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FACILITATED TRANSPORT OF 6-MERCAPTOPURINE AND 6-THIOGUANINE AND NON-MEDIATED PERMEATION OF 8-AZAGUANINE IN NOVIKOFF RAT HEPATOMA CELLS AND RELATIONSHIP TO INTRACELLULAR PHOSPHORIBOSYLATION

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6-Mercaptopurine and 6-thioguanine strongly inhibited the zero-*trans* entry of hypoxanthine into Novikoff rat hepatoma cells which lacked hypoxanthine/guanine phosphoribosyltransferase, whereas 8-azaguanine had no significant effect. 6-Mercaptopurine was transported by the hypoxanthine carrier with about the same efficiency as its natural substrates (Michaelis-Menten constant = 372 ± 23 μM; maximum velocity = 30 ± 0.7 pmol/μl cell H_2O per s). 8-Azaguanine entry into the cells, on the other hand, showed no sign of saturability and was not significantly affected by substrates of the hypoxanthine/guanine carrier. The rate of entry of 8-azaguanine at 10–100 μM amounted to only about 5% of that of hypoxanthine transport and was related to its lipid solubility in the same manner as observed for various substances whose permeation through the plasma membrane is believed to be non-mediated. Only the non-ionized form of 8-azaguanine (pK_a = 6.6) permeated the cell membrane.

Studies with wild type Novikoff cells showed that permeation into the cell was the main rate-determining step in the conversion of extracellular 8-azaguanine to intracellular aza-GTP and its incorporation into nucleic acids. In contrast, 6-mercaptopurine was rapidly transported into cells and phosphoribosylated; the main rate-determining step in its incorporation into nucleic acids was the further conversion of 6-mercaptopurine riboside 5′-monophosphate.

Introduction

Many purine analogs are highly toxic to tumor cells and are used in cancer therapy [1]. Although the exact mode of their action is not always clear, it is known that the active forms of these analogs are their nucleotide derivatives and that they are converted to nucleotides via the normal salvage pathways of the cell (see Ref. 2).

There is general agreement that 8-azaguanine, 6-thioguanine and 6-mercaptopurine are phosphoribosylated solely by hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8) and that the resistance of mutants of various cell lines and tumors to high concentrations of these purine analogs is due to loss of active enzyme [3,4]. However, the analogs differ in effectiveness as selective agents for the isolation of transferase-deficient mutants [3–5], mutants have been isolated that fail to exhibit cross-resistance [6,7], and mutants have been isolated that are partially resistant to 8-azaguanine in spite of the presence of functional transferase and the ability of the cells to grow in a medium containing amethopterin, hypoxanthine and thymidine (HAT medium) [3,4,8–10]. The mechanisms responsible for these observations have not been elucidated. They could be related to differences in mode of permeation of these analogs into the cell and/or in their substrate specificity for hypoxanthine/guanine phosphoribosyltransferase [7]. These considerations led to the present investigation in which we examined the mode of permeation of

Abbreviations: DMO, 5,5′-dimethyl-2,4-oxazolidinedione; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

these purine analogs into Novikoff cells and the relationship between rates of permeation and intracellular phosphoribosylation.

Little information was available on the mode of permeation of these purine analogs. Initially it was suggested that 6-mercaptopurine enters cells by passive diffusion [11]. On the other hand, the report [12] of the isolation of an 8-azaguanine-resistant subclone of Chinese hamster ovary cells which seems to be deficient in purine transport * rather than in transferase activity suggested that 8-azaguanine enters cells via the hypoxanthine-guanine transport system. Our present results, however, contradict both of these conclusions. They demonstrate that 8-azaguanine is, at best, a very poor substrate for the hypoxanthine-guanine carrier of Novikoff cells [13,14]. It probably enters the cells mainly by non-mediated permeation, and slow permeation seems to be limiting its incorporation into nucleic acids. In contrast, 6-thioguanine and 6-mercaptopurine have approximately as high an affinity for the transport system as do the normal substrates, hypoxanthine and guanine, and intracellular metabolism is limiting their incorporation into nucleic acids.

Materials and Methods

Cell culture. Novikoff rat hepatoma cells (subline N1S1-67) and an azaguanine-resistant hypoxanthine/guanine phosphoribosyltransferase-deficient) subline thereof [13,15] were propagated in Swim's medium 67 in suspension culture as described previously [16]. Cells were enumerated with a Coulter counter and cell viability was assessed by staining with trypan blue. For measuring nucleobase transport or uptake *, cells were harvested from mid to late exponential phase cultures and suspended in basal medium 42B (BM42B; Ref. 17) or glucose-free BM42B to $(1–4) \cdot 10^7$ cells/ml. Where indicated cells were depleted of ATP by preincubation in glucose-free BM42B containing 5 mM KCN and 5 mM iodoacetate at 37°C for 10–15 min [18]. Cultures were examined for mycoplasma by the uridine/uracil incorporation method [19]. No mycoplasma contamination was detected.

Measurement of purine accumulation in hypoxanthine/guanine phosphoribosyltransferase-deficient cells. Time courses of accumulation of purines into cells in suspension for periods of 60 to 120 s at 25°C in the zero-*trans* ** mode were measured by a rapid mixing/sampling technique described in detail elsewhere [21,22]. In the procedure cell suspension and a solution containing radioactively labeled purine are mixed at short intervals in a ratio of 7.35 to 1 with a dual syringe apparatus and substrate uptake is terminated by collecting the cells by centrifugation through a silicone oil mixture in an Eppendorf microcentrifuge. The cell pellets are then analyzed for radioactivity. To determine time courses of 8-aza[^{14}C]guanine accumulation stretching over 10–20 min, cell suspensions were supplemented with substrate and after various times of incubation the cells from duplicate 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. Intracellular concentrations of isotope were obtained by correcting for substrate trapped in the extracellular space of the pellet, as estimated by use of [*carboxyl*-^{14}C]inulin, and normalized to intracellular H_2O volume [21]. The time course of carrier-mediated intracellular substrate accumulation to transmembrane equilibrium in the zero-*trans* mode is described in the case of a completely symmetrical transporter by the following integrated rate equation [14,22]:

$$S_{2,t} = S_1 \left[1 - \exp\left(- \frac{tV + (1 + (S_1/K))\, S_{2,t}}{K + 2S_1 + (S_1^2/K)} \right) \right] \quad (1)$$

where $S_{2,t}$ = concentration of substrate inside the cell at time t ($S_{2,0} = 0$); S_1 = exogenous substrate concentration (and is taken as constant); V = maximum

* 'Transport' denotes solely the transfer of unmodified exogenous substrate across the cell membrane as mediated by a saturable, selective carrier. 'Uptake' denotes the transfer of radioactivity from exogenous labeled substrate to intracellular space or components regardless of metabolic conversions.

** As defined by Eilam and Stein [20], 'zero-*trans*' designates the transport of a substrate from one side of the membrane to the other side, where its concentration is zero. 'Equilibrium exchange' designates the unidirectional flux of radiolabeled substrate from one side to the other side of the membrane, where substrate is held at equal concentration. Arbitrarily, we designate the outside and inside faces of the membrane as 1 and 2, respectively.

velocity, and K = Michaelis-Menten constant. K and V for hypoxanthine and 6-mercaptopurine transport were estimated by fitting Eqn. 1 by the method of least squares to zero-*trans* entry data pooled for seven or eight substrate concentrations. Use of the symmetrical model was justified because other studies * have shown that the initial velocities of hypoxanthine zero-*trans* entry and exit and inward and outward equilibrium exchange in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells are equivalent. To analyze time courses of nucleobase uptake at a single concentration, Eqn. 1 was fitted to the data, whereby K was fixed at its experimentally determined value to yield an estimate of V. In both cases initial zero-*trans* velocities (v_{12}^{zt}) were calculated for a given concentration (S_1) as the initial slope of the best-fitting curve, $S_1V/(K + S_1)$. All computations were conducted as described by Wohlhueter et al. [22] with some refinements to be described in a separate publication **. Computed values are stated ± S.E. of estimate.

