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Inward fluxes of adenosine in erythrocytes and cultured cells measured by a quenched-flow method

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Dilazep, a vasodilator previously recognized as an inhibitor of adenosine permeation, very rapidly blocked the uptake of adenosine by cultured L5178Y cells, and accordingly was used as a quencher in a simple quenched-flow system for measuring cellular uptake of nucleosides during very short intervals. Time courses of cellular uptake of adenosine, assayed during intervals between 0.05 and 0.5 s with the quenched-flow system, were linear and defined initial rates of adenosine uptake. The latter are rates of inward transport of adenosine. Kinetic constants for that process in cultured S49 cells determined with the quenched-flow procedure were similar to those determined with an assay dependent on manual timing. In studies of adenosine uptake kinetics in human erythrocytes at 22°C and 37°C in which the quenched-flow procedure was used, time courses of adenosine uptake were linear at both temperatures and defined initial uptake rates; kinetic constants (means ± S.E.M.) at 22°C ($n = 8$) were K_m 25 ± 14 μM and $V_{max.}$ 15 ± 5 pmol/s per μl of cell water and at 37°C ($n = 3$) were K_m 98 ± 17 μM and $V_{max.}$ 80 ± 9 pmol/s per μl of cell water.

In animal cells, nucleoside-specific transport elements of the plasma membrane mediate the entry and exit of nucleoside molecules [for reviews, see Plagemann & Wohlhueter (1980) and Young & Jarvis (1983)]. In cells of several types under conditions in which intracellular metabolism of nucleoside permeants is absent or suppressed, nucleoside transport is a reversible 'facilitated diffusion' process and nucleoside transporters are seen to have low specificity with respect to the base portion of the nucleosides accepted as substrates (Cass & Paterson, 1972; Plagemann *et al.*, 1978).

Several pentofuranosides of S^6-substituted 6-thiopurines and of N^6-substituted adenine derivatives are potent inhibitors of nucleoside transport (Paterson *et al.*, 1983*a*,*b*). Of these, the most extensively studied is NBMPR. This inhibitor binds tightly (K_D 0.1–1 nM), but reversibly, to plasma-membrane sites that are part of, or are associated with, the nucleoside transport mechanism. NBMPR occupancy of high-affinity sites in human erythrocytes correlates with loss of transporter function (Cass *et al.*, 1974). Several vaso-active compounds (dilazep, hexobendine, dipyridamole) are also potent inhibitors of nucleoside transport and of NBMPR binding (Pohl & Brock, 1974; Kolassa *et al.*, 1978; Lum *et al.*, 1979; Paterson *et al.*, 1980, 1983*a*,*b*). Inhibitors of nucleoside transport (NBMPR, hydroxynitrobenzylthioguanosine and dipyridamole) have been used in kinetic studies of nucleoside permeation as stopping reagents to end intervals of nucleoside uptake by cells (Cass & Paterson, 1972; Cabantchik & Ginsburg, 1977; Bowen *et al.*, 1979; Harley *et al.*, 1982). Nucleoside transport mechanisms of two types have been recognized, distinguishable by their sensitivities to NBMPR (Belt, 1983; Paterson *et al.*, 1983*a*; Plagemann & Wohlhueter, 1984).

Current approaches to the measurement of nucleoside influx in permeant-metabolizing cells depend on the concept that the initial rate of cellular uptake is intrinsically the rate of inward transport of the permeant (Harley *et al.*, 1982). Time courses for cellular accumulation of nucleoside permeants, which are frequently non-linear, are used to determine initial uptake rates. Because of the rapidity of nucleoside transport, this approach has required that time courses of nucleoside uptake must be definitive during the first few seconds after flux initiation. This point is

Abbreviations used: NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine); Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

illustrated by the non-linear time courses reported by Lum *et al.* (1979) and Harley *et al.* (1982) for the uptake of adenosine by cultured cells and by Goldman *et al.* (1981) for the uptake of fluorodeoxyuridine by Ehrlich ascites-carcinoma cells.

Conventional methods of ending intervals of cell exposure to labelled permeant include (*a*) rinsing with cold medium (Heichal *et al.*, 1979), (*b*) centrifugal pelleting of cells under oil (Wohlhueter *et al.*, 1978), (*c*) dilution of isotopically labelled permeant with non-labelled permeant, followed by pelleting under oil (Strauss *et al.*, 1980), and (*d*) rapid exposure of cells to inhibitors of nucleoside transport, followed by pelleting under oil (Harley *et al.*, 1982). At their technical limits, some of these methods may yield intervals of permeant exposure as brief as 1 or 2 s. In the present paper, we describe a simple quenched-flow system that permitted uptake of [^{3}H]adenosine by suspended cells during intervals of 0.05–0.5 s. This method enabled determination of kinetic constants for adenosine influx at 37°C. Some of the data presented here have been reported at symposia (Paterson *et al.*, 1983*a*,*b*)

Materials and methods

Erythrocytes and cultured cells

Erythrocytes from heparinized blood samples were washed three times with phosphate-buffered saline (Dulbecco & Vogt, 1954), and the buffy coats were discarded.

