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# Parasite-induced processes for adenosine permeation in mouse erythrocytes infected with the malarial parasite *Plasmodium yoelii*

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In mouse erythrocytes harbouring the malarial parasite *Plasmodium yoelii*, three processes contributed to inward fluxes of adenosine, one of which is attributed to the native nucleoside transporter, because of the inhibitory effects of nitrobenzylthioinosine (NBMPR). New (parasite-induced) permeation processes of low NBMPR-sensitivity were (i) saturable fluxes with preference for the D enantiomer (D-Ado) and (ii) apparently unsaturable fluxes that proceeded by a channel-like route without enantiomeric selectivity. Parasite-induced fluxes of L- and D-Ado were similarly inhibited by furosemide [$IC_{50}$ (concn. causing half-maximal inhibition) 15–17 $\mu$M], whereas D-Ado fluxes in uninfected erythrocytes were 10-fold less sensitive.

## INTRODUCTION

During intra-erythrocytic development of malarial parasites (*Plasmodium* spp.) changes in host-cell plasma membranes occur that include insertion of parasite proteins [1] and alterations in membrane permeability to small organic solutes [2,3]. Salvage of host purines by malarial parasites, which do not synthesize purines [4], obviously occurs during the intra-erythrocytic stages of the parasite cycle. Part of the salvage process, utilization of plasma purine nucleosides, proceeds by the membrane transport and enzymic machinery of the erythrocyte. Nucleosides enter erythrocytes of mice and humans by facilitated diffusion nucleoside-transport (NT) systems that are of high sensitivity to nitrobenzylthioinosine (NBMPR) [$IC_{50}$ (concn. causing half-maximal inhibition) < 30 nM] [5–7], a potent NT inhibitor that is bound tightly by the nucleoside transporter glycoprotein [8]. We have reported the presence of NT activity of low NBMPR-sensitivity in mouse erythrocytes infected with *P. yoelii* [6]. Nucleoside uptake of low NBMPR-sensitivity has also been observed in human erythrocytes infected with *P. falciparum* [9,10].

In the present study we show that 'physiological adenosine' (D-Ado) enters mouse erythrocytes infected with *P. yoelii* by an NT system that resembles the native transporter of mouse erythrocytes, and by two parasite-induced processes of low NBMPR-sensitivity. One of the induced processes has channel-like properties in being unsaturable and permeable to both D-Ado and the L enantiomer (L-Ado), in contrast with the native NT system, which has high, but not absolute, enantiomeric selectivity [11].

## EXPERIMENTAL

### Maintenance of parasites

*P. yoelii* 17XL parasites were passaged in 18–25 g female Ha/ICR mice (Health Sciences Laboratory Animal Services, University of Alberta) by intraperitoneal inoculation with $10^4$ *P. yoelii*-infected mouse erythrocytes (pRBCs). Parasitaemias were monitored by determining the pRBC fraction in Giemsa-stained smears of tail blood samples.

### Preparation of pRBC-enriched fractions

pRBC preparations in which the fraction of parasitized cells was 90% or greater were made as described previously [6], using a modification of the discontinuous Percoll/saline gradient described by Knight & Sinden [12]. Briefly, blood cells obtained by cardiac puncture from anaesthetized, parasite-infected mice were washed with Tris-buffered (pH 7.4) saline (TBS) and layered over two-step (50%, 65%) Percoll gradients in 15 ml centrifuge tubes. After centrifugation (1450 *g*, 10 min), pRBC-enriched fractions were recovered from the interface between the Percoll fractions. Erythrocytes from uninfected mice (RBCs) were pelleted through identical Percoll gradients. Cells were washed to remove Percoll and resuspended in TBS for use in transport experiments. Purity of the pRBC-enriched fractions was evaluated by means of Giemsa-stained smears.

### Nucleoside-flux measurements

Rates of nucleoside entry into erythrocytes were measured at 22 °C by established procedures [13] in which intervals of permeant influx were initiated by the rapid addition of 100 $\mu$l of cell suspension to an equal volume of $^3$H-labelled permeant in TBS in 1.5 ml microcentrifuge tubes, and ended by pelleting cells (Eppendorf model 5412 microcentrifuge; 16000*g*; 30 s) under 100 $\mu$l of a silicone oil/paraffin oil solution [density ($\rho$) = 1.03 g/ml]. Addition of cell suspension and centrifuge switch-on were performed in response to metronome signals. Under these conditions, permeant entry during the cell pelleting period was equivalent to that during 2 s of incubation at the maximum uptake rate, so that nominal intervals of cell exposure to permeant were increased by 2 s [14]. The $^3$H content of cell pellets was determined as described in [13].