Initial velocities of non-saturable 8-azaguanine permeation (v_0) were estimated graphically from linear portions of accumulation curves.

Measurement of 8-azaguanine efflux from hypoxanthine/guanine phosphoribosyltransferase-deficient cells. The cells were preloaded by incubation in glucose-free BM42A, pH 6.0, containing aza[^{14}C]guanine at 37°C for 40 min. After equilibration at 25°C exit was measured by the rapid kinetic technique whereby samples of suspension of preloaded cells were mixed in a ratio of 1 to 7.35 with BM42B, pH 7.5, containing supplements as indicated in the appropriate experiments. Exit was terminated by collecting the cells by centrifugation through the oil and the cell pellets were analyzed for radioactivity as in uptake experiments.

Measurement of long-term uptake † *of purines into cells capable of phosphoribosylation.* The experiments were conducted essentially in the same manner as described for 8-azaguanine uptake into hypoxanthine/guanine phosphoribosyltransferase-deficient cells over periods of 10 to 20 min. The cells from duplicate 0.5-ml of suspension containing labeled substrate were collected by centrifugation through oil and analyzed for radioactivity (total cell-associated radioactivity). Parallel samples of suspension were rapidly frozen in an ethanol/solid CO_2 bath and later analyzed for radioactivity in acid-insoluble material as described previously [17].

When fractionation of intracellular radioactivity was required, labeled cells were centrifuged through oil directly into an acid solution which was further processed and chromatographed as described previously [13].

Determination of octanol buffer partition coefficient (Z_{oct}) †† *as function of pH.* Volumes of 1 ml of octanol were mixed with 1 ml of basal solution adjusted to various pH values and containing (2–6) · 10^5 cpm of radiolabeled substance. The mixtures were incubated on a gyrotory shaker at 37°C for 24 h. After allowing the phases to separate, duplicate 0.2-ml samples of each phase were analyzed for radioactivity. Z_{oct} represents the ratio of the radioactivity in the octanol phase/radioactivity in the buffer phase.

Materials. Materials were purchased as follows: Unlabeled purines and purine analogs from Sigma Chemical Co., St. Louis, MO; [G-^{3}H]hypoxanthine and [8-^{14}C]hypoxanthine from Amersham/Searle, Arlington Heights, IL; 8-aza[2-^{14}C]guanine from ICN Pharmaceuticals, Inc., Irvine, CA and Research Products International Corp., Elk Grove Village, IL; 6-mercapto[8-^{14}C]purine from Research Products International Corp. and New England Nuclear; and cytochalasin B from Aldrich Chemical Co., Milwaukee, WI. Dipyridamole was a gift from Geigy Pharmaceuticals, Yonkers, NY.

Chromatographic analyses with several solvent systems indicated a purity of the 8-aza[2-^{14}C]guanine and 6-mercapto[8-^{14}C]purine from the two sources of greater than 95%.

Results

Facilitated transport of 6-mercaptopurine and 6-thioguanine

Hypoxanthine transport in hypoxanthine/guanine

* Plagemann, P.G.W. and Wohlhueter, R.M., in preparation.

** Wohlhueter, R.M., Erbe, J. and Plagemann, P.G.W., in preparation.

† See footnote (*) in page 50.

†† Partition coefficients have generally been designated K [23]. For clarity, we have used Z, since K has been used to designate a fundamental Michaelis constant for the transporter with respect to a given substrate.

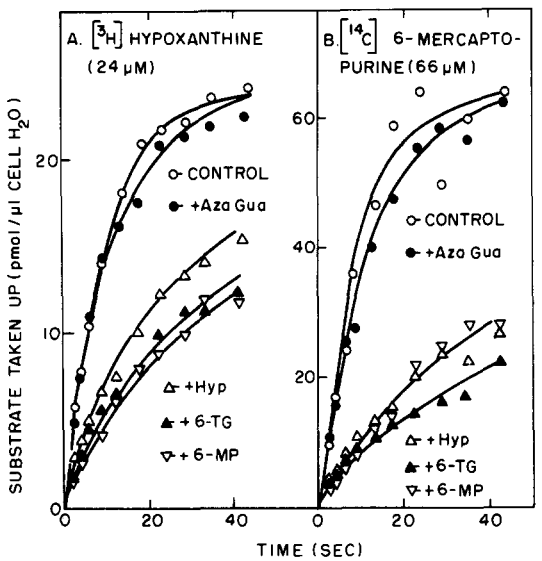

Fig. 1. Effect of other purines on the zero-*trans* influx of hypoxanthine (A) and 6-mercaptopurine (B) in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells. The zero-*trans* accumulation of 24 μM [G-^{3}H]hypoxanthine (38 cpm/pmol) or 66 μM 6-mercapto[8-^{14}C]purine (5.2 cpm/pmol) at 25°C were measured by the rapid kinetic technique as described in Materials and Methods. Unlabeled nucleobases were added as indicated simultaneously with labeled substrate. Final concentrations of added unlabeled 8-azaguanine (AzaGua), hypoxanthine (Hyp), 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP) were in (A) 1.0, 1.0, 0.5, and 0.5 mM, respectively, and in (B) 1.2 mM for all.

phosphoribosyltransferase-deficient Novikoff cells is very rapid (see Fig. 1A, control). In the first-order range of concentration the half time ($t_{1/2}$) for equilibration of hypoxanthine across the membrane of these cells at 25°C falls between 8 and 12 s [13]. We have, therefore, estimated initial transport velocities by computing the initial slope of the time course of hypoxanthine accumulation to transmembrane equilibrium.

Fig. 1A also shows that 6-thioguanine and 6-mercaptopurine strongly inhibited the influx of 24 μM [^{3}H]hypoxanthine. In fact, the rate of accumulation of radioactive hypoxanthine was reduced by 6-thioguanine and 6-mercaptopurine (81–85%) about as effectively as by unlabeled hypoxanthine. In contrast, 1 mM 8-azaguanine had no significant effect on hypoxanthine transport. Similarly, 1.2 mM hypoxanthine, 6-thioguanine and 6-mercaptopurine reduced to about the same extent (84–90%) the zero-*trans* influx of 66 μM 6-mercapto[^{14}C]purine, whereas 8-azaguanine had no significant effect (Fig. 1B). Furthermore, preloading either ATP (and PRPP)-depleted wild type or hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells with 5 mM 8-azaguanine or adenine (which is transported by a different carrier than hypoxanthine [13]) failed to result in countertransport of 2 μM [^{14}C]hypoxanthine, whereas the preloading of the cells with 5 mM 6-thioguanine, 6-mercaptopurine or hypoxanthine resulted in a transient intracellular accumulation of labeled hypoxanthine to a maximum concentration 3- to 6-fold of that in the medium (data not shown; see Ref. 13 for procedure). The results suggested that the hypoxanthine carrier efficiently transports 6-thioguanine and 6-mercaptopurine, but not 8-azaguanine.

