Biochimica et Biophysica Acta, 1070 (1991) 157–162

BBAMEM 75428

Adenine and hypoxanthine transport in human erythrocytes: distinct substrate effects on carrier mobilty

Martin Kraupp, Richard Marz, Gerhard Prager, Werner Kommer, Mehrdad Razavi, Mehrdad Baghestanian and Peter Chiba

Institut für Medizinische Chemie der Universität Wien, Wien (Austria)

(Received 21 May 1991)

Key words: Carrier mobility; Adenine transport; Hypoxanthine transport; Transport kinetics; (Human erythrocyte)

Transport of adenine and hypoxanthine in human erythrocytes proceeds via two mechanisms: (1) a common carrier for both nucleobases and (2) unsaturable permeation 4–5-fold faster for adenine than for hypoxanthine. The latter process was resistant to inactivation by diazotized sulfanilic acid. Carrier mediated transport of both substrates was investigated using zero-trans and equilibrium exchange protocols. Adenine displayed a much higher affinity for the carrier ($K_m \approx 5$–8 μM) than hypoxanthine ($K_m \approx 90$–120 μM) but maximum fluxes at 25 °C were generally 5–10-fold lower for adenine ($V_{max} \approx 0.6$–1.4 pmol/μl per s) than for hypoxanthine ($V_{max} \approx 9$–11 pmol/μl per s). The carrier behaved symmetrically with respect to influx and efflux for both substrates. Adenine, but not hypoxanthine reduced carrier mobility more than 10-fold. The mobility of the unloaded carrier, calculated from the kinetic data of either hypoxanthine or adenine transport, was the same thus providing further evidence that these substrates share a common transporter and that their membrane transport is adequately described by the alternating conformation model of carrier-mediated transport.

Introduction

Purine nucleobases enter cells by a nonconcentrative facilitated diffusion transport system [1–3]. Numerous kinetic studies have been carried out on adenine and hypoxanthine transport * in human red blood cells [4] and in animal cells [5,6], however, with conflicting results. According to one study [4] adenine transport in erythrocytes did not display saturation kinetics but behaved as a first-order process, indicating that the carrier has a very low affinity towards adenine as substrate. In contrast hypoxanthine transport showed hyperbolic saturation kinetics and was found to be well described by the alternating conformation model as formulated by Lieb and Stein [7]. But in mutual inhibition experiments the order of affinity was reversed: adenine was a much stronger inhibitor of hypoxanthine transport than vice versa [4]. Counterflow experiments revealed yet another inconsistency: transient accumulation of both hypoxanthine and adenine could be demonstrated after preloading with hypoxanthine but not with adenine.

A recent investigation demonstrated that adenine and hypoxanthine transport shows a biphasic behavior which was interpreted as two parallel processes: a saturable and an unsaturable transport component. K_m values of adenine and hypoxanthine zero-trans influx were calculated by numerical subtraction of the unsaturable flux component and correlated well with the respective K_i values from mutual inhibition experiments. The authors concluded that adenine and hypoxanthine share the same carrier, with adenine having a much lower K_m and V_{max} than hypoxanthine. The unsaturable process was interpreted as simple diffusion and was several-fold faster for adenine than for hypoxanthine [8]. This interpretation, however, seems inconsistent with the similar octanol/water partition coefficients for hypoxanthine and adenine reported previously [4]. The rate constant of the unsaturable adenine flux was also higher than expected for a simple diffusion process, suggesting either an additional mediated process or an altered kinetic behavior of the carrier with increasing substrate concentration.

* Transport: transfer of unmodified substrate across the cell membrane; uptake: accumulation of extracellular substrate in cells regardless of intracellular metabolism.

Correspondence: M. Kraupp, Institut für Medizinische Chemie der Universität Wien, Währingerstrasse 10, A-1090 Vienna, Austria.

The question whether these kinetically defined processes are indeed independent was not addressed, however, the interpretation of the transport data rests on this assumption. The large differences in V_{max} values of the saturable process further indicate that either the carrier might behave asymmetrically in some respect or that the alternating conformation model of facilitated diffusion does not describe this process adequately.

In order to adress these questions we investigated the kinetics of adenine and hypoxanthine transport in detail. Not only zero-trans * influx but also efflux and equilibrium exchange experiments were carried out, so that a complete description of this transport system in terms of the alternating conformation model of carrier mediated transport as formulated previously [7] was possible. Inactivation studies provided an additional criterion to discriminate between saturable and unsaturable nucleobase transport.

