0.0188	2.92
dAMP→dAdo	N.D.	3.20
Ado→Ino	2.02	3.08
dAdo→dIno	1.27	1.89
AMP→IMP	0.119	1.09
dAMP→dIMP	0.00335	N.D.
Ado→Ade	N.D.	N.D.
dAdo→Ade	N.D.	N.D.
Ino→IMP[a]	0.122	0.681
dIno→dIMP[a]	0.00251	N.D.

During preparation of the cell-free extracts, as described in Materials and Methods, the pelleted particulate fractions were washed twice in 10 ml of enzyme buffer, resuspended in 1 ml of buffer and tested for enzymic activities as for Table 1. N.D., not detectable.
The substrate concentration was 600 μM.