In a more detailed analysis we determined the effect of 570 μM 6-thioguanine on the zero-*trans* influx of hypoxanthine at 8 concentrations (10–280 μM) in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells. Computer fits of Eqn. 1 to the complete time courses of hypoxanthine accumulation to transmembrane equilibrium at the 8 substrate concentrations (data not shown) yielded best fitting parameters of $K = 412 \pm 18$ μM and $V = 56 \pm 1$ pmol/μl cells H_2O per s ($r_{y,\hat{y}} = 0.9969$) and $K = 1904 \pm 607$ μM and $V = 44 \pm 10$ pmol/μl cell H_2O per s ($r_{y,\hat{y}} = 0.9048$) in the absence and presence of 6-thioguanine, respectively. As observed for other substrates of the hypoxanthine carrier [13], the presence of 6-thioguanine mainly caused an increase in K, but also a slight reduction in V (mixed type inhibition). A plot of K/V against inhibitor concentration yielded a $K_{i,slope}$ (see Ref. 24) of about 200 μM for the inhibition of hypoxanthine transport by 6-thioguanine.

Representative initial time courses of intracellular accumulation of various concentrations of 6-mercaptopurine in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells are illustrated in Fig. 2. The kinetic parameters for the zero-*trans* influx of 6-mercaptopurine were estimated by fitting Eqn. 1 to the complete time courses of accumulation of six concentrations of substrate to transmembrane equilibrium. The best fitting parameters ($K = 372 \pm 23$ μM; $V = 30 \pm 0.7$ pmol/μl cell H_2O per s) were similar to those for hypoxanthine transport in these

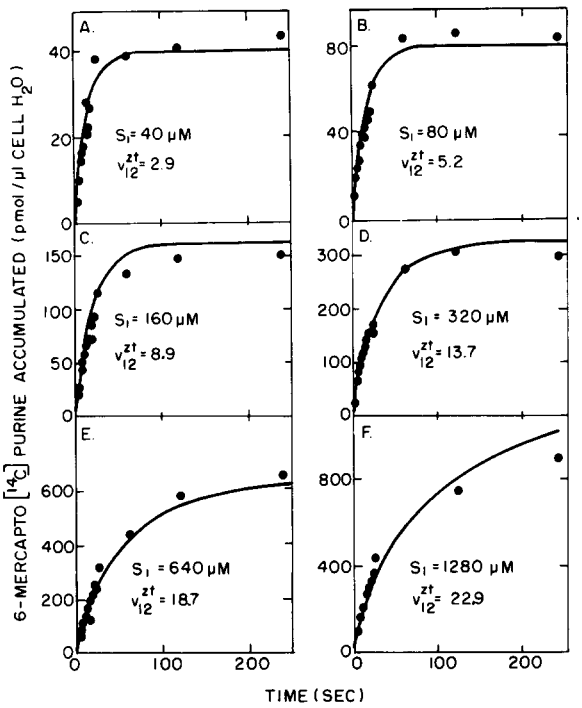

Fig. 2. Zero-*trans* influx of 6-mercaptopurine in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells at 25°C. Time courses of radioactivity accumulation to transmembrane equilibrium from mercapto[^{14}C]purine (336 cpm/μl, irrespective of concentration) were determined by the rapid kinetic technique as described in Materials and Methods. Radioactivity/cell pellet was corrected for substrate trapped in extracellular space (3.3 μl/cell pellet) and converted to pmol/μl cell H_2O on the basis of an intracellular H_2O space of 25 μl cell pellet. Eqn. 1 was fitted to the pooled data. The best fitting parameters were $K = 372 \pm 23$ μM, and $V = 30 \pm 0.7$ pmol/μl cell H_2O per s (correlation coefficient $r_{y,\hat{y}} = 0.9769$).

cells (noted above; Refs. 13, 14). The zero-*trans* influx of 320 μM 6-mercaptopurine was about the same at various pH values between 5.8 and 7.8 (data not shown) as previously reported for hypoxanthine [34].

Non-mediated permeation of 8-azaguanine

Compared to hypoxanthine and 6-mercaptopurine, 8-azaguanine entered hypoxanthine/guanine phosphotransferase-deficient Novikoff cells only very slowly, and its accumulation varied greatly as a function of extracellular pH (pH_e), decreasing progressively with an increase in pH_e between 5.5 and 8.0 (Fig. 3). At a pH below about 6.8, radiolabeled 8-azaguanine accumulated to intracellular levels in excess of exogenous levels regardless of the temperature of incubation. In fact, at pH 5.8 the maximum intracellular concentration of 8-azaguanine was 2- to 3-times that in the medium, whereas at pH_e 7.9 it was only 10% of that in the medium. Both the rate of entry and the final intracellular steady-state concentration varied in the same manner as a function of pH_e. When pH_e was changed to 7.4, after 10 min of incubation at pH_e of about 6.0, the intracellular concentration of 8-azaguanine decreased rapidly to a lower steady-state level (Fig. 3B).

These results can be understood as a consequence of the ionization of 8-azaguanine ($pK_a = 6.6$; Ref. 25), if it is assumed that the cationic species prevalent at higher pH is excluded from passing the cell membrane, while the non-ionic species is equilibrated across the membrane. Such differential permeability of non-ionic and ionic species is well known for various weak acids and bases and serves as basis of their utility in measuring intracellular pH (pH_i) in bacteria and eucaryotic cells [26,27]. The total concentration of the substances in the intracellular (C_i) and extracellular (C_e) spaces are a function of pH_i, pH_e and pK_a according to the following equation:

$$\frac{C_i}{C_e} = \frac{10^{(pH_i - pK_a)} + 1}{10^{(pH_e - pK_a)} + 1} \qquad (2)$$

This equation has been derived from those presented in other publications [26,27] to facilitate computer simulations of the relationship between C_i/C_e, pH_i and pH_e. The theoretical relationship between C_i/C_e, pH_e and pH_i for 8-azaguanine is illustrated in Fig. 4B. $C_i/C_e = 1$ only when $pH_i = pH_e$ and C_i/C_e is exquisitely sensitive to changes in pH_e and pH_i, especially when pH_i is high relative to pH_e.

The C_i/C_e ratios and corresponding pH_i estimated from the data in Fig. 3A and those fom another similar experiment (data not shown) are plotted against pH_e in Fig. 4C and are consistent with the model.

From these results alone it cannot be decided whether the cationic form of 8-azaguanine is excluded from passing the membrane because of the specificity of a transporter for the non-ionized form or because passage is not mediated by a carrier and thus has to occur through the lipid bilayer. Our over-