Materials and methods

Preparation of human red blood cells

Fresh blood from healthy volunteers was heparinized and centrifuged at 3000 rpm. Supernate and buffy layer were removed and the pellet resuspended three times in at least 5 volumes of saline. The tube was rocked gently at room temperature for at least 10 min before centrifugation to ensure complete removal of endogenous purines (e.g. uric acid). The pellet obtained after the final wash was either resuspended in an equal volume of saline to give a hematocrit of 50% or used directly as 'loosely packed cells'.

Transport studies

Influx. Transport was assayed as described [8] with slight modifications. Erythrocytes (20 μl, 50% hematocrit) were placed in a 1.5 ml Eppendorf tube and the incubation started by rapid addition of 80 μl labeled substrate in saline at 25°C. Transport was stopped by adding 700 μl of papaverine (20 mM) in saline cooled in a freezing mixture to about −2°C. Cooling the stop solution to temperatures below 0°C significantly improved its performance. Within 10 s 0.2 ml silicone oil were added and the cells separated immediately from the medium by centrifugation through oil. For equilibrium exchange influx protocols, cells at a hematocrit of 50% were preincubated with unlabeled substrate at 4°C.

Efflux. One volume of loosely packed cells were preincubated at 4°C for up to 120 min with one volume of saline containing the labeled substrate. The cells were pelleted in an Eppendorf centrifuge for 15 s and the supernate removed. With a positive displacement pipette, 5 μl of these 'tightly packed cells' were placed into a 1.5 ml centrifuge tube. Efflux was started for zero-trans protocols by the addition of 95 μl of saline. At the start of efflux the extracellular concentration of the preloaded substrate was less than 2% of the intracellular concentration under these conditions. For equilibrium exchange experiments, efflux was started by mixing cells with saline containing unlabeled substrate at the intracellular concentration.

Time measurements

Incubation times were determined using an electronic clock (Breitenbach and Heller, Vienna, Austria) with a precision of 0.01 s. The clock was started and stopped by microswitches attached to the plunger of the pipettes used to dispense the start and stop solutions.

Sample processing

Aliquots for radioactivity measurements were taken from the supernatant, the remainder was removed by suction and the tubes rinsed with water. The cell pellet was hemolyzed by adding 0.45 ml H_2O and extracted with perchloric acid (0.5 M final concn.). After centrifugation the radioactivity in the clear supernate was measured by liquid scintillation counting in a Packard CA2000 counter (Packard Instruments) with a constant counting error of 1%.

Metabolism

After equilibration with radioactive permeant, 5 μl of tightly packed cells were extracted with 50 μl of 1M perchloric acid and neutralized with $KHCO_3$. The extract was applied to Kieselgel TLC sheets and chromatographed using adenine, adenosine, hypoxanthine, inosine, AMP and IMP as carriers [11]. The fractions were identified under UV light, cut out, eluted with 0.01 M HCl and the radioactivity was determined by liquid scintillation counting.

Data analysis

Cell volume and extracellular space of cell pellets were determined with ^3H_2O and [^{14}C]inulin [9].

To characterize carrier-mediated nucleobase transport we follow the nomenclature and definition of parameters as formulated by Lieb and Stein [7].

$$v_{12} = (S_1 - S_2)/(K \cdot R_{00} + R_{12} \cdot S_1 + R_{21} \cdot S_2 + S_1 \cdot S_2 \cdot R_{cc}/K) \quad (1)$$

Eqn. 1 describes net flux (v_{12}) from compartment 1 to 2 in the presence of substrate (S_1, S_2) in both compart-

* Zero-trans: transport of substrate from one side of the membrane (cis) to the other side (trans) where its initial concentration is zero. 'Equilibrium-exchange': unidirectional flux of labeled substrate to the other side of the membrane where unlabeled substrate is present at an equal concentration.

ments. This carrier model is characterized by four resistancies (R_{12}, R_{21}, R_{ee}, R_{00}) and a carrier substrate dissociation constant K. Three resistancies (R_{12}, R_{21}, R_{ee}) correspond to the reciprocals of the maximum transport velocities measured under zero-trans influx (R_{12}), efflux (R_{21}) and equilibrium exchange (R_{ee}) conditions. The fourth (R_{00}) can be calculated, since $R_{12} + R_{21} = R_{ee} + R_{00}$. In our use of Eqn. 1, compartment 1 is extracellular and compartment 2 intracellular. In zero-trans influx experiments all terms containing S_2 vanish and the same is true for zero-trans efflux experiments with terms containing S_1. In both cases division by the remaining resistance reduces Eqn. 1 to the hyperbolic term of Eqn. 2.

