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Short communication

Rapid radioassay for metabolites of adenosine and deoxyadenosine in erythrocytes

Eve Szabados, Richard I. Christopherson*

Department of Biochemistry, University of Sydney, Sydney NSW 2006, Australia

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Abstract

A radioassay has been developed to quantify the uptake and initial metabolism of adenosine (Ado) or deoxyadenosine (dAdo) by human erythrocytes. Cell suspension and [^{3}H]Ado are mixed at 3-s intervals with a novel dual-syringe apparatus, and uptake and metabolism of Ado is stopped by centrifuging the cells through a dibutylphthalate layer into perchloric acid. The neutralized cell extract is analyzed by two-dimensional chromatography on poly(ethyleneimine)-cellulose plates by two procedures using combinations of solvents optimised for the separation of nucleosides and nucleobases, and for nucleotides derived from the exogenous [^{3}H]Ado.

1. Introduction

Intracellular Ado can either be phosphorylated to AMP or deaminated to inosine as shown in Fig. 1.

Wohlhueter et al. [1] designed a dual-syringe apparatus where two plastic syringes are held by adaptors and the syringe plungers are attached to a guide rod grooved evenly to ensure constant volumes at each delivery. Using this device, suspended cells were mixed with radiolabelled substrate and separated from the substrate within seconds by centrifuging the cells through oil. Radioactivity in the cell pellet was equated to total substrate uptake. To study the uptake and phosphorylation of radiolabelled Ado by erythrocytes, Plagemann et al. [2] used the same apparatus but centrifuged cells through Dow Corning 550 silicone fluid–light mineral oil (84:16, v/v, final density 1.034 g/ml) layer into trichloroacetic acid. The acid-soluble cell extract was analyzed by thin-layer chromatography to separate and quantify intracellular Ado, AMP, ADP and ATP. A number of HPLC procedures are available for quantification of nucleotides [3], nucleosides and nucleobases [4] using their UV absorbance, but such procedures lack the sensitivity of TLC with ^{3}H-labelled metabolites.

There is currently no sensitive TLC procedure which enables quantification of all cellular metabolites derived from Ado. Such metabolites could be separated by chromatography on poly(ethyleneimine)-cellulose plates where ionic solvents act as anion exchangers separating nucleotides according to their charge. At pH 8, the charge differences between the various nucleosides are small and their net charges reflect the phosphate content of the molecule [5]. R_F

* Corresponding author.

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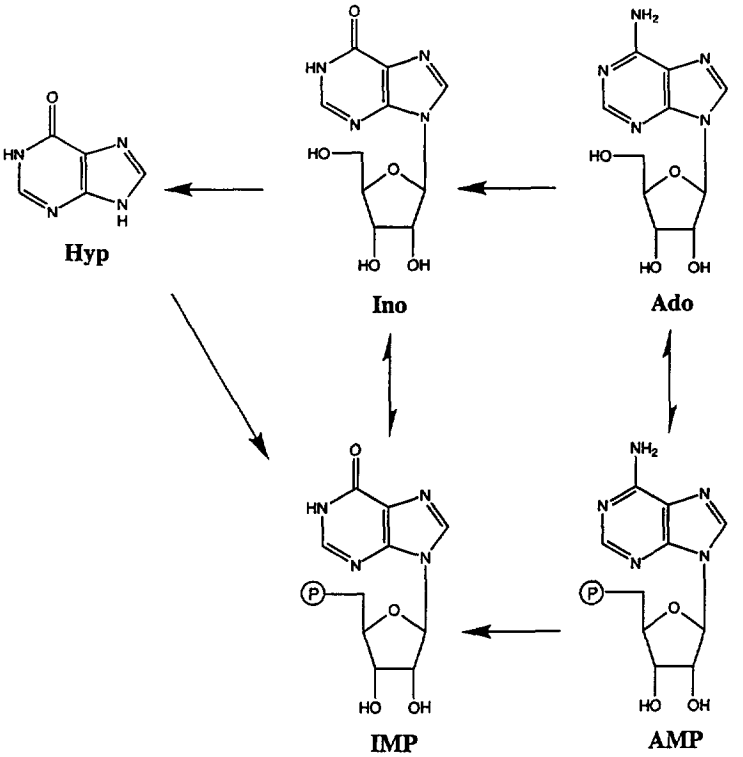

Fig. 1. Metabolism of Ado.

