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MEDIATED TRANSPORT OF NUCLEOSIDES IN HUMAN ERYTHROCYTES

SPECIFIC BINDING OF THE INHIBITOR NITROBENZYLTHIOINOSINE TO NUCLEOSIDE TRANSPORT SITES IN THE ERYTHROCYTE MEMBRANE

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SUMMARY

Transport of purine and pyrimidine nucleosides in human erthrocytes, which occurs by facilitated diffusion, is inhibited by 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine) and related compounds. The present studies with nitrobenzylthioinosine indicated that inhibition of nucleoside transport resulted from binding of this compound to nucleoside transport sites in the erythrocyte membrane. Two modes of retention of nitrobenzylthioinosine by intact erythrocytes were apparent: saturable binding with an apparent dissociation constant of 10^{-9} M and non-saturable intracellular accumulation of inhibitor. High affinity binding of nitrobenzylthioinosine was significantly reduced by the related transport inhibitors, 6-(methylthio)-9-β-D-ribofuranosylpurine and 2-amino-6-((2-hydroxy-5-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine. The high affinity binding sites were identified as transport sites by demonstrating that inhibition of uridine transport was proportional to the number of high affinity sites occupied by nitrobenzylthioinosine. The results presented indicate that nitrobenzylthioinosine interacts specifically with nucleoside transport elements of the human erythrocyte.

INTRODUCTION

Transport of nucleosides across the plasma membrane of human erythrocytes was previously shown [1–3] to be a mediated process that exhibited the properties of facilitated diffusion as defined by Stein [4]. Cass and Paterson [2, 5] analyzed the

Abbreviations: nitrobenzylthioguanosine, 2-amino-6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine; nitrobenzylthioinosine, 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine; hydroxynitrobenzylthioguanosine, 2-amino-6-((2-hydroxy-5-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine; methylthioinosine, 6-(methylthio)-9-β-D-ribofuranosylpurine; nitrobenzylthioguanine, 2-amino-6-((4-nitrobenzyl)thio)-purine; TES, *N*-tris(-hydroxymethyl)methyl-2-aminoethane sulfonic acid; nitro[7″-^{14}C]benzylthioinosine, 6-((4-nitro-[7-^{14}C]benzyl)thio)-9-β-D-ribofuranosylpurine; nitrobenzylthio[8-^{14}C]inosine, 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosyl-[8-^{14}C]-purine.

substrate specificity of the nucleoside transport mechanism of erthrocytes by comparing the abilities of external nucleosides to accelerate efflux of intracellular uridine. The variety of ribo- and deoxyribonucleosides of both purines and pyrimidines accepted as permeant* suggested a mechanism for nucleoside transport in the erythrocyte with broad specificity toward the base moiety of the permeant molecule [2, 5].

Nucleoside transport in human erythrocytes and Ehrlich ascites tumor cells is inhibited by 6-thiopurine ribonucleosides with a variety of hydrophobic substituent groups on the sulfur atom [2, 5–7]. The most potent of these inhibitors were 2-amino-6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine (nitrobenzylthioguanosine), 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine), and 6-((2-hydroxy-5-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine (hydroxynitrobenzylthioguanosine). Inhibition appeared to be specific for transport of nucleosides since uptake of uracil, D-ribose, or L-leucine by erythrocytes was unaffected, and the aglycone, 2-amino-6-((4-nitrobenzyl)thio)-purine (nitrobenzylthioguanine), had no effect on the transport of uridine [7].

Earlier studies indicated that nitrobenzylthioinosine interacted with erythrocyte ghosts [8]. Two modes of interaction were observed: a saturable, high affinity binding and a non-saturable accumulation of nitrobenzylthioinosine proportional to its concentration in the medium. The high affinity binding of nitrobenzylthioinosine was reversible, involved no detectable chemical transformation, and was postulated by Pickard et al. [8] to represent binding to nucleoside transport sites in the erythrocyte membrane. This postulate was tested in the present work. The equivalence of saturable binding of nitrobenzylthioinosine to intact erythrocytes with that to unsealed erythrocyte ghosts was demonstrated, indicating that nitrobenzylthioinosine binds to sites located in the erythrocyte membrane. To show that nitrobenzylthioinosine was bound specifically to nucleoside transport sites, graded inhibition of uridine transport was related to the amount of nitrobenzylthioinosine bound to the membranes of inhibited erythrocytes. The strict proportionality observed between fractional inhibition of uridine transport and the amount of saturably bound inhibitor identified the high affinity sites as part of the nucleoside transport mechanism of the human erthrocyte.