$$d[S]/dt = V_{max} \cdot [S]/(K_M + [S]) + \text{constant} \cdot [S] \quad (2)$$

Knowledge of all R and K_M values allows calculation of K, since e.g. in the case of zero-trans influx $K_M = K \cdot R_{00}/R_{12}$ [7].

$$S_{in}/S_{out} = 1 - \exp(k \cdot t) \quad (3)$$

$$S_{in}/S_{t=0} = \exp(-k \cdot t) + S_{equ}/S_{t=0} \quad (4)$$

Initial rates were obtained by fitting Eqns. 3 and 4 to time courses of influx and efflux of labeled substrates using non-linear least squares regression. Initial rates were calculated from the slopes at $t = 0$ s. The kinetic parameters V_{max}, K_M and the constant were determined by non-linear least-squares regression analysis of initial rate data using Eqn. 2.

Non-linear least-squares regression was carried out using subroutine CURVFIT which is a Fortran implementation of Marquard's algorithm as described in Ref. 12.

Chemicals

[2-^{3}H]Adenine, [G-^{3}H]hypoxanthine, ^3H_2O, [^{14}C]inulin were purchased from Amersham. Papaverine-HCl, unlabeled nucleobases and nucleosides were obtained from Sigma. Diazotized sulfanilic acid (DASA) was prepared before each experiment [13]. Plastic backed Kieselgel-TLC sheets (Kieselgel F-60) were from Merck (Darmstadt, F.R.G.), silicone oil AR200 was purchased from Wacker Chemie (Munich, F.R.G.), Ready Solve scintillation cocktail was from Beckman Instruments.

Results and Discussion

Time course of influx and efflux

Initial rates of adenine influx and efflux were determined for concentrations ranging from 0.3 to 3500 μM. A typical K_M experiment was performed at 15 concentrations. Representative time courses of [^{3}H]adenine influx and efflux under zero-trans conditions are illustrated in Fig. 1. The figure also demonstrates why our method of calculating initial rates is necessary. Transport is so rapid at low concentrations that no initial linear portion could be measured. In influx experiments 60% of the equilibrium value is reached after 4 s at 25°C and a concentration of 1.5 μM (Fig. 1A).

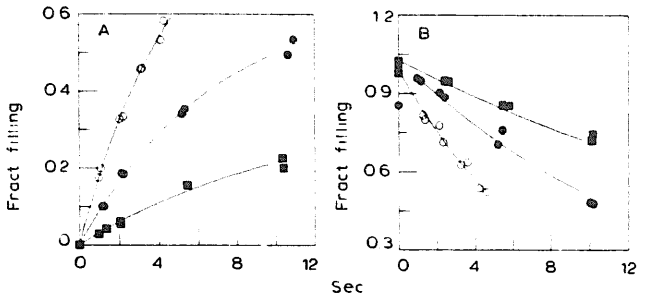

Fig. 1. Time-course of zero-trans influx (A) and efflux (B) of adenine. Influx and efflux were assayed as described under materials and methods. (A) Fractional filling is the ratio of the intracellular concentration at time = t to the equilibrium concentration (○, 1.52 μM; ●, 10.3 μM; ■, 72.3 μM). (B) Cells were preincubated at 4°C with [^{3}H]adenine for 4 min (○, 3 μM) and 10 min (●, 26 μM; ■, 101 μM) respectively, before efflux was started. Here fractional filling is defined as the ratio of the intracellular concentration of labeled permeant at time = t to that at zero-time.

In the case of efflux (Fig. 1B) an additional problem arises. At low substrate concentrations a considerable fraction of labeled substrate is metabolized even at 4°C during preincubation (Table I). Since this reduces the concentration of free intracellular adenine, initial efflux rates were calculated using the actual substrate concentrations derived from the TLC data.

Although exponential equations are only applicable to time courses of first-order reactions, Fig. 1 shows that for the short observed time intervals they provide good approximations to time courses of influx and efflux at concentrations both below and above the K_m. Calculating the slope of these curves at time zero gives reliable estimates of initial transport rates.

Similar experiments were performed with hypoxanthine as substrate for concentrations between 5 and 5200 μM. Data analysis was done as described for

TABLE I

Metabolism of adenine in erythrocytes at 4°C

Cells (50% hematocrit) were equilibrated at 4°C with radioactive substrate for 4 to 10 min, depending on the concentration of adenine, and analyzed as described in Materials and Methods.