values therefore depend both on the salt concentration and pH of the solvent. For separation of nucleosides and nucleobases, Bochner and Ames [5] used 1.2 *M* ammonium sulfate (pH 3.5) which gave R_F values decreasing with the hydrophobicity of the molecule due to a salting-out effect. Ribo- and deoxyribonucleosides or nucleotides are resolved with solvents containing borate [6] which forms complexes with *cis*-glycols (ribonucleosides or ribonucleotides) adding a negative charge to these compounds and retarding their migration. We have developed a radioassay for quantification of metabolites of Ado or dAdo in cell extracts using a novel dual-syringe apparatus and unique two-dimensional chromatographic separations. This procedure has been used for detailed analysis of the initial metabolism of exogenous Ado by human erythrocytes.

2. Experimental

2.1. Materials

[2-^{3}H]Ado (26,000 Ci/mol, 38.5 μM) was purchased from Amersham International (Amersham, UK), poly(ethyleneimine)-cellulose thin-layer chromatography plates were from Macherey-Nagel (Duren, Germany). Hanks' balanced salt solution and dibutylphthalate were supplied by Sigma (St. Louis, MO, USA). All other reagents were of the highest analytical grade available.

2.2. Assembly of dual-syringe apparatus

The following components were purchased from the Hamilton Company (Reno, NV, USA). A 1.0-ml fixed-needle syringe (Model 1001, No.

81317) was fitted with a repeating dispenser with a 50-tooth rack (Model PB600-1, No. 83700), and a 10.0-ml fixed-needle syringe (Model 1010, No. 81617) was fitted with a repeating dispenser (Model PB600-10, No. 83701). The two syringes were then bolted together (Fig. 2) by aligning the two repeating dispensers back-to-back with the push buttons directly opposite so they could be squeezed rapidly, simultaneously and repetitively (up to 50 times). Each of 50 presses of the two repeating dispensers delivered 200 μl of cell suspension (erythrocytes) and 20 μl of nucleoside ([^{3}H]Ado) which were mixed in a Y-shaped tubing connected to the syringe needles.

2.3. Short-term incubation of cell suspensions with [^{3}H]Ado

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 $4.6 \cdot 10^9$ cells/ml in Hanks' balanced salts solution. Dibutylphthalate (700 μl, density 1.04 g/ml) was layered onto 200 μl of 0.8 *M* $HClO_4$ with 25% (v/v) glycerol (required to maintain the acid as the lowest layer) in 1.5-ml Eppendorf tubes (without lids) in the rotor of a micro-centrifuge. At 3-s intervals, 200-μl aliquots of cell suspension at 37°C were mixed with [^{3}H]Ado (20 μl, 550 μ*M*, 500 Ci/mol) and layered onto the dibutylphthalate layer of sequential Eppendorf tubes. The dual-syringe apparatus was used to deliver 200 μl cell suspension and 20 μl [^{3}H]Ado simultaneously with mixing at each step (Fig. 2). The longest time-sample was dispensed first and the centrifuge was started (8500 *g*, 1 min) 1 s after delivery of the last mixture of cells plus [^{3}H]Ado, giving a total elapsed time of 3 s for the final sample. All the cells sedimented into the perchloric acid layer in 2 s. The acid-soluble extract was removed and neutralized by vortex-mixing for 1 min with an equal volume of 0.5 *M* trioctylamine in 1,1,2-trichlorotrifluoroethane [7]. The two phases were separated by centrifugation (9000 *g*, 5 min) and the upper aqueous layer was removed and stored at −20°C for subsequent analysis by TLC.

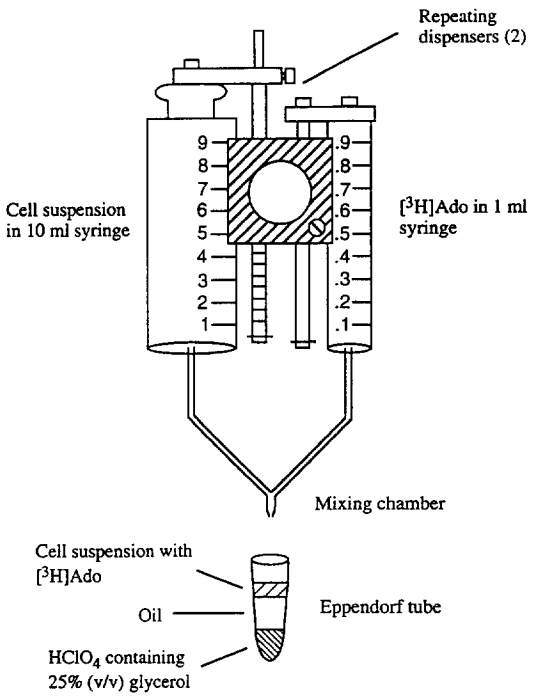

Fig. 2. Dual-syringe apparatus for rapid aliquotting and mixing of cell suspension (200 μl) and [^{3}H]Ado (20 μl).