S49 and L5178Y mouse lymphoma cells were propagated in Fischer's medium supplemented with 10% horse serum, 2 mM-Hepes (pH 7.4) and antibiotics (penicillin G_1, 100 units/ml; streptomycin, 100 μg/ml). Cultures were re-started from frozen stocks at 2-month intervals. For experiments, cultures were expanded to 800 ml in roller bottles rotated at 1.5 rev./min. Cell concentrations were kept below 6×10^5 cells/ml to ensure exponential proliferation.

Nucleoside uptake

In assays of adenosine uptake, cells were suspended in 'transport medium', which consisted of $NaHCO_3$-free Fischer's medium with 20 mM-Hepes (pH 7.4, 20°C). To obtain time courses of adenosine uptake, cells in replicate incubation mixtures (either in centrifuge tubes or in the flow system) were permitted brief graded intervals of [^{3}H]adenosine uptake that were ended by dilazep blockade of nucleoside transport.

Assays with manual timing used 1.5-ml plastic centrifuge tubes in which 100 μl of transport medium containing [^{3}H]adenosine (2–3 μCi) was layered over 100 μl of an oil mixture (Harley *et al.*, 1982). Intervals of permeant uptake were begun by adding 100 μl of cell suspension (2×10^6–5×10^6 cells in transport medium), and ended (after metronome-timed intervals) by addition of 200 μl of transport medium containing 0.7–1.3 mM-dilazep. Immediately after the addition of the stopping reagents, assay mixtures were centrifuged (16000 g, 30 s) to pellet cells under the oil. Zero-time values for cell-associated ^{3}H were determined by (i) adding cells to medium containing both [^{3}H]adenosine and stopping reagent, and (ii) immediately pelleting cells under oil.

In the quenched-flow system, intervals of permeant uptake were initiated by mixing a stream (Fig. 3*a*) of cell suspension (2×10^7–3×10^7 cultured cells/ml or 1×10^9–2×10^9 erythrocytes/ml) with a stream of medium containing [^{3}H]adenosine (8–30 μCi/ml). The mixed streams flowed at fixed velocity in reaction lines (the lengths of which determined exposure intervals) before mixing with a stream of the quenching reagent, 1.0 mM-dilazep. The quenched stream was sampled after discarding the first 1 ml of effluent to obtain cells (pelleted under oil) for assay of ^{3}H content.

To determine the ^{3}H content of cell pellets, supernatant fractions were removed by suction and the centrifuge tubes were rinsed above the oil layer with water, which, together with most of the oil, was removed by suction. Cultured cell pellets were dissolved in 0.2 ml of 1% Triton X-100, the centrifuge tubes were placed in counting vials and the tube contents were mixed with 8 ml of scintillant (Pande, 1976) for ^{3}H assay.

Erythrocyte pellets in their centrifuge tubes were dissolved in 0.4 ml of 1% Triton X-100 and, 15 min after mixture of tube contents with 0.75 ml of 5% (w/v) trichloroacetic acid, the tubes were centrifuged (16000 g, 2 min). The acid extracts were sampled for assay of ^{3}H content.

Data analysis

Water space and extracellular space of cell pellets were determined with ^3H_2O and [U-^{14}C]-sucrose in manually timed assays of adenosine uptake. Cellular content of permeant is reported as [Internal]/[External], that is, as the ratio of the concentration of adenosine and its metabolites (based on ^{3}H content) in cell water to that in the extracellular medium. Time courses reported here begin with values of this ratio in which the numerator is the zero-time concentration of cell-associated [^{3}H]adenosine determined by exposing cells to medium containing both [^{3}H]adenosine and stopping reagent. Zero-time values of the ratio were independent of permeant concentration and were 0.03–0.05, and, in one case, 0.10.