Ade (μM)	CPM				% adenine
	nucleotides	Hyp + Ino	Ado	Ade	
0.4	1218	218	77	2893	66
1.3	666	158	113	7358	89
2.8	444	154	182	8158	91
8.3	151	151	128	7426	95
28.3	60	137	280	7288	94

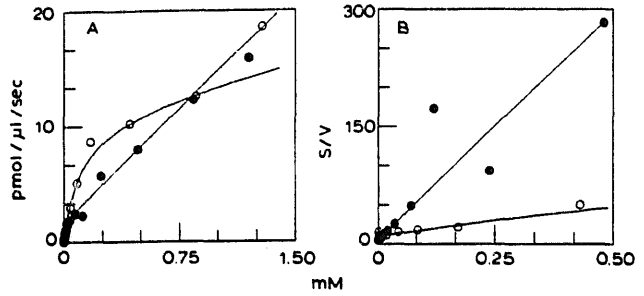

Fig. 2. Kinetics of zero-trans influx of adenine and hypoxanthine. Calculation of initial rates and kinetic parameters was carried out as described in Materials and Methods (○, hypoxanthine; ●, adenine). (A) Plot of initial rates versus concentration. (B) $[S]/v$ versus [S] plot of the data shown in (A) after subtraction of the fitted linear component.

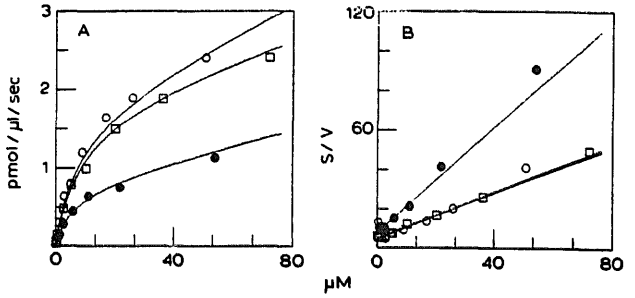

Fig. 3. Kinetics of adenine transport. Initial transport rates of zero-trans influx (□), zero-trans efflux (○) and equilibrium-exchange (●) were estimated as described for Figs. 1 and 2 and the kinetic parameters were calculated by nonlinear regression of Eqn. 2. (A) Plot of initial rates versus concentration. (B) $[S]/v$ versus [S] plot of the data shown in (A) after subtraction of the fitted linear component.

adenine except no correction for metabolism was necessary due to the higher concentrations employed (data not shown).

Zero-trans and equilibrium exchange kinetics of hypoxanthine and adenine transport

The concentration dependence of influx and efflux of adenine and hypoxanthine was determined under zero-trans and equilibrium-exchange conditions. In all experimental protocols the kinetics of adenine and hypoxanthine transport could be resolved into two components: (1) a saturable hyperbolic component, and (2) a nonsaturable linear component (Figs. 2A, 3A). The kinetic parameters including the linear term were estimated by fitting Eqn. 2 directly to the data. After subtraction of the fitted linear component, the data conformed to simple Michaelis-Menten kinetics as shown for instance by the linearity of $[S]/v$ vs. [S] plots (Figs. 2B, 3B).

The data for adenine compiled in Table II show that the V_{max} values for zero-trans influx and efflux are equal, i.e. the carrier displays directional symmetry. The same is true for hypoxanthine, however, the maximum velocity of hypoxanthine transport is roughly 7-fold higher than that of adenine. V_{max} values for hypoxanthine equilibrium exchange agree well with maximum zero-trans fluxes, showing that hypoxanthine as substrate has only little, if any, influence on carrier mobility *. This is not the case for adenine (Fig. 3, Table II). The carrier displays directional symmetry with adenine as substrate but is slowed down under equilibrium exchange conditions. Thus the mobility of the carrier is reduced when loaded with adenine as shown by a comparison of the maximum velocities of zero trans and equilibrium exchange fluxes (Table II).

The carriers resistance for a complete round trip without substrate, R_{00}, was calculated using the relationship $R_{12} + R_{21} = R_{ee} + R_{00}$. The data in Table III show that adenine causes an almost 15-fold reduction of carrier mobility ($R_{ee}/R_{00} \approx 15$) whereas the resistancies for hypoxanthine transport are not significantly different from R_{00}. Since R_{00} is an inherent property of the carrier independent of any substrate present, it should be the same whether calculated from the pa-

* Mobility: a macromolecular conformational shift of the carrier by which the substrate binding site is transferred from one side of the membrane to the other side regardless whether substrate loaded or empty.