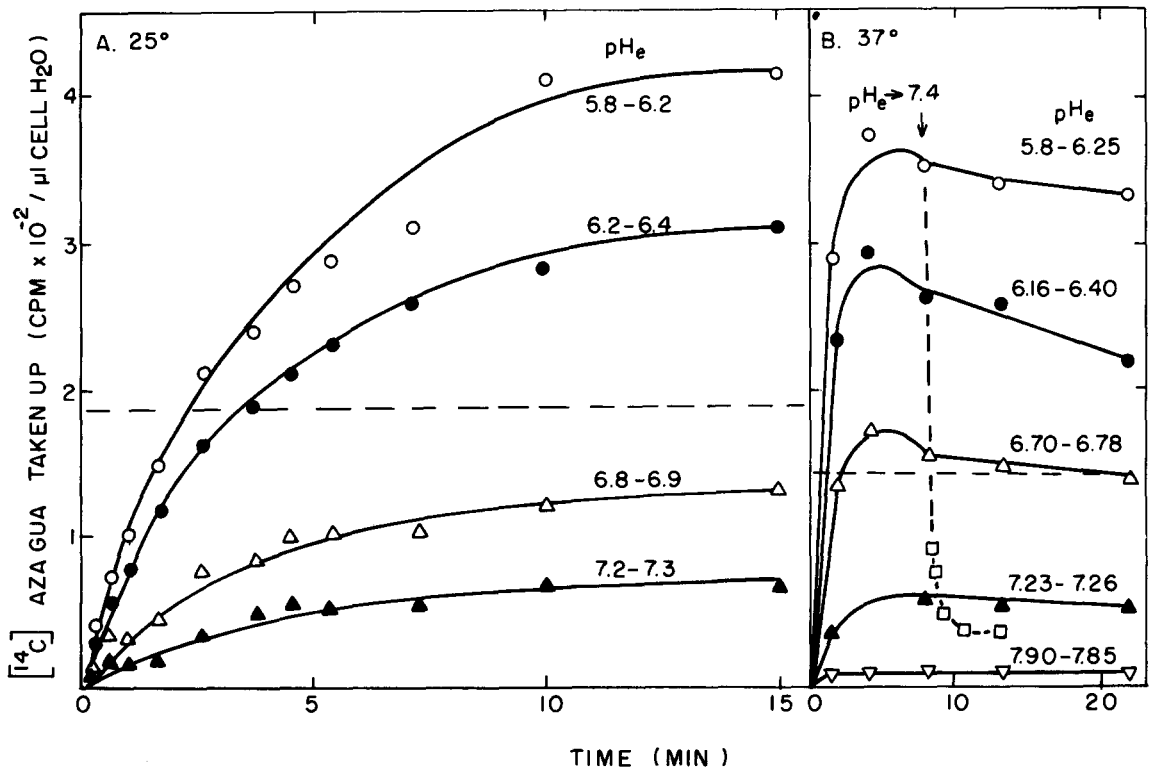

Fig. 3. Effect of pH_e on the accumulation of 8-azaguanine by hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells at 25°C (A) and 37°C (B). Uptake of 4 μM 8-aza[2-^{14}C]guanine (48 cpm/pmol) was measured as described in Materials and Methods. The cells were suspended in glucose-free BM42B containing 20 mM Hepes and adjusted to the indicated pH_e. The values shown represent the pH of the suspensions at the beginning and the end of the incubation period. After 10 min of incubation the pH of a portion of the pH-6 suspension was adjusted to 7.4 by the addition of 1 N NaOH and samples of cells monitored for cell associated radioactivity (□- - - - -□). All points represent averages of duplicate samples. The broken lines indicate the intracellular radioactivity concentration equivalent to that in the medium.

all results support the latter view (see below). The permeability coefficient (P) of a substance through a lipid bilayer is a function of the solubility of the substance in membrane lipids relative to that in an aqueous medium (Z) *, the diffusion constant for its movement within the membrane (D_{mem}) and the reciprocal of the thickness of the membrane (l) [23,28]:

$$P = \frac{Z \times D_{mem}}{l} \qquad (3)$$

where D_{mem} is a complex function of the molecular properties of the substance such as molecular weight and shape. Thus, for uncharged substances with similar molecular dimensions the lipid solubility of a substance is the main determinant of its rate of non-mediated permeation into cells [23,28]. Charged groups impede permeation because of lowering the lipid solubility of a substance. We have assessed the lipid solubility of substances by their solubility in octanol relative to that in an aqueous buffer solution (Z_{oct}), since previous results indicated that octanol possesses solubility properties similar to those of the lipids extracted from Novikoff cells [29]. Fig. 4A shows that the lipid solubility of 8-azaguanine as gauged by Z_{oct}, represents a Henderson-Hasselbach curve for a substance with $pK_a = 6.6$ (Fig. 4A also depicts Z_{oct} for hypoxanthine vs. pH), and predicts a difference in permeability of non-ionic and ionic forms of at least 100-fold, as required by the weak

* See footnote (††) on p. 51.

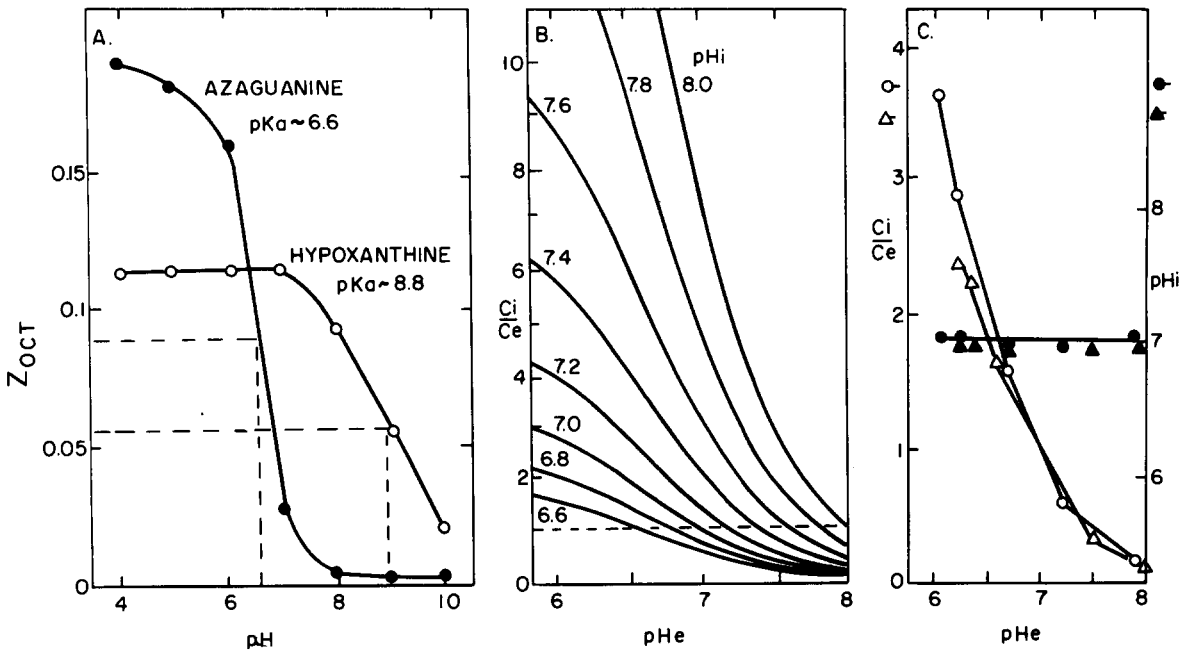

Fig. 4. Octanol partition coefficient (Z_{oct}) for 8-azaguanine and hypoxanthine as a function of pH (A) and the theoretical (B) and experimental (C) relationships between the ratio of intracellular to extracellular concentration (C_i/C_e) of 8-azaguanine ($pK_a = 6.6$) and pH_i and pH_e. Z_{oct} was determined as described in Materials and Methods. The curves in (B) of C_i/C_e as a function of pH_e were generated according to Eqn. 2 with pH_i fixed at the indicated values. The C_i/C_e ratios and pH_e values in (C) were estimated from the results depicted in Fig. 3B (at 5 min of incubation) and those of a second experiment conducted in the same manner. The pH_i values were calculated by substituting the experimentally determined values of C_i/C_e and pH_e into the following equation:

$$pH_i = pK_a + \log(C_i/C_e\,(1 + 10^{(pH_e - pK_a)}) - 1).$$

acid model. The actual rate of permeation of the ionic species can be estimated only roughly from the data of Fig. 3B ($pH_e = 7.90$ to 7.85), but was less than about 1/50 that for the non-ionized species ($pH_e = 5.8$ to 6.25). In relation to the data in Fig. 3 it also needs to be emphasized that the initial rate of entry of aza[^{14}C]guanine reflects the proportion of substrate that is non-ionized at the different pH_e, whereas the final intracellular steady concentration is determined by differences between pH_e and pH_i.