2.4. Thin-layer chromatography of ^{3}H-labelled nucleosides and hypoxanthine

A 20-μl sample of the neutralized cell extract was applied in 5-μl aliquots, drying at room temperature between applications, to a poly(ethyleneimine)-cellulose plate (10 × 10 cm). The glycerol was removed by immersion of the plate in anhydrous 2-propanol containing 1.2 g Tris base/l for 10 min. The plate was allowed to dry before development of the first dimension at room temperature with 200 m*M* LiCl saturated with H_3BO_3 (pH 3.5)–ethanol, (1:1, v/v) (modification of Ref. [8]). After drying and washing in 2-propanol–Tris, the chromatogram was developed at right-angles with 1.2 *M* ammonium sulfate (pH 3.5; [5]). Washing chromatograms twice with 2-propanol–Tris removed 13% of Hyp

(hypoxanthine), 4% of dIno (deoxyinosine) and 3% of Ino (inosine); Ado and dAdo were unaffected.

2.5. Thin-layer chromatography of ^{3}H-labelled nucleotides

The glycerol in the neutralized extracts was removed with 2-propanol–Tris as described above. Cell extracts (20 μl) were analyzed on poly(ethyleneimine)-cellulose plates (20 × 20 cm) at 4°C 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 12 cm (modification of Ref. [9]). The chromatograms were desalted and neutralized by immersion in 2-propanol–Tris 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 12 cm (modification of Ref. [9]).

The positions of purine derivatives after two-dimensional chromatography were determined by visualization of appropriate marker compounds (Ado, dAdo, Ino, dIno, Hyp or ATP, dATP, ADP, dADP, AMP, dAMP, IMP; 5 nmol) under ultraviolet light at 254 nm (Fig. 3). Marker compounds were mixed with ^{3}H-labelled cell extracts prior to chromatography, the separated spots were excised and counted in 5 ml of scintillation cocktail (3.0 g 2,5-diphenyloxazole/l toluene) using a Pharmacia Wallac 1410 liquid scintillation counter with an efficiency of 18.3% for ^{3}H. The dpm values for purine metabolites were converted to intracellular concentrations using a cellular volume of 0.090 pl for erythrocytes [10].

3. Results and discussion

The initial uptake of [^{3}H]Ado by erythrocytes is faster than its subsequent metabolism (Fig. 4). Initially, the intracellular concentration of [^{3}H]Ado is higher than the concentration of its metabolites. Intracellular [^{3}H]Ado is deaminated via Ado → Ino → Hyp (Fig. 4a) or phosphorylated via Ado → AMP → ADP → ATP (Fig. 4b). The novel dual-syringe apparatus used was constructed from two Hamilton syringes attached to repeating dispensers fastened together (Fig. 2). This apparatus is easily constructed from commercially available components. Dibutylphthalate (1.04 g/ml) was used in preference to the Dow Corning 550 silicone fluid–light mineral oil (84:16, v/v, 1.03 g/ml, [1]) as the oil phase. We found that oil mixtures with different densities are suitable for different cell types; dibutylphthalate–mineral oil (86:14, v/v, 1.01 g/ml) is suitable for human CCRF-CEM