To extract initial rates from time courses obtained with manual timing, parabolas were fitted to the data and initial rates were obtained from the coefficients of the first-order term

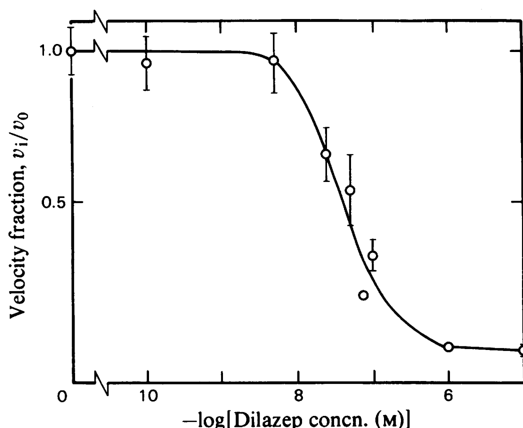

Fig. 1. *Inhibition of adenosine permeation in L5178Y cells by dilazep*

Replicate mixtures containing 4×10^7 cells in 1.0ml of transport medium with the specified concentrations of dilazep were incubated for 10min at 22°C. Cells were then assayed for their ability to take up [2-^{3}H]adenosine (final concn. 15μM) from their own dilazep-containing incubation medium during 3s intervals at 22°C. Assay mixtures in triplicate were assembled as in the manually timed assays (see the Materials and methods section), and the interval of uptake was started by the addition of dilazep-treated cells to an equal volume of 30μM-[2-^{3}H]-adenosine in dilazep-containing incubation medium (reserved from the preceding step). Then, 3s later, to end adenosine uptake, an equal volume of 1mM-dilazep in transport medium was added and the cells were at once pelleted under oil. Cell pellets were assayed for their ^{3}H content as described in the Materials and methods section. The rate of adenosine uptake ($\pm$S.D.) in the absence of dilazep was 7.8 ± 0.6pmol/s per μl of cell water. Means $\pm$ S.D. are plotted. The velocity fraction, v_i/v_0, represents the ratio of rates in the presence (v_i) and the absence (v_0) of dilazep.

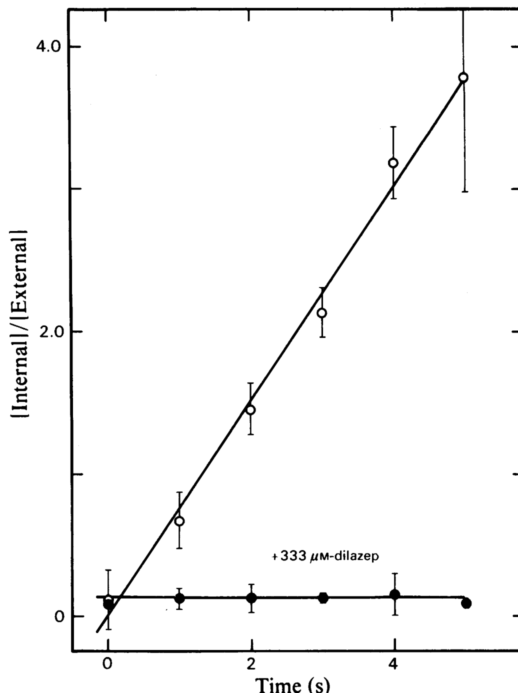

Fig. 2. *'Instantaneous' inhibition of adenosine permeation by dilazep*

L5178Y cells in replicate assay mixtures were exposed to 0.1μM-[^{3}H]adenosine for the indicated intervals in the absence (○) or presence (●) of 333μM-dilazep. Assays of cellular uptake of [^{3}H]-adenosine were conducted with timing by metronome signals as described in the Materials and methods section. Uptake intervals were initiated by addition of cell suspension and were ended by addition of 0.7mM-dilazep. The ordinate parameter, [Internal]/[External], is the ratio of the concentration of adenosine and its metabolites (based on ^{3}H content) in cell water to that in the medium; the zero-time value of this parameter is defined in the Materials and methods section. Means of triplicate assays $\pm$ S.D. are plotted.

(Harley *et al.*, 1982). Time courses obtained by the flow method were virtually linear, and slopes were obtained by fitting straight lines to the data.

From data relating transport rates and adenosine concentrations, kinetic constants were calculated by using an iterative weighted least-squares algorithm (Cleland, 1967) for fitting data to a non-linear form of the Michaelis–Menten equation.

Chemicals

[2-^{3}H]Adenosine (18Ci/mmol) and [G-^{3}H]tubercidin (20Ci/mmol) were purchased from Moravek Biochemicals, Brea, CA, U.S.A., and ICN Pharmaceuticals, Irvine, CA, U.S.A., supplied 2′-deoxy[8-^{3}H]adenosine (13Ci/mmol). After storage, these radiochemicals were re-purified by liquid chromatography using a C_{18} μBondapak column (Waters) eluted with methanol/water solutions. [U-^{14}C]Sucrose (584Ci/mol) and ^3H_2O were purchased, respectively, from Amersham, Oakville, Ont., Canada, and New England Nuclear (Canada), Montreal, Que., Canada. Dilazep [3,4,5-trimethoxybenzoic diester with tetrahydro-1*H*-1,4-diazepine-1,4(5*H*)-dipropanol] was a gift from F. Hoffmann–LaRoche and Co., Basle, Switzerland. Tubercidin (7-deaza-adenosine) was provided by the Upjohn Co., Kalamazoo, MI, U.S.A., through the courtesy of Dr. G. L. Neil.