TABLE II

Kinetic parameters of adenine and hypoxanthine transport

Initial rates and kinetic parameters of adenine and hypoxanthine transport were estimated as described for Figs. 1 and 2. All experiments were carried out at 25°C. Averages and standard deviations were calculated from three independent experiments. In the case of equilibrium exchange, data from influx and efflux experiments were pooled.

Substrate	Protocol	K_M (µM)	V_{max} (pmol/µl per s)	Constant (10^{-3} s^{-1})
Ade	zt-influx	8.3± 1.8	1.84±0.4	14.6±4
	zt-efflux	11.6± 6.0	1.31±0.4	11.9±7
	equ. exch.	4.9± 0.7	0.61±0.1	9.3±2
Hyp	zt-influx	127.7± 4.7	13.1 ±1.1	3.3±0.1
	zt-efflux	140.8±68.4	11.4 ±3.8	2.8±1.0
	equ. exch.	133.6± 2.7	10.5 ±0.8	1.2±0.8

TABLE III

Parameters characterizing the alternating conformation carrier model

Resistancies and K values were calculated as described in Materials and Methods using the data of Table III.

Substrate	K	R_{12}	R_{21}	R_{cc}	R_{00}
Adenine	72 ± 140	0.83 ± 0.18	0.84 ± 0.28	1.55 ± 0.22	0.11 ± 0.399
Hypoxanthine	140 ± 20	0.077 ± 0.0064	0.071 ± 0.0036	0.096 ± 0.0072	0.077 ± 0.103

rameters of adenine or hypoxanthine transport, if both substrates share the same carrier and if the alternating conformation model for transport holds [7]. Table III shows that the two R_{00} values agree well, confirming the existence of a shared transport system.

Effects of substrates on carrier mobility have been described for several well characterized transport systems as for instance the nucleoside carrier [14–17]. However, in most cases substrates have been found to cause an acceleration of carrier mobility. This can be shown directly by demonstrating trans acceleration, i.e. the effect of an unlabeled substrate exclusively on one (trans) side of the membrane on the transport of a labeled substrate which is exclusively on the other (cis) side. Trans acceleration is an additional kinetic criterion for the alternating conformation carrier model since pore and channel models do not predict this behavior [18].

Our data imply that adenine should act as a strong trans inhibitor of other substrates as for instance hypoxanthine. However, trans inhibition is more difficult to demonstrate than trans stimulation. Even after short incubation times both substrates will be on either side of the membrane causing mutual competitive inhibition. To overcome this problem we chose low adenine concentrations to minimize its unmediated influx, and higher hypoxanthine concentrations to minimize competitive inhibition of hypoxanthine transport by intracellular adenine. The initial rates of hypoxanthine efflux measured under these conditions are given in Table IV. Even at the low extracellular concentrations of adenine used, a significant reduction of hypoxanthine efflux occurred. These results also explain why counterflow of hypoxanthine and other purine compounds into cells preloaded with high concentrations of adenine could not be observed in previous work [1,2,4]. Under these experimental conditions adenine acts as a strong trans inhibitor of purine base influx rather than as a stimulator of apparent uphill-transport. In addition, due to its high affinity for the carrier, low extracellular adenine concentrations will also cause competitive inhibition of nucleobase influx.

TABLE IV

Transinhibition of hypoxanthine efflux by adenine

Cells were preincubated for 60 min at 4°C with three concentrations of [^{3}H]hypoxanthine as described in Fig. 1 and under Materials and Methods. Efflux was started by mixing 5 μl of tightly packed cells with 95 μl of saline containing the indicated concentrations of unlabeled adenine. Initial efflux rates were calculated from the time courses as described in Materials and Methods.

Hyp (μM)	pmol/μl per s (% Inhibition)		
	0 μM Ade	5 μM Ade	10 μM Ade
50	4.5 (0)	3.9 (12)	3.6 (19)
100	7.7 (0)	6.8 (12)	4.3 (44)
200	13.5 (0)	9.7 (28)	7.0 (48)

Unmediated transport of nucleobases

The nature of the unsaturable transport component is still unknown. Rate constants measured in various experimental protocols and in both directions did not differ significantly for a given substrate. Adenine was consistently 4–5-fold faster than hypoxanthine which is unexpected for a simple diffusion process considering the similar octanol/water partition coefficients. An attempt to distinguish between either simple diffusion and unspecific leakage transport or altered kinetic behavior of the same carrier due to increasing substrate concentration was made. The effects of pH and of the protein modifying agent DASA on zero-trans influx of adenine at concentrations of 1 μM and 1000 μM were investigated. Varying the pH from 5.5 to 8.0 had no significant effect on adenine influx at both concentrations (data not shown).