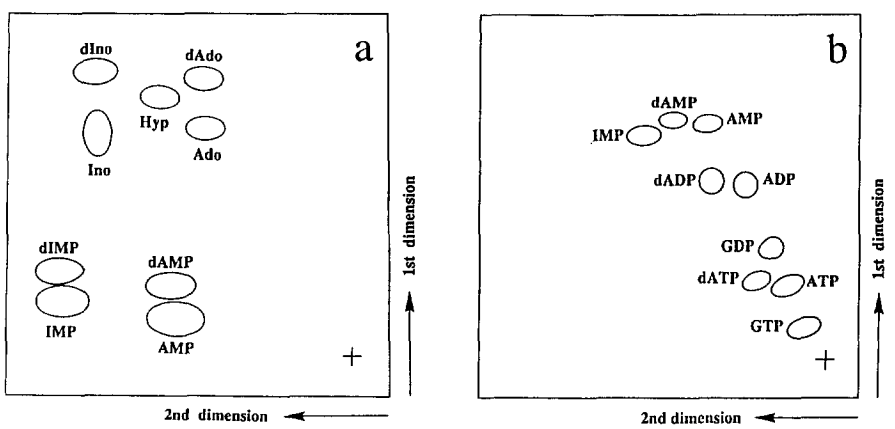

Fig. 3. Two-dimensional chromatography of metabolites derived from Ado or dAdo on poly(ethyleneimine)-cellulose. (a) Separation of nucleosides and hypoxanthine; (b) separation of nucleotides. Further details appear in Section 2.

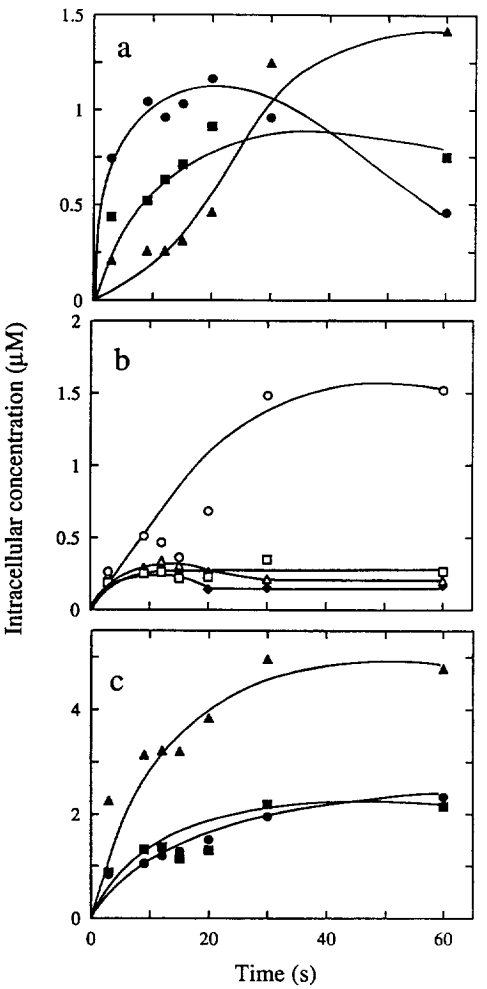

Fig. 4. Initial metabolism of [^{3}H]Ado by human erythrocytes. (a) ●, Ado; ■, Ino; ▲, Hyp; (b) ○, ATP; □, ADP; △, AMP; ◆, IMP; (c) ●, Ino + Hyp + IMP; ■, ATP + ADP + AMP; ▲, Ino + Hyp + IMP + ATP + ADP + AMP. Cell suspensions were incubated at 37°C, centrifuged through oil into perchloric acid at the indicated times, and analyzed by two-dimensional thin-layer chromatography as described in Section 2.

leukemia cells. Anhydrous methanol, routinely used in the separation of nucleotides for desalting chromatograms between the first and second dimensions, was found to remove nucleosides and bases from the plates. Anhydrous 2-propanol containing 1.2 g Tris base/l was used to remove the glycerol from the cell extracts after application of samples to plates and to desalt the chromatograms between development of the first and second dimensions. The two-dimensional thin-layer chromatographic procedures (Fig. 3) which separate all the purine nucleotides, nucleosides and Hyp derived from Ado or dAdo, utilise unique combinations of developing solvents which have been optimised for these separations. These solvents could be modified for analysis of short-term metabolism of other purine nucleosides by many cell types. This procedure for rapid sampling and two-dimensional chromatographic analysis has also been used to study the uptake and short-term metabolism of Ado and dAdo by human CCRF-CEM leukemia cells. It could be used as a diagnostic test for Ado deaminase deficiency using erythrocytes or lymphocytes from immunodeficient patients. The absence of Ado deaminase results in severe combined immunodeficiency because the only route for dAdo deamination (dAdo → dIno → Hyp) is blocked. The dAdo accumulated is converted to adenine deoxyribonucleotides (dAdo → dAMP → dADP → dATP) with dATP accumulating to millimolar concentrations in cells. The procedures described here for analysis of the initial metabolism of dAdo would provide a rapid assessment of a patient's capability to deaminate dAdo in intact blood cells.

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