Results

Dilazep, an inhibitor of adenosine permeation (Pohl & Brock, 1974), is the dihydrochloride salt of an *NN′*-bis-substituted derivative of homopiper-

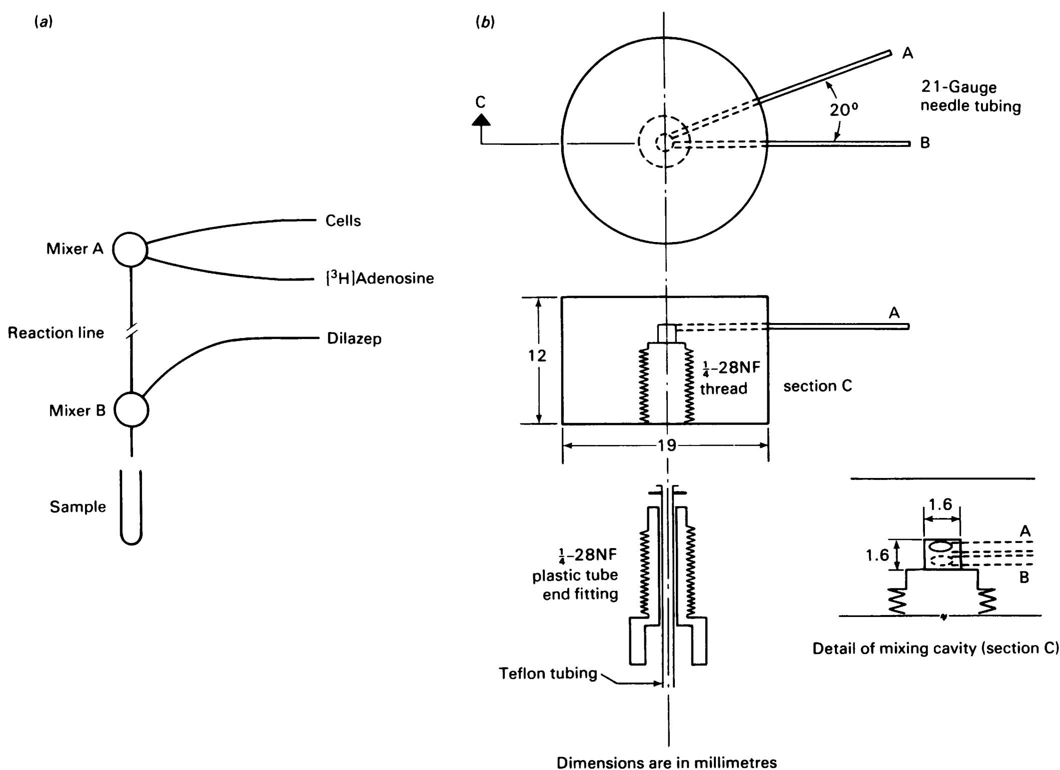

Fig. 3. *Quenched-flow system to permit brief fixed intervals of adenosine uptake by suspended cells*
Part (*a*): streams of transport medium containing cells (2×10^7–4×10^7/ml), [^{3}H]adenosine or 1 mM-dilazep were conducted in plastic tubing (0.8 mm internal diam.) from 10 ml Hamilton syringes operated simultaneously by a Harvard model 940 syringe pump set at top speed. Mixer A combined streams of cells and [^{3}H]adenosine to begin intervals of permeant uptake, and the resulting cell–permeant stream was combined in Mixer B with the dilazep stream to end the uptake intervals. The flow rate through the reaction line was 21.6 ml/min. Intervals of permeant uptake were calculated from the flow rate and the volume of the system in which permeant uptake occurred (the sum of the mixer half-volumes plus the volume of the reaction line). Reaction lines were of such lengths that uptake intervals between 0.05 and 0.5 s were obtained; uptake intervals were changed by using reaction lines of different lengths. Part (*b*): mixers A and B were identical. Mixer bodies, constructed of Perspex plastic [poly(methyl methacrylate], had the dimensions shown above. In the mixers, jets (21-gauge needle tubing) directed the two reactant streams tangentially to opposite sides of a cylindrical mixing cavity (volume about 3 μl). The flanged end of the reaction line, held in place by a threaded plastic fitting, formed the floor of the mixing cavity. The Teflon tubing was 1.5 mm external diam., 0.8 mm internal diam.

azine and, accordingly, is highly soluble in water. For these reasons, dilazep was tested as a quencher in the development of a quenched-flow method for the assay of adenosine transport. The experiment of Fig. 1 demonstrated that dilazep is a potent inhibitor of adenosine uptake by cultured L5178Y mouse lymphoma cells. The cellular uptake of adenosine during the 3 s assay intervals was decreased by 50% by dilazep concentrations of about 40 nM.