Cell water space was determined as the difference between the [$^3$H]water and [$^{14}$C]sucrose spaces of cell pellets, measured after exposure of cells to those agents under flux assay conditions.

### Materials

L-Ado [15] was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, U.S.A.; the L-Ado preparation was both chemically and enantiomerically

Abbreviations used: NT, nucleoside transport; NBMPR, nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9-$\beta$-D-ribofuranosylpurine}; D-Ado, 'physiological adenosine' (9-$\beta$-D-ribofuranosyladenine); L-Ado, 9-$\beta$-L-ribofuranosyladenine; RBCs, uninfected mouse erythrocytes; pRBCs, mouse erythrocytes infected with *Plasmodium yoelii*; TBS, Tris-buffered saline (166 mM-NaCl/10 mM-glucose/10 mM-Tris/HCl, pH 7.4 at 22 °C); $IC_{50}$, concentration causing half-maximal inhibition.

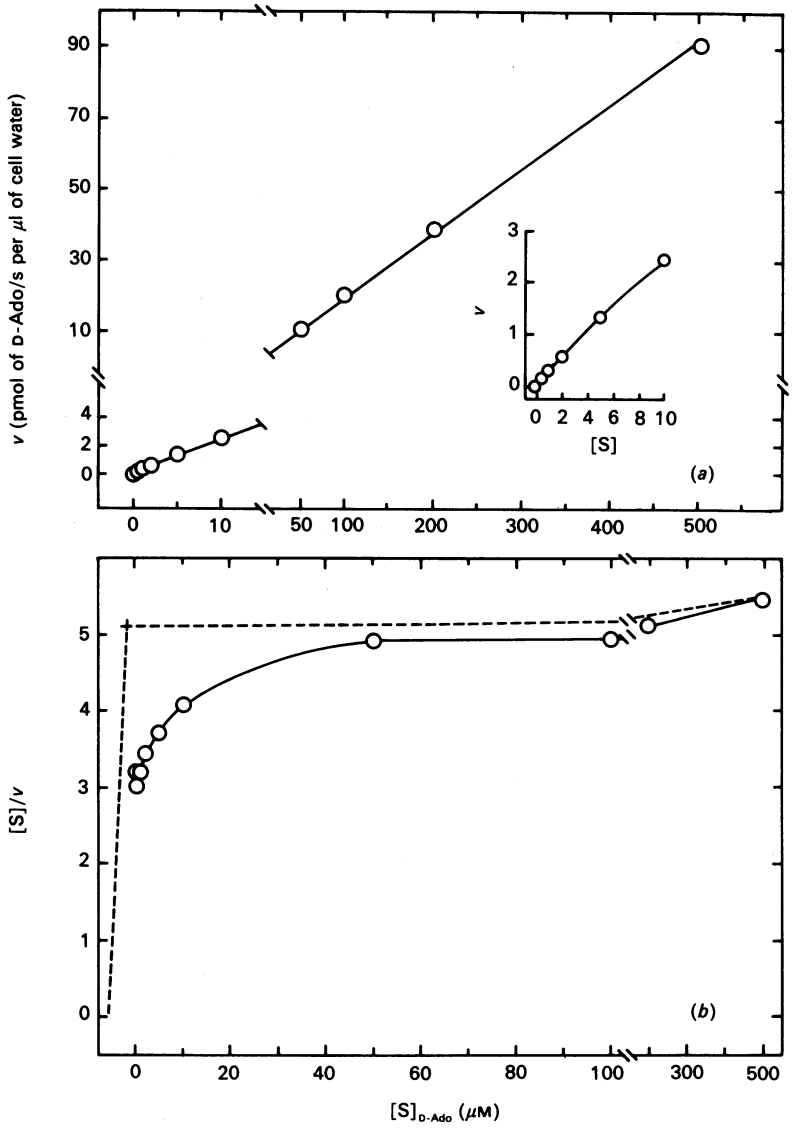


**Fig. 1. Concentration-dependence of D-Ado fluxes in pRBCs**

Inward fluxes of D-[$^3$H]Ado in suspensions of pRBCs ($10^8$ cells/ml) were measured during 3 s intervals in the presence of 1 $\mu$M-NBMPR. Data shown are means for five replicate assays. (*a*) Shows a direct plot of the rate–concentration data, whereas (*b*) shows an [S]/*v*-versus-[S] plot. Kinetic constants were determined by non-linear-regression analysis of the data using a model-fitting computer program [17]. The data in (*b*) are consistent with a two-component curve for which linear asymptotes are shown (broken lines).