In the following experiments we measured aza-[^{14}C]guanine accumulation in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells at pH_e 5.8 to 6.2, the lowest pH at which the cells can be maintained over the period of the experiment without risk of significant cell damage. At this pH at least 80% of the 8-azaguanine was non-ionized (Fig. 4A). Fig. 5A shows the initial entry velocity of 8-aza-guanine entry was only 5% of that of hypoxanthine under identical experimental conditions. Uptake of radioactivity from 10 μM aza[^{14}C]guanine was not affected by the addition of 1 mM unlabeled 8-aza-guanine. (Fig. 5B). In additional experiments no sign of saturation was detectable up to a concentration of 2 mM 8-azaguanine, the highest concentration of 8-azaguanine that could be tested in our experimental design without encountering solubility problems.

The slow entry of 8-azaguanine was not a peculiar property of the hypoxanthine/guanine phosphoribo-syltransferase-deficient Novikoff cells. Azaguanine accumulation was similarly slow or even slower in ATP (PRPP)-depleted wild-type Novikoff cells and in ATP-depleted Chinese hamster ovary, mouse P388 and L cells, and in hypoxanthine/guanine phospho-ribosyltransferase-deficient Novikoff cells was not affected by ATP depletion (data not shown).

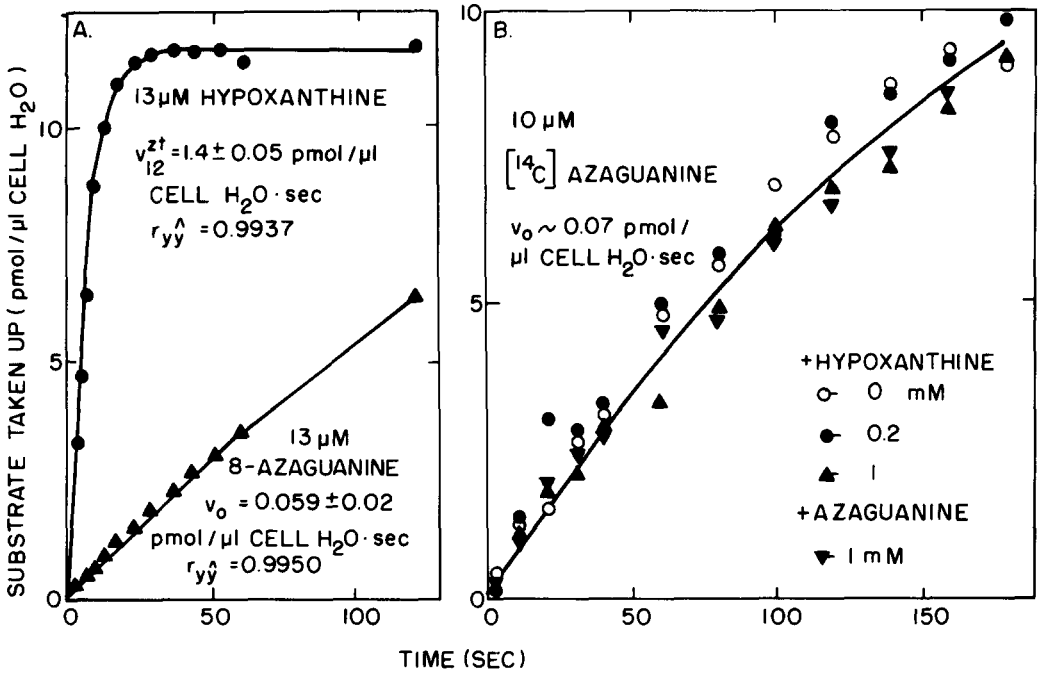

Fig. 5. Comparison of hypoxanthine and 8-azaguanine accumulation in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells (A) and effects of 8-azaguanine concentration and hypoxanthine on the accumulation of radiolabeled 8-azaguanine. A. The uptake of 13 μM [^{3}H]hypoxanthine (35 cpm/pmol) and of 13 μM aza[^{14}C]guanine (26 cpm/pmol) at 25°C and pH 6.2 were determined by the rapid kinetic technique and the initial uptake velocities estimated as described in Materials and Methods. The experiment in (B) was conducted in the same manner, except that the aza[^{14}C]guanine concentration was 10 μM (25 cpm/pmol) and unlabeled 8-azaguanine or hypoxanthine were added to the indicated final concentrations simultaneously with labeled 8-azaguanine.

The rate of entry of non-ionized 8-azaguanine was compatible with that expected for a substance with its lipid solubility, if entry were non-mediated. This is indicated by comparing its k/Z ratio to that of L-glucose, cytosine [30], orotate [31], and 5,5'-dimethyl-2,4-oxazolidinedione (DMO), the permeation of all of which appears to be non-saturable (Table I). As indicated by the similarity of the k/Z ratios for these substances as well as 8-azaguanine, their rates of permeation are directly proportional over a 1 000-fold range to their lipid solubilities. In contrast, the k/Z ratio for hypoxanthine (whose permeation is certainly mediated) lies about two orders of magnitude higher. The rate constant k is used here instead of the permeability constant P (see Eqn. 3) which is normalized to area, because the surface area of the cultured cells is unkwown, and it is proportional to P.

The uptake of 10 μM aza[^{14}C]guanine was not inhibited by the presence of hypoxanthine in the medium (Fig. 5B), nor stimulated by the presence of unlabeled 8-azaguanine on the *trans* side of the membrane (Fig. 6A). In addition, comparison of the data in Fig. 6A and B shows that the rate of exit of 8-azaguanine was comparable to its rate of entry taking into account that pH_i was about 6.9 to 7.1 (Fig. 4B) and that at this pH only about 30% of the intracellular labeled 8-azaguanine was not ionized (Fig. 4A) and thus available for exit. 8-Azaguanine exit was also not affected by the presence of substrate or 1 mM hypoxanthine on the *trans* side of the membrane (Fig. 6B). It should be emphasized that in all the experiments discussed already extreme care was taken to assure that pH_e was identical in the suspensions undergoing different treatments. As shown already, small differences in pH_e can have marked effects on both the rate of permeation and the final steady-state concentration of radioactivity.

Entry of 8-azaguanine into hypoxanthine/guanine

TABLE I

PARTITION COEFFICIENTS, MOLECULAR WEIGHTS, AND FIRST ORDER RATE CONSTANTS FOR PERMEATION OF VARIOUS SUBSTANCES

Z = partition coefficient: Substrate concentration in octanol/aqueous buffer solution as determined by Graff [30]. Data are from Graff et al. [30], Fig. 4, and unpublished results. Z for 8-azaguanine and 5',5'-dimethyl-2,4-oxazolidinedione (DMO) is at pH < 6.0, for hypoxanthine between pH 4 and 7, all others are independent of pH between 6 and 8. k = apparent first order rate constant at 37°C: Values for L-glucose and cytosine are from Graff et al. [30]; that for orotate from Wohlhueter et al. [31]; that for 8-azaguanine has been calculated from data in the present study ($v_0 = S_1 k$); that for DMO was determined in the same manner as for 8-azaguanine at pH 5.8–6.0 and represents unpublished data. k for hypoxanthine was calculated as V/K corrected to 37°C ($k_{25°C} \times 3$) on the basis of known temperature dependence of its transport (Ref. 15; and unpublished data).