Fig. 2 shows that the time course of adenosine uptake by the cultured cells intersected virtually at zero time with that of cells exposed simultaneously to adenosine and dilazep, indicating that dilazep blocked adenosine permeation almost instantaneously. An 'instantaneous' blockade of adenosine permeation in HeLa cells may be similarly achieved with NBMPR (Harley *et al.*, 1982).

In an experiment similar to that of Fig. 2 (results not shown), the addition of dilazep (final concn. 25 μM) blocked the uptake by cultured S49 mouse lymphoma cells of 0.3 μM-[^{3}H]tubercidin. The blockade was complete, and the intersection of the time courses had an abscissa value ($\pm$S.E.M.) of -0.03 ± 0.02 s.

Because blockade of adenosine permeation by dilazep was rapid and complete, 1 mM-dilazep was employed as a quenching reagent in a simple quenched-flow system (Fig. 3) intended to permit cellular uptake of adenosine during intervals of less than 1 s. In this system, rapid streams of cell sus-

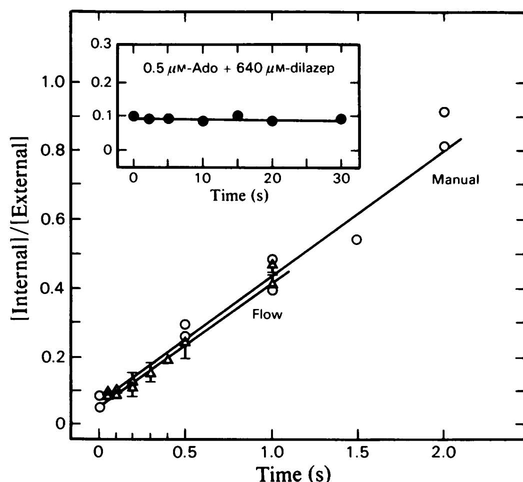

Fig. 4. *Assays of adenosine uptake by cultured S49 mouse lymphoma cells conducted manually and by a quenched-flow method*

The indicated intervals of adenosine uptake were begun by mixing cells in suspension with 0.5 μM-[^{3}H]adenosine in transport medium and ended by quenching transport with 1.3 mM-dilazep in transport medium. Assay components were combined manually with metronome timing or by the quenched-flow procedure. Cells from the same batch were used for both procedures, which were conducted at 22°C. The ordinate parameter, [Internal]/[External], is the ratio of intracellular and extracellular concentrations of ^{3}H (in adenosine equivalents) derived from [^{3}H]adenosine and is described more fully in the Materials and methods section. Data obtained with the flow system (△) are means ± S.D. calculated from triplicate determinations of cellular ^{3}H content from dilazep-quenched samples. The manually obtained data (○) represent single assays. The inset illustrates the cellular uptake (manual assay) of adenosine (Ado) in the presence of 640 μM-dilazep.

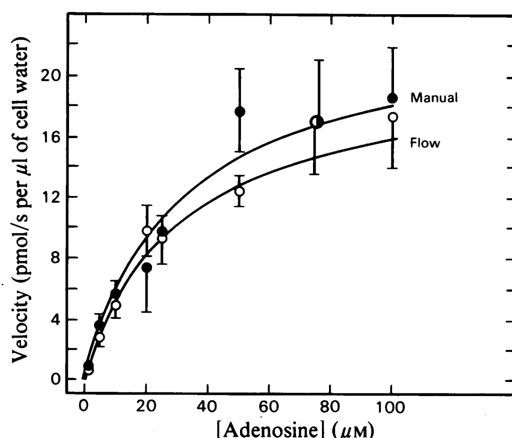

Fig. 5. *Adenosine transport in cultured L5178Y cells*

Time courses of cellular uptake of adenosine (at the indicated concentrations) were determined by the manual and flow methods with dilazep quenching in both, as in Fig. 4. Initial rates of adenosine uptake (defined as transport rates) were calculated from these time courses; plotted are means (±S.D.) from four experiments in which the two uptake assays were compared. By fitting the Michaelis–Menten equation to the rate data, the following kinetic constants (±S.E.M.) were obtained.