DASA was chosen because of its unspecific reactivity towards amino acid side chains although it also may react with bilayer lipid. It has been used in several studies to inactivate ecto-enzymes since it reacts preferentially with the exofacial domains of membrane proteins due to the charged sulfonic acid group [19–21].

Pretreatment with DASA led to a concentration dependent inactivation of the nucleobase carrier as shown by the decreased initial rates of adenine transport at 1 μM (Fig. 4A). The initial transport rate in untreated cells in this experiment was 0.434 pmol/μl per s while treatment with 0.2 mM and 1.0 mM DASA led to decreased transport rates of 0.284 (65%) and 0.108 pmol/μl per s (25% of control), respectively. Transport of 1000 μM adenine, i.e. unmediated entry was unaffected by this treatment (Fig. 4B) demonstrating that the general permeability properties of the lipid bilayer were not altered. This is further evidence that the unsaturable transport component is a mechanism

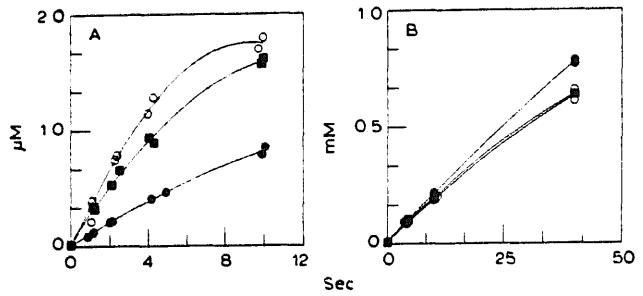

Fig. 4. Effect of DASA on adenine influx. Red blood cells (20% hematocrit) were incubated in Hepes-buffered saline (pH 7.4) (○) or in buffered saline with DASA at concentrations of 0.2 mM (■) and 1.0 mM (●), respectively, at 37 °C for 30 min. Cells were washed and resuspended in saline to give a final hematokrit of 50%. Zero-trans influx of adenine was assayed at concentrations of 1 μM (A) and 1000 μM (B).

distinct from the saturable component, i.e. is not due to an altered kinetic behavior of the nucleobase carrier at high substrate concentrations. Although this resistance to inactivation by DASA is consistent with unmediated diffusion through the lipid bilayer, it remains unclear why there should be an almost 5-fold difference in the diffusion rate for adenine and hypoxanthine.

It has been argued that ratios of first order rate constants over octanol/water partition coefficients (k/Z) are a useful criterion to distinguish between carrier mediated transport and nonmediated permeation through cell membranes [4]. Ratios for substrates, for which carrier mediated transport has been demonstrated, are higher than those for substrates like L-glucose or cytosine which are thought to enter cells solely by unmediated permeation. Table V shows that the diffusional component of hypoxanthine transport can be explained by unmediated permeation, since its k/Z value is comparable to those of cytosine and L-glucose. The value for the unsaturable component of adenine transport is so much higher that it can not be explained alone by simple diffusion through the lipid bilayer.

Although diffusion may not contribute significantly to overall transport of adenine at physiological concentrations, over 90% of transmembrane flux of adenine is due to diffusion at adenine concentrations higher than 1000 μM. At these concentrations, which have usually been employed in previous studies [1–4], carrier-mediated transport is completely masked by diffusion and transport asymmetries could therefore not be detected.

The careful kinetic characterization reported in this paper serves to reconcile the contradicting results found in the literature. While our primary data agree well with those published previously [8] we were able to provide a consistent interpretation. Adenine and hypoxanthine clearly share a common transport system. This had previously been obscured by two facts: adenine decreases carrier mobility, and at unphysiologically high substrate concentrations a second process, which shows no saturation characteristics, predominates.

TABLE V

Octanol / water partition coefficients, water solubilities and rate constants for diffusion of adenine, hypoxanthine, cytosine and L-glucose

	$10^3 * k$	Z_{OCT}	k/Z
Adenine	11.2 [a]	0.105 [b]	0.107
Hypoxanthine	2.43 [a]	0.115 [b]	0.021
Cytosine	0.55 [b]	0.035 [b]	0.016
L-Glucose	0.034 [b]	0.00158 [b]	0.022

[a] Data represent averages of all experiments.
[b] Data from Ref. 4.

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