Substrate	Molecular weight	Z (± S.E.)	k (min^{-1})	k/Z
L-Glucose	180	0.00158 ± 0.00040	0.00404	2.6
Cytosine	111	0.0352 ± 0.00067	0.173	4.9
Orotate	156	0.00438 ± 0.00049	0.0060	1.4
DMO	129	0.983 ± 0.078	4.8	4.9
8-Azaguanine (non-ionized)	150	0.173 ± 0.064	0.3–0.8	1.7–4.6
Hypoxanthine	136	0.115 ± 0.009	24	210

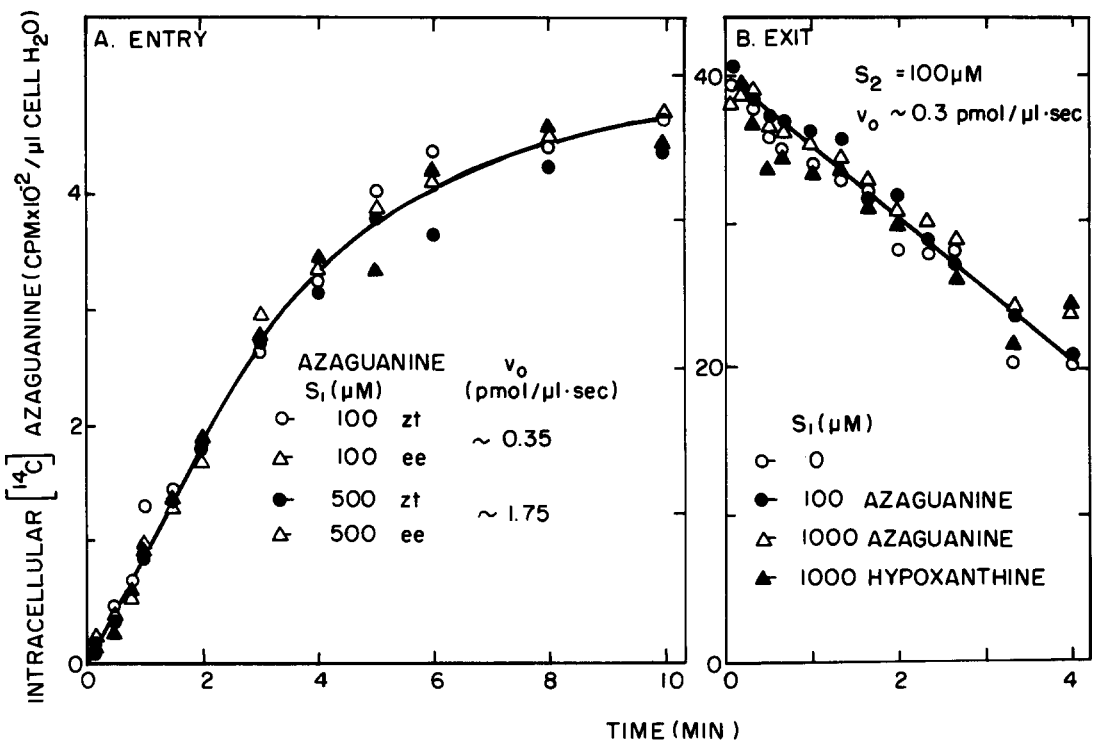

Fig. 6. Effect of substrate on the *trans* side on 8-azaguanine entry into (A) or exit from (B) hypoxanthine/guanine phosphoribosyltransferase-deificient Novikoff cells at 25°C. 8-Azaguanine accumulation and exit from preloaded cells were measured as described in Materials and Methods. In (A) uptake of 100 or 500 µM aza[^{14}C]guanine (420 cpm/µl, irrespective of concentration) were measured at pH 6.0 into cells that had been (equilibrium exchange, ee) or had not been (zero-*trans*, zt) preincubated at pH 6.0 with the same concentration of unlabeled 8-azaguanine at 37°C for 40 min. In (B) the cells were preloaded with 100 µM [^{14}C]azaguanine (28 cpm/pmol) at 37°C for 40 min and exit was measured into BM42B, pH 7.5, which was supplemented where indicated with 100 or 1 000 µM unlabeled 8-azaguanine or 1 000 µM hypoxanthine.

phosphoribosyltransferase-deficient Novikoff cells was temperature dependent. The initial velocities of entry of 5 μM 8-azaguanine (43 cpm/pmol) at 37, 25, 16 and 6°C (pH 6.2) were 0.027, 0.013, 0.0079 and 0.0029 pmol/μl cell H_2O per s, respectively. An Arrhenius plot of the data (not shown) was approximately linear and yielded an activation energy of about 12 kcal/mol). A similar experiment with ATP-depleted, wild-type Novikoff cells yielded an activation energy about 17 kcal/mol. The initial rate of 10 μM 8-azaguanine was inhibited about 70 to 80% and 15 to 20% by 20 μM dipyridamole and 20 μM cytochalasin B, respectively (data not shown).

Relationships between permeation and intracellular phosphoribosylation of 8-azaguanine and 6-mercaptopurine

We have demonstrated previously [13] that the uptake of labeled hypoxanthine above equilibrium concnetrations by wild type Novikoff cells (see Fig. 7A, control) and other types of cultured cells, in which hypoxanthine is readily phosphoribosylated, reflects the accumulation of nucleotides and that phosphoribosylation is the primary rate determinant in the uptake of hypoxanthine over a 10–20-min time period ('long-term uptake'). 8-Azaguanine (500 μM) had relatively little effect on this conversion of hypoxanthine to nucleotides, whereas 100 μM 6-mercaptopurine or 6-thioguanine was strongly inhibitory (Fig. 7A). The lack of effect of 8-azaguanine extended to other 8-azapurine nucleobases (Fig. 7B). Thus 8-azapurine nucleobases seem to be poor substrates for hypoxanthine/guanine phosphoribosyltransferase under physiological conditions relative to 6-mercaptopurine and 6-thioguanine, which readily compete with hypoxanthine as phosphoribosyl acceptors.

Fig. 8A illustrates that the long-term uptake of 10 μM aza[^{14}C]guanine by wild type Novikoff cells at 37°C and pH 7.0 was much slower than that of hypoxanthine (cf. Fig. 7A). In fact, the rate of uptake of 8-azaguanine measured over a 1-h period was similar to the initial velocity of 8-azaguanine entry into cells as measured in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells under equivalent conditions of pH, temperature and substrate concentration (Figs. 5 and 6). Furthermore, chromatographic analysis of the acid-soluble pool of the cells

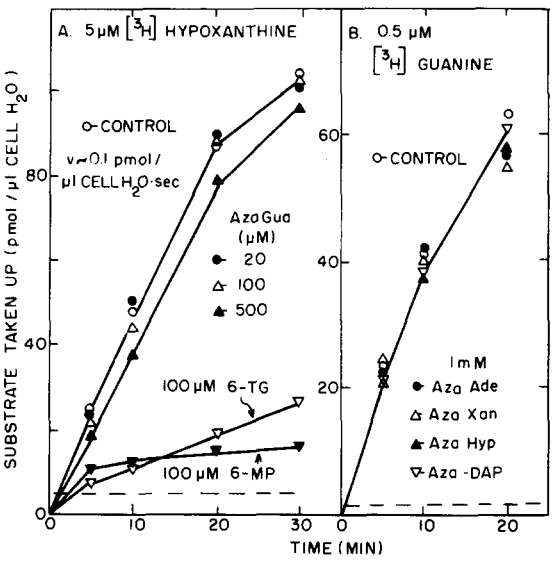

Fig. 7. Effect of 6-thioguanine, 6-mercaptopurine and various azapurine analogs on the long-term uptake of hypoxanthine (A) and guanine (B) in wild type Novikoff cells at 37°C. Samples of a suspension of cells in BM42B were supplemented with 5 μM [^{3}H]hypoxanthine (60 cpm/pmol) or 0.5 μM [^{3}H]guanine (600 cpm/pmol) and where indicated with 6-thioguanine (6-TG), 6-mercaptopurine (6-MP), 8-azaguanine (AzaGua), 8-azaadenine (AzaAde), 8-azaxanthine (AzaXan), 8-azahypoxanthine (AzaHyp) or 8 aza-2,6-diaminopurine (AzaDAP). Substrate uptake was measured as described in Materials and Methods. The broken lines indicate the intracellular concentration of substrate equivalents equal to that in the medium.