Assay method	$V_{max.}$ (μM)	K_m (pmol/s per μl of cell water)
Manual	30 ± 6	23.0 ± 3.0
Flow	29 ± 4	20.5 ± 1.4

pension and of [^{3}H]adenosine-containing medium meet in a mixing chamber to start uptake, which proceeds as the cell-permeant mixture flows through a reaction line to a second mixing chamber, in which it is mixed with a stream of quencher (1 mM-dilazep in transport medium) to end transporter activity. The interval of adenosine uptake is determined by the flow velocity and the volume of the reaction line. Cells from samples of the quenched stream are immediately pelleted under oil for analysis of ^{3}H content.

Crucial parts of the quenched-flow system are the two identical mixers detailed in Fig. 3(*b*). Jets direct the two reactant streams tangentially into a cylindrical mixing cavity, imparting opposed rotational motions to the streams, as in previously described mixers (Gibson & Milnes, 1964; Thayer & Hinckle, 1975). When solutions of Bromcresol Purple and HCl were mixed in this system operating with a flow velocity of 21.6 ml/min in the reaction line, formation of the yellow colour, which indicated mixing, took place within the mixing cavity. At lower flow velocities, mixing was not complete within the cavity. We have assumed that the time required for mixing in this system could be neglected in determining uptake intervals because (*a*) mixing times were calculated to be in the order of 10 ms and (*b*) deviations from instant mixing in the starting and stopping mixers would tend to be compensatory, although the velocity of flow through the latter is greater than through the former.

In the experiment of Fig. 4, assays of adenosine uptake by S49 cells by the quenched-flow procedure, or by a conventional method with manually timed addition of dilazep as a stopper [as in previous experiments that used NBMPR quenching (Harley *et al.*, 1982)], yielded similar time courses of adenosine uptake.

Fig. 5 summarizes several experiments that compared kinetic constants for adenosine transport in L5178Y cells obtained with manual and flow assays. Transport rates saturated as the external adenosine concentration was increased and the two assay methods yielded similar kinetic

Table 1. *Kinetic constants for inward transport of adenosine and related compounds in quenched-flow studies*
The kinetic constants listed for Expt. 1 were derived from the data of Fig. 6. Transport rates were obtained from time courses of cellular uptake of [^{3}H]adenosine at 22°C as in Figs. 5 and 6 (procedure A) or as in Fig. 2 (procedure B), and kinetic constants (±S.E.M.) were extracted from rate–concentration data as in Figs. 5 and 6.

Experiment	Cells	Procedure	K_m (μM)	$V_{max.}$ (pmol/s per μl of cell water)	Competitive inhibition K_i (μM)
1. [^{3}H]Adenosine influx with or without 50 μM-tubercidin as competing inhibitor	L5178Y	A	15±2	15±2	27±3
2. [^{3}H]Adenosine influx with or without 2′-deoxyadenosine (10, 20, 50, 100 μM) as competing inhibitor	Human erythrocytes	B	23±1	13±1	31±2
3. [^{3}H]2′-Deoxyadenosine influx	L5178Y	A	27±4	17±2	

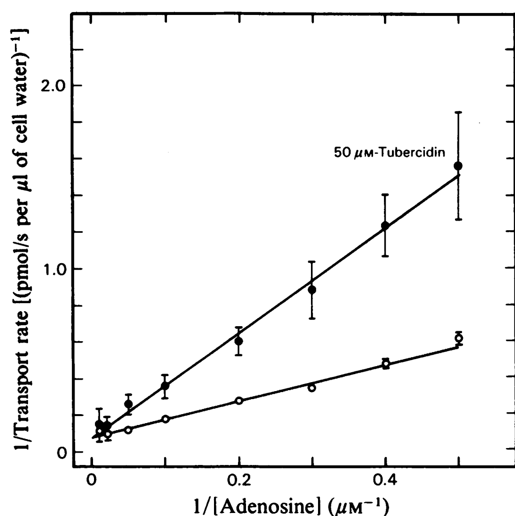

Fig. 6. *Competitive inhibition by tubercidin of adenosine transport by cultured L5178Y cells*
Initial rates of adenosine uptake (transport rates) by L5178Y cells were determined as in Fig. 5 by the quenched-flow procedure. Assays were conducted in transport medium containing [^{3}H]adenosine at the concentrations indicated without (○) and with (●) 50 μM-tubercidin. The lines shown were obtained by fitting a reciprocal form of the Michaelis–Menten equation to the rate data, and the kinetic constants (±S.E.M.) so obtained are listed in Table 1. Error bars represent S.D.

constants. The constants reported in Fig. 5 are similar to those obtained previously for these and other cells (Paterson *et al.*, 1981; Harley *et al.*, 1982) with manual assays that employed NBMPR as a stopping reagent, and are similar to values reported by Chello *et al.* (1983) for adenosine transport in L1210 cells.