pure [11]. A sample of this material was tritiated (33 Ci/mmol) by Moravek Biochemicals, Brea, CA, U.S.A. [$^3$H]Nucleosides were purified by h.p.l.c. before use. D-[2,8-$^3$H]Ado (33 Ci/mmol), $^3H_2O$ (100 mCi/ml) and [U-$^{14}$C]sucrose (499–671 mCi/mmol) were from Moravek Biochemicals, ICN Radiochemicals (Irvine, CA, U.S.A.) and DU PONT Canada (NEN Products, Lachine, Que., Canada) respectively, and NBMPR was prepared in this laboratory [16]. Percoll was from Pharmacia Canada, Dorval, Que., Canada. Non-radioactive nucleosides, furosemide and phlorizin were from Sigma Chemical Company, St. Louis, MO, U.S.A.

## RESULTS

In RBCs, a single component of NBMPR inhibition of D-Ado influx was evident from concentration–effect relationships for inhibition by NBMPR of 1 $\mu$M-D-Ado permeation, indicating that a single NT system of high NBMPR-sensitivity ($IC_{50}$ 1.3 nM) mediated D-Ado influx in those cells. In contrast, two or more components of D-Ado entry that differed in sensitivity to NBMPR were apparent in pRBCs. The evident presence of an influx component of high NBMPR-sensitivity in pRBCs ($IC_{50}$ < 1 nM) suggested that the native transporter was functional in the infected cells. In addition, a major parasite-induced D-Ado-influx component(s) of low NBMPR-sensitivity was present in pRBCs ($IC_{50}$ 47–54 $\mu$M). These results are in agreement with a previous report describing the expression of a major D-Ado-permeation process of low NBMPR-sensitivity in pRBCs [6].

The concentration-dependence of D-Ado fluxes in RBCs differed from that in pRBCs. In RBCs, a single saturable component of D-Ado influx was expressed ($K_m$ 7.2 ± 1.2 $\mu$M and $V_{max.}$ 5.5 ± 0.5 pmol/s per $\mu$l of cell water; means ± S.E.M.). In the presence of 1 $\mu$M-NBMPR, D-Ado influx in RBCs was abolished. Thus D-Ado fluxes in pRBCs were assayed in the presence of 1 $\mu$M-NBMPR in order to preclude D-Ado entry via the native NT system in those cells. Under these conditions, two com-

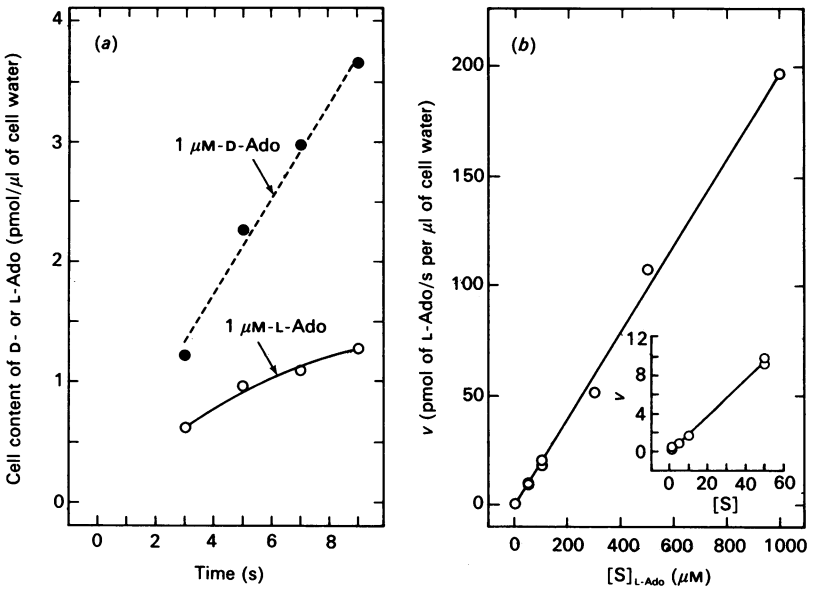


**Fig. 2. L-Ado fluxes in pRBCs**

(*a*) Replicate assay mixtures containing $10^8$ cells/ml and 1 μM-D- or -L-[$^3$H]Ado were incubated at 22 °C for the intervals specified, which were started by addition of cell suspension to complete the assay mixtures, and ended by centrifugal pelleting of the cells under oil. The intervals shown represent the time between addition of cell suspension and centrifuge switch-on, plus 2 s, the time required (in uptake-rate equivalents) to clear cultured cells from similar assay mixtures [14]. Data shown are means of duplicate or triplicate determinations. (*b*) Inward fluxes of L-[$^3$H]Ado in cell suspensions ($10^8$ cells/ml) were measured during 3 s intervals. The inset shows fluxes measured at low concentrations of L-Ado. The data shown are the combined results (means for duplicate or triplicate assays) from two separate experiments.