indicated that the intracellular concentration of free 8-azaguanine remained very low throughout the entire incubation period; it had attained a steady state within 1 min of incubation which did not exceed 10% of the extracellular concentration (Fig. 8A). Such low steady-state levels of free substrate can only be maintained if the capacity of the cells to phosphoribosylate the substrate exceeds its rate of entry [13,32]. Small amounts of labeled aza-GMP were detected at 1 min of incubation, but at later time points the amounts of label in aza-GMP were so low relative to the total radioactivity of the acid extract that they could not be quantitated adequately. The main intracellular labeled component was aza-GTP and the curve for the accumulation of radioactivity in total cell material reflected the sum of the radioactivity in aza-GTP and in nucleic acids. Com-

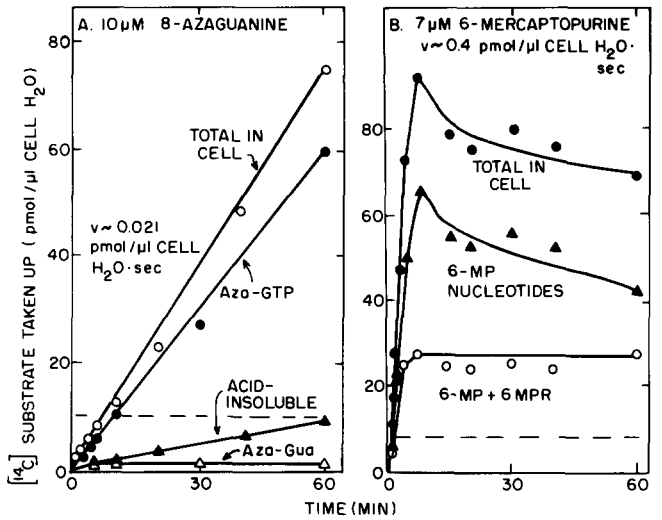

Fig. 8. Uptake of [^{14}C]azaguanine at 37°C (A) and of mercapto[^{14}C]purine at 25°C (B) by wild type Novikoff cells. The uptake of 10 μM aza[^{14}C]guanine (10 cpm/pmol) and of 7 μM mercapto[^{14}C]purine (29 cpm/pmol) into total cell material and their incorporation into acid-insoluble material were determined as described in Materials and Methods. In addition, the acid soluble pools were extracted from samples of cells and analyzed chromatographically. The intracellular concentrations of aza-GTP and 8-azaguanine (AzaGua) in (A) and of 6-mercaptopurine (6-MP) plus 6-mercaptopurine riboside (6-MPR) and of 6-mercaptopurine nucleotides in (B) were calculated on the basis of the chromatographic separations and the total intracellular concentrations of substrate equivalents. The broken lines indicate the intracellular concentations of substrate equivalents equal to those in the medium.

bined these results suggest that, at this concentration of 8-azaguanine, permeation was the main rate-determining step in its incorporation into the nucleotide pool.

The situation was quite different with 6-mercaptopurine. The uptake of 7 μM mercapto[^{14}C]purine was about 20 times more rapid than that of 8-azaguanine and about as rapid as that of hypoxanthine, so that the intracellular concentration of 6-mercaptopurine equivalents exceeded that in the medium within 10 sec of incubation at 25°C (data not shown). Uptake was linear for about 4 min and then ceased abruptly (Fig. 8B). A similar time course of net uptake was observed in four independent experiments with 6-mercaptopurine concentrations ranging from 1 to 160 μM. Chromatographic fractionation of the acid-soluble cell contents showed that within 20 s of incubation 6-mercaptopurine nucleotides represented the primary intracellular components. The main nucleotides formed comigrated with IMP and 6-mercaptopurine riboside 5′-phosphate (6-thio-IMP). Thus, the cessation in 6-mercaptopurine uptake manifest by 4 min (Fig. 8B) was most likely related to a slow conversion of 6-thio-IMP to 6-thio-GMP or of the further phosphorylation of 6-thio-IMP or 6-thio-GMP [33]. Chromatographic analyses in other solvents indicated substantial formation of 6-mercaptopurine riboside (or a substance comigrating with it). Resolution of nucleoside and base, however, was not adequate to allow accurate quantitation. The riboside(s) probably arose from the degradation of the monophosphates since little, if any, ribosides accumulated in hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells (data not shown). The incorporation of 6-mercaptopurine into nucleic acid by Novikoff at 25°C was negligible and also very slow at 37°C (data not shown).

Discussion

Our results demonstrate that 6-mercaptopurine and 6-thioguanine enter Novikoff cells by facilitated diffusion. Both seem to be transported by the same carrier as hypoxanthine and with similar efficiency. This conclusion is suggested by the similarity of the apparent Michaelis-Menten constants and maximum

velocities for the transport of 6-mercaptopurine and hypoxanthine, and by the similarity of these Michaelis-Menten constants to the apparent K_i of inhibition of the zero-*trans* influx of hypoxanthine by 6-thioguanine. Furthermore, as shown in Fig. 1A, unlabeled 6-mercaptopurine, 6-thioguanine and hypoxanthine reduced the entry of labeled hypoxanthine to about the same extent. Zero-*trans* influx of the nucleobases was determined in hypoxanthine/guanine phosphoribosyltransferase-deficient cells so that these measurements were not complicated by intracellular phosphoribosylation of the bases. Our finding of equivalent zero-*trans* influx and efflux and inward and outward equilibrium exchange of hypoxanthine in these cells rules out a *trans*-effect and indicates that the transporter exhibits directional symmetry and equal mobility of loaded and empty carrier, just as reported for the nucleoside transporter [14,22].

On the other hand, 8-azaguanine is, at best, a very poor substrate for this transporter. This conclusion is indicated by the finding that 8-azaguanine enters the cells less than 5% as rapidly as hypoxanthine or 6-mercaptopurine, that 8-azaguanine has no effect on hypoxanthine transport and that hypoxanthine and other substrates of the hypoxanthine carrier have neither a significant effect on 8-azaguanine permeation nor effect its countertransport (Figs. 1, 5 and 6). Our data cannot prove unequivocally that 8-azaguanine enters cells mainly or exclusively by non-mediated permeation, but they are fully consistent with this conclusion. In particular, (i) 8-azaguanine uptake shows no saturability, up to the highest feasible concentration of 2 mM, and (ii) the rate of permeation of its non-ionic form is related to its lipid solubility by the same proportionality as observed for a number of other substances whose permeation is believed to be non-mediated [30,31]. To a first approximation these results are also comparable to those observed for the permeation of low molecular weight non-electrolytes into plant cells and human red cells [23,28].

This relationship between permeability and lipid solubility seems to pertain also to the ionic form of the weak acid 8-azaguanine; both parameters are much decreased in comparison to the non-ionic form. A similar correlation between Z and rate of permeation as a function of pH has been observed for DMO *. It should be pointed out, of course, that discrimination against ionic species is not diagnostic of non-mediated permeation: the non-ionic form of 5′-fluorouracil ($pK_a = 8.0$) is a substrate of the pyrimidine transporter of Novikoff cells, while the ionic form is not [31].

Some properties of 8-azaguanine permeation as reported here are not necessarily indicative of non-mediated permeation, but are not inconsistent with the interpretation. For example, a high activation energy does not distinguish between facilitated transport and non-mediated permeation; both processes are similarly affected by temperature [14,23,28,30]. Because Z is relatively independent of temperature, it has been suggested [23,28] that the main effect of temperature on non-mediated permeation is on D_{mem} (see Eqn. 3), probably as a result of its effect on the viscosity of the membrane lipids. Nor is an inhibition of permeation of 8-azaguanine by dipyridamole and cytochalasin B decisive evidence for or against mediation. These compounds inhibit permeation of L-glucose (which is almost certainly not mediated) as well as permeation via several distinct carriers, as those for hexoses, nucleosides and nucleobases. The mechanistic basis for these diverse inhibitory effects are simply not understood [14]. One might conceive, however, that interaction of these lipophilic substances with hydrophobic surfaces of integral membrane proteins might impede facilitated transport and non-mediated permeation alike.