Table 1 reports kinetic constants for the inward transport of adenosine in cultured L5178Y cells and erythrocytes and for the inhibition of these processes by tubercidin and 2′-deoxyadenosine, respectively, determined with the quenched-flow procedure. In these experiments, transport rates were determined as initial rates of uptake measured from time courses. Expt. 1 (Table 1 and Fig. 6) showed that tubercidin is a competitive inhibitor [K_i 27±3 μM (S.E.M.)] of adenosine influx, as previously reported (Harley *et al.*, 1982). Rate–concentration data from this experiment are shown in Fig. 6. In a similar study (Expt. 2, Table 1), 2′-deoxyadenosine competitively inhibited [K_i 31±2 μM (S.E.M.)] the inward transport of adenosine in human erythrocytes. Table 1 (Expt. 3) reports kinetic constants for the inward transport of 2′-deoxyadenosine by L5178Y cells. Thus the affinities of tubercidin, 2′-deoxyadenosine and adenosine for the nucleoside transport mechanism are similar in L5178Y cells. The kinetic constants reported here for adenosine permeation in L5178Y cells are in good agreement with those obtained with our previously described procedure for measuring nucleoside uptake rates (Harley *et al.*, 1982).

Because the time courses of cellular adenosine uptake during intervals of 0–0.5 s appeared to be linear, and to define initial rates of uptake, a simplified procedure was used to measure the latter in human erythrocytes. Fig. 7 shows that (*a*) time courses for adenosine uptake by erythrocytes during intervals of 0.05, 0.3 and 0.5 s were linear, and (*b*) those time courses at various adenosine concentrations had a common origin on the ordinate at zero time. This result indicated that the slopes of these lines were a direct measure of transport rates which, as Fig. 7 shows, saturated as the adenosine concentration was increased. The very high rates of adenosine transport found in erythrocytes at 37°C (5 times higher than at 22°C) probably reflect transport rates *in vivo*. Thus the quenched-flow method appears to be adequate for measurement of the high rates of adenosine uptake that occur in cells at 37°C. Mean values (±S.E.M.)