ponents of parasite-induced D-Ado influx were evident (Figs. 1*a* and *b*): (i) a saturable component with high affinity for D-Ado ($K_m$ 5.7±0.9 μM and $V_{max.}$ 0.75±0.12 pmol/s per μl of cell water) and (ii) an apparently unsaturable component.

The cell water spaces in pRBCs (45.2±1.5 μl/$10^9$ cells; $n$ = 21) were about 1.5-fold larger than those of RBCs (29.7 ±1.3 μl/$10^9$ cells; $n$ = 18). When expressed in terms of cell number, the total inward flux of 1 μM-D-Ado in pRBCs (16.3 ±1.3 pmol/s per $10^9$ cells; $n$ = 17) did not differ significantly from that in RBCs (14.4±1.3 pmol/s per $10^9$ cells; $n$ = 8). The contribution of the native NT system to 1 μM-D-Ado influx in pRBCs, determined as the difference between D-Ado flux in the presence or absence of 1 μM-NBMPR, was about 30% of the total inward flux.

Parasite-induced adenosine permeation activity was explored further in studies that employed L-Ado, a poor permeant for the native NT system [11]. Time courses for inward fluxes of adenosine in pRBCs are shown in Fig. 2(*a*). Whereas entry of 1 μM-L-Ado in RBCs was almost undetectable (0.011± 0.002 pmol/s per μl of cell water), as we have shown previously [11], L-Ado fluxes in pRBCs were substantial (0.23±0.07 pmol/s per μl of cell water), being about half of those of the D-enantiomer (0.40±0.03 pmol/s per μl of cell water). Despite the substantial L-Ado fluxes, the parasitized cells were virtually impermeable to 3 μM-sucrose (results not shown). Exploration of the concentration-dependence of L-Ado influx in pRBCs (Fig. 2*b*) failed to reveal a saturable component, suggesting that a 'channel-like' entry process accounted for the rapid L-Ado fluxes in those cells. The failure of L-Ado fluxes in pRBCs to saturate under these conditions showed (i) that L-Ado was not a substrate for the native NT system in pRBCs, nor a substrate for the parasite-induced saturable nucleoside-entry process, and (ii) that the latter process was stereoselective. In pRBCs, the apparently linear dependence of L-Ado influx on permeant concentration ([S]/$v$ = 5.0; Fig. 2*b*) was similar to that for the unsaturable component of D-Ado influx in the presence of NBMPR (Fig. 1*b*), suggesting that L-Ado fluxes and unsaturable D-Ado fluxes in pRBCs were kinetically similar and were probably attributable to the same process.

Phlorizin, an inhibitor of sugar transport, and furosemide, an inhibitor of anion transport, are agents that have been shown to suppress malarial-parasite growth *in vitro* [18]. In the present study, these substances were tested as inhibitors of L-Ado influx in pRBCs. Whereas 100 μM-phlorizin was a weak inhibitor, 100 μM-furosemide decreased the flux of 1 μM-L-Ado by about 70% (result not shown). In Fig. 3, the concentration–effect relationship for inhibition by furosemide of the NT system of RBCs is compared with the inhibition of L-Ado and NBMPR-insensitive D-Ado fluxes in pRBCs. In the latter cells, furosemide was a potent inhibitor of L-Ado fluxes ($IC_{50}$ 15 μM) and of D-Ado fluxes in the presence of 1 μM-NBMPR ($IC_{50}$ 17 μM). Under the latter condition, D-Ado fluxes in pRBCs were attributable mainly (> 80%) to the unsaturable entry process. The similar inhibition by furosemide of L-Ado fluxes and unsaturable D-Ado fluxes in pRBCs further supports the notion that both fluxes were attributable to the same process. In contrast, 10-fold higher concentrations of furosemide were required to inhibit entry of D-Ado via the NT system in RBCs ($IC_{50}$ 200 μM) (Fig. 3).