If the entry of 8-azaguanine is non-mediated, as we propose, a defect in the hypoxanthine carrier would not be expected to endow cells with a selective resistance to 8-azaguanine, and the temperature sensitive defect in 8-azaguanine incorporation by the azaguanine-resistant clone of Chinese hamster ovary cells isolated by Harris and Whitmore [12] must have causes other than a high temperature sensitivity of the hypoxanthine carrier. For example, it could be due to some alteration in the substrate specificity or pH dependence (see below) of hypoxanthine/guanine phosphoribosyltransferase.

The present results also help to define the relationship between permeation and phosphoribosylation of purine analogs. Entry into the cells is the primary rate determinant in the incorporation of 8-azaguanine into the nucleotide pool and into nucleic acids (Fig. 8A), whereas overall uptake of 6-mercaptopurine is not limited by influx or phosphoribosylation, but by the

* Plagemann, P.G.W. and Wohlhueter, R.M., in preparation.

conversion of 6-thio-IMP to 6-thio-GMP or by the phosphorylation of 6-thio-IMP or 6-thio-GMP (Fig. 8B) [34]. 8-Azaguanine appears to compete only ineffectively in vivo for phosphoribosylation by phosphoribosyltransferase in comparison to hypoxanthine, 6-mercaptopurine and 6-thioguanine, as judged from the fact that 8-azaguanine has far less effect on the long term uptake of [^{3}H]hypoxanthine than does 6-mercaptopurine, 6-thioguanine or unlabelled hypoxanthine [7] (Fig. 7). The inefficient phosphoribosylation of 8-azaguanine in whole cells is probably a consequence of the largely anionic state of this compound at intracellular pH. Hypoxanthine/guanine phosphoribosyltransferase of human erythrocytes seems to accept only non-ionized purines as substrates; thus the apparent pH optima with 8-azaguanine, 6-mercaptopurine, 6-thioguanine, and guanine as substrate coincide with their pK_a values [35]. At an intracellular pH of 7.0 and a total concentration of 50–100 μM, 8-azaguanine was found to be only 1–5% as efficient a substrate for hypoxanthine/guanine phosphoribosyltransferase than is hypoxanthine. We have confirmed these relationships for hypoxanthine/guanine phosphoribosyltransferase from Novikoff cells with hypoxanthine and 8-azaguanine (data not shown).

The observed differences in substrate affinity of 8-azaguanine, on the one hand, and of 6-mercaptopurine and 6-thioguanine, on the other, toward transport carriers and toward hypoxanthine/guanine phosphoribosyltransferase may account for some of the unexplained differences in response of cells to treatment with these purine analogs. Such differences need to be considered in the evaluation of the nature of resistance and cross-resistance of mutants to these drugs and in the clinical application of these and other substrate analogs.

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References

1 McElwain, T.J. (1976) in Scientific Foundations of Oncology (Symington, T. and Carter, R.L., eds.), pp. 633–640, Year Book Medical Publishers, Inc., Chicago
2 Henderson, J.F. and Paterson, A.R.P. (1973) Nucleotide Metabolism, pp. 128–130, and 266, Academic Press, New York
3 Brockman, R.W. (1974) in Antineoplastic and Immunosuppressive Agents (Sartorelli, A.C. and Johns, D.G., eds.) Handbook Expt. Pharmacol., 38/1, 352–410
4 Thompson, L.H. (1973) Methods Cell Biol. 6, 209–281
5 Nikaido, O. and Fox, M. (1976) Mutat. Res. 35, 279–287
6 Fox, M. and Radacic, M. (1978) Mutat. Res. 49, 275–296
7 Van Diggelen, O.P., Donahue, T.F. and Skin, T.F. (1979) J. Cell. Physiol. 98, 59–72
8 Fox, M., Boyle, J.M. and Fox, B.W. (1976) Mutat. Res. 35, 289–310
9 Beaudet, A.L., Roufa, D.J. and Caskey, C.T. (1973) Proc. Natl. Acad. Sci. USA 70, 320–324
10 Morrow, J., Colofiore, J. and Rintoul, D. (1973) J. Cell. Physiol. 81, 97–110
11 Bieber, A.L. and Sartorelli, A.C. (1964) Cancer Res. 24, 1210–1215
12 Harris, J.F. and Whitmore, G.F. (1974) J. Cell. Physiol. 83, 43–52
13 Marz, R., Wohlhueter, R.M. and Plagemann, P.G.W. (1979) J. Biol. Chem. 254, 2329–2338
14 Plagemann, P.G.W. and Wohlhueter, R.M. (1980) Curr. Top. Membrane Transp. 14, 225–330
15 Zylka, J.M. and Plagemann, P.G.W. (1975) J. Biol. Chem. 250, 5756–5767
16 Ward, G.A. and Plagemann, P.G.W. (1969) J. Cell. Physiol. 73, 213–231
17 Plagemann, P.G.W. (1971) J. Cell. Physiol. 77, 213–240
18 Plagemann, P.G.W., Marz, R. and Erbe, J. (1976) J. Cell. Physiol. 89, 1–18
19 Schneider, E.L., Stanbridge, E.J. and Epstein, C.J. (1974) Exptl. Cell. Res. 84, 311–318
20 Eilam, Y. and Stein, W.D. (1974) Methods Membrane Biol. 2, 283–354
21 Wohlhueter, R.M., Marz, R., Graff, J.C. and Plagemann, P.G.W. (1978) Methods Cell Biol. 20, 211–236
22 Wohlhueter, R.M., Marz, R. and Plagemann, P.G.W. (1979) Biochim. Biophys. Acta 553, 262–283
23 Lieb, W.R. and Stein, E.D. (1971) Curr. Top. Membrane Transp. 2, 1–39
24 Segel, I.W. (1975) Enzyme Kinetics, pp. 170–178, John Wiley and Sons, Inc., New York
25 Parks, R.E., Jr. and Agarwal, K.C. (1975) in Antineoplastic and Immunosuppressive Agents (Eichler, O., Farah,

A., Herken, H. and Welch, A.D., eds.), Handbook Exptl. Pharmacol. 38/2, 458–467
26 Cohen, R.D. and Isles, R.A. (1975) Crit. Rev. Clin. Lab. Sci. 6, 101–143
27 Waddel, N.J. and Bates, R.G. (1979) Physiol. Rev. 49, 285–329
28 Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 1–95
29 Plagemann, P.G.W., Graff, J.C. and Wohlhueter, R.M. (1977) J. Biol. Chem. 252, 4191–4201
30 Graff, J.C., Wohlhueter, R.M. and Plagemann, P.G.W. (1977) J. Biol. Chem. 252, 4185–4190
31 Wohlhueter, R.M., McIvor, R.S. and Plagemann, P.G.W. (1980) J. Cell. Physiol. 104, 309–319
32 Wohlhueter, R.M. and Plagemann, P.G.W. (1980) Int. Rev. Cytol. 64, 171–240
33 Zimmermann, T.P., Chu, L.C., Bugge, C.J.L., Nelson, D.J., Miller, R.L. and Elion, G.B. (1974) Biochem. Pharmacol. 23, 2736–2749
34 Nelson, J.A., Carpenter, J.W., Rose, L.M. and Adamson, D.J. (1975) Cancer Res. 35, 2872–2878
35 Kong, C.M. and Parks, R.E., Jr. (1964) Mol. Pharmacol. 10, 648–656