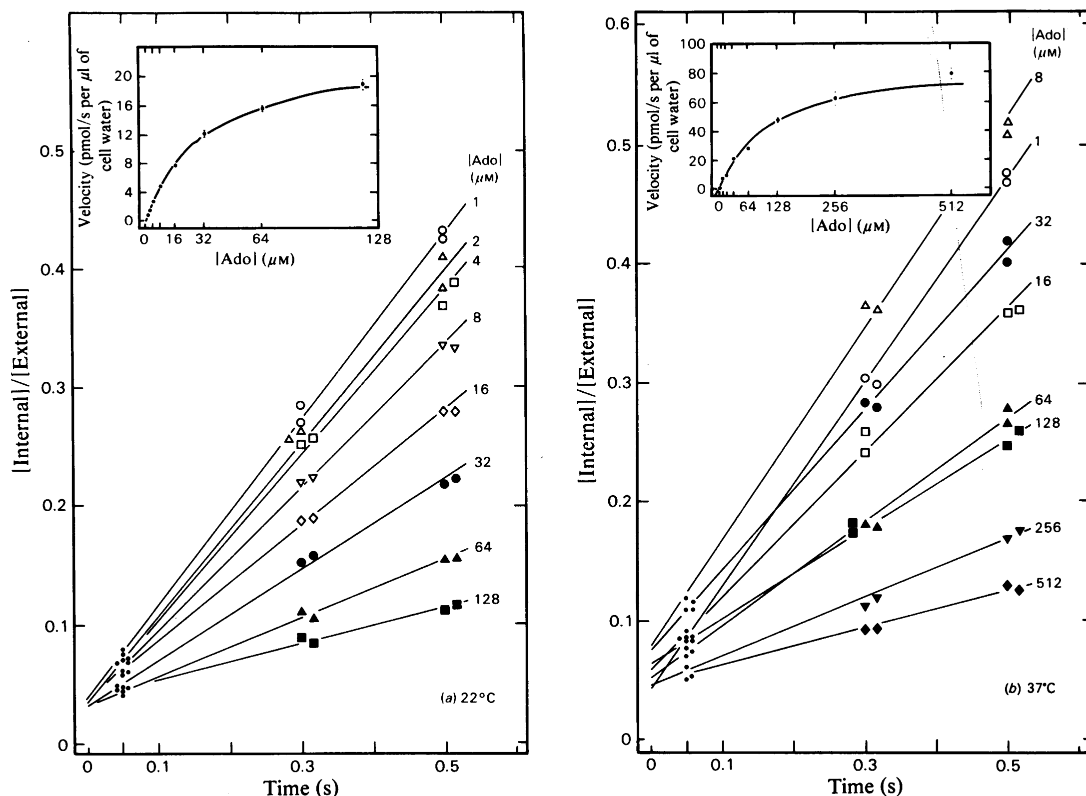

Fig. 7. *Use of an abbreviated quenched-flow procedure in a kinetic study of adenosine transport in human erythrocytes*
Time courses of [^{3}H]adenosine uptake by erythrocytes were obtained by the quenched-flow procedure. Quenched-flow assays for each condition were repeated; where single symbols are shown, the two data points fall within the symbol; for visibility, some data points have been plotted beside their time values (0.05, 0.3 or 0.5s). The ordinate parameter, [Internal]/[External], is the ratio of the intracellular and extracellular concentrations of ^{3}H (in adenosine equivalents) derived from [^{3}H]adenosine and is explained more fully in the Materials and methods section. The time courses shown for each adenosine (Ado) concentration are lines fitted by a least-squares method, and rates of adenosine uptake derived from these data are plotted in the insets. The lines shown in the insets represent the Michaelis–Menten equation fitted to the rate data, and the kinetic constants so derived are listed in Table 2. The same cell preparation was used for the experiments conducted at 22°C (*a*) and 37°C (*b*); the latter were conducted in a 37°C room. Error bars represent S.D..

of the kinetic constants for the inward transport of adenosine by human erythrocytes at 22°C were K_m 25±14 µM and $V_{max.}$ 15±5 pmol/s per µl of cell water ($n = 8$), and the constants for transport at 37°C were K_m 98±17 µM and $V_{max.}$ 80±9 pmol/s per µl of cell water ($n = 3$). The kinetic constants, each from a separate experiment, from which these means were calculated are listed in Table 2.

Discussion

Quenched-flow systems have been useful in following the progress of rapid biochemical reactions and of ion fluxes during millisecond intervals [see Cash & Hess (1981) for examples]. Our use of a quenched-flow procedure to measure cellular uptake of adenosine during intervals of 0.05–0.5 s has yielded time courses of adenosine uptake that appear to define initial uptake rates. In the quenched-flow procedure, uptake intervals are a fixed and intrinsic characteristic of each configuration of the flow apparatus, provided that flow velocities are constant. Precise manual timing is difficult with uptake intervals less than 1 s, whereas the quenched-flow method provides reproducible intervals of cellular nucleoside uptake of less than 0.5 s. The quenched-flow procedure may be useful in characterizing very rapid influx processes and those in which the time course of uptake shows early departure from linearity.

These experiments with the quenched-flow

Table 2. *Kinetic constants from quenched-flow studies of the inward transport of adenosine in human erythrocytes*

Transport rates were derived from time courses compiled from quenched-flow assays of adenosine uptake during graded intervals as in Figs. 5 and 6 (procedure A) or during three intervals as in Fig. 7 (procedure B). Kinetic constants (±S.E.M.) were obtained from the rate data as in Fig. 5.

Donor	Assay temperature (°C)	Procedure	K_m (μM)	$V_{max.}$ (pmol/s per μl of cell water)
1	22	A	28±4	18±2
2	22	A	17±1	14±1
	22	A	14±1	12±1
3	22	A	56±8	17±2
	22	A	14±3	12±2
4	22	A	17±3	7±1
5	22	B	23±1	13±1*
	37	B	107±30	82±17
6	22	B	30±1	23±1
	37	B	109±20	88±12
7	37	B	79±8	70±5

* Also reported in Table 1, Expt. 2.

procedure have substantiated earlier kinetic studies of adenosine influx in cultured cells in which uptake intervals were ended with the manually timed addition of NBMPR to the assay system. The present study also shows that time courses of adenosine uptake from 0 to 0.5 s define initial rates of adenosine uptake by human erythrocytes at 22°C and 37°C and by cultured L5178Y cells and S49 cells at 22°C. Changing the assay temperature from 22°C to 37°C decreased the apparent affinity (K_m) of adenosine for the transport mechanism of erythrocytes, but increased the maximum velocity of transport. The net effect of these changes would appear to be a minor increase in adenosine permeation rates at physiological concentrations (<1 μM).

The K_m values for the inward transport of adenosine in cultured cells reported here and previously (Harley *et al.*, 1982) are in general agreement with those found by Chello *et al.* (1983), but are several-fold lower than the values reported by Lum *et al.* (1979). These differences probably have a technical basis.

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