## DISCUSSION

The low NBMPR-sensitivity of parasite-induced adenosine-permeation processes distinguished them from the native NT system, which was blocked by NBMPR concentrations as low as 10 nM. While recognizing the need for further characterization of the induced non-stereoselective entry of adenosine, we have termed this process 'channel-like', because the process discriminated against sucrose entry and was inhibited by furosemide,

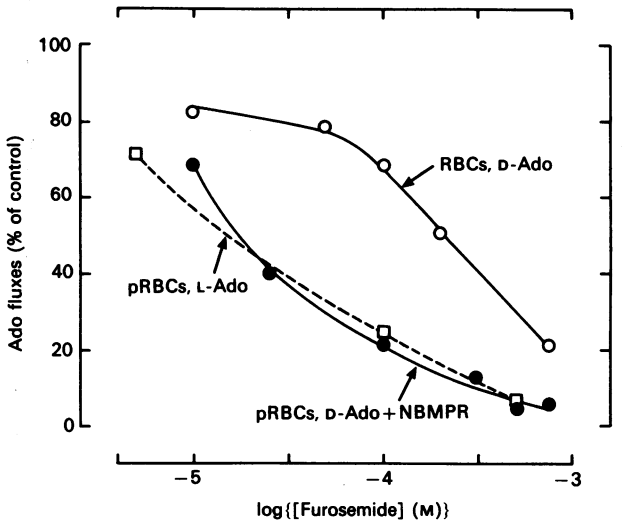


**Fig. 3. Inhibition of D- and L-Ado fluxes by furosemide in RBCs and pRBCs**

Cells ($2 \times 10^8$/ml) were incubated with furosemide at the concentrations shown for 15 min at 22 °C before addition of furosemide-containing solutions of 1 μM (final concn.)-D- or -L-[$^3$H]Ado and, in some samples, 1 μM (final concn.)-NBMPR to the assay mixtures. Initial rates of permeant entry were determined from time courses of D- or L-Ado influx measured as in Fig. 2(*a*). Data shown are means for duplicate or triplicate assays. Influx rates in the absence of furosemide (control values) were (i) pRBCs, L-Ado: 6.1 pmol/s per $10^9$ cells; (ii) pRBCs, D-Ado in the presence of 1 μM-NBMPR: 7.4 pmol/s per $10^9$ cells; and (iii) RBCs, D-Ado: 11 pmol/s per $10^9$ cells.

properties that suggest participation of a membrane-spanning protein, rather than non-specific diffusion. Whether channel or transporter models are appropriate remains to be established [19].

The non-stereoselective nucleoside-entry process identified in the present study, in being relatively insensitive to phlorizin, differs from the process by which amino acids, anions and hexitols enter human erythrocytes infected with *P. falciparum* [2,20]. Phlorizin was a potent inhibitor ($IC_{50}$ 17 μM) of sorbitol entry in *P. falciparum*-infected erythrocytes [20], but a weak inhibitor of L-Ado fluxes in *P. yoelii*-infected mouse erythrocytes (the present study). Furosemide, a potent inhibitor of channel-like adenosine fluxes in *P. yoelii*-infected mouse erythrocytes (the present study) was a poor inhibitor of anion fluxes in *P. falciparum*-infected human erythrocytes, although it inhibited parasite growth in those cells [18]. It is not known if the channel-like entry process for nucleosides is expressed in *P. falciparum*-infected erythrocytes or if the *P. yoelii*-induced channel accepts permeants other than nucleosides.

The inhibition by furosemide of a substantial adenosine flux component in pRBCs suggests that the loop diuretic may be interacting with a parasite-induced protein component of the erythrocyte membrane. Expression of the channel-like nucleoside-entry process in pRBCs may be related to the cell-volume increases associated with intra-erythrocytic parasite development ([21]; the present study), since furosemide inhibits (i) the electroneutral co-transport of $K^+/Cl^-$ ions induced in erythrocytes by osmotic swelling [22], and (ii) the channel-like nucleoside permeation induced by osmotic swelling in eel erythrocytes that is also sensitive to other loop diuretics [23]. The latter system also accepts neutral amino acids and sugars as permeants and participates in amino-acid-mediated cell-volume regulation [23,24].

Although proliferation of malarial parasites in mouse erythrocytes dramatically changed adenosine-flux characteristics in the erythrocytes, total inward fluxes of D-Ado per cell were similar in pRBCs and RBCs in media containing 1 μM-D-Ado. The native NT system was active in pRBCs, although D-Ado fluxes by this route were substantially less than in uninfected cells. Together these findings suggest that a substantial fraction of the native NT elements of the erythrocyte membrane may be modified during parasite development, although it is possible that parasite-specified NT polypeptides may also be incorporated into the host erythrocyte membrane.

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