MATERIALS AND METHODS

Whole blood, stored at 4 °C in acid citrate–dextrose solution (U.S.P.) for 21–28 days, was obtained from the Red Cross Society Blood Transfusion Service, Edmonton, Alberta. Unless otherwise noted, erythrocytes were washed 3 times in buffered saline (140 mM NaCl, 1.4 mM $MgSO_4$, and 18 mM *N*-tris(hydroxymethyl)-2-aminoethane sulfonic acid (TES) at pH 7.4) with centrifugation at $1700 \times g$ for 15 min. Washed erythrocytes were "loaded" with uridine by incubating a 50% suspension of cells in buffered saline containing 10.5 mM [2-^{14}C]- or [5-^{3}H]uridine for 40 min at 37 °C, conditions sufficient to achieve equal internal and external uridine concentrations of 6 mM [2]. Incubations were terminated by centrifugation ($1700 \times g$, 15 min), and extracellular radioactivity provided a measure of the intracellular concentration of uridine.

* Permeant refers to compounds that are transported across biological membranes by mediated processes.

Rates of equilibrium exchange diffusion of uridine in the presence and absence of nitrobenzylthioinosine were determined at 25 °C using cells prepared as described above. Flasks containing 0.25-ml portions of packed uridine-loaded cells* were prepared and reactions were initiated by the rapid addition of 2.5 ml buffered saline containing (a) 6 mM non-radioactive uridine or (b) 6 mM non-radioactive uridine and 0.01–1.0 μM nitrobenzylthioinosine. Reactions were terminated [2, 3] at 5- or 10-s intervals by rapid addition of 2.5-ml portions of stopping solution (20 μM nitrobenzylthioinosine in buffered saline). For reaction intervals of zero, 20 μM nitrobenzylthioinosine was added to loaded cells 10 s before the addition of 6 mM uridine. Separate reaction mixtures were prepared for each time point, and immediately after terminating the reaction, portions of each mixture were centrifuged with di-1-butylphthalate ($1700 \times g$, 2 min) to obtain cell-free medium [1,10]. The hematocrit of each reaction mixture was determined. Radioactivity in samples of cell-free medium was assayed using Bray's counting fluid [11] and a liquid scintillation system. Samples containing both ^{3}H and ^{14}C were assayed using the combustion technique described below. Initial rates of outward transport of uridine were obtained from time courses of appearance of extracellular radioactivity in the medium; straight lines were fitted to the data by the method of least squares.

To measure binding of nitrobenzylthioinosine, washed erythrocytes suspended in 10 vol. of buffered saline containing nitro[7''-^{14}C]benzylthioinosine or nitrobenzylthio[8-^{14}C]inosine [8] were incubated with shaking for 15 min at 25 °C. Cells were collected and washed once in 5 vol. of buffered saline using centrifugation at $1700 \times g$ for 15 min. When cells loaded with 6 mM uridine were assayed, all solutions in which cells were incubated or washed contained 6 mM uridine. Under these conditions, binding was maximal within 5 min. For each reaction mixture, the equilibrium concentration of nitrobenzylthioinosine was obtained from measurement of radioactivity in the first supernatant after centrifugation using Bray's fluor [11] or Aquasol (New England Nuclear) and a liquid scintillation system. The amount of nitrobenzylthioinosine associated with washed cell sediments was determined by combusting 0.4-ml portions which were previously distributed on 12 mm $\times$ 25 mm absorbant strips** and dried. Dried cell samples were converted by combustion in a Packard Model 305 oxidizer to water and CO_2, which were collected separately in appropriate fluors [12] for counting in a liquid scintillation system. Recovery of radioactivity from combusted samples was $> 98\%$, and spillover of either ^{14}C or ^{3}H was less than 0.5% when doubly labeled samples were combusted. The number of cells in each combustion sample was obtained by suspending a portion of each cell sediment in a measured volume of buffered saline for enumeration with a Model F Coulter counter.

To establish that nitrobenzylthioinosine was bound to membrane-associated sites, the retention of radioactive nitrobenzylthioinosine by erythrocyte ghosts prepared by osmotic lysis [9] was compared to retention by intact erythrocytes. Erythrocytes were washed 3 times in isotonic sodium phosphate (310 mosM, pH 7.4) and then incubated in 10 vol. of isotonic sodium phosphate containing radioactive nitrobenzylthioinosine (0.005–1.0 μM) for 15 min at 25 °C. After incubations were ter-

* There are approx. $1 \cdot 10^{10}$ cells per ml of packed cells.

** "Telfa" pads; Kendall Co. (Canada), Toronto, Ontario.

minated by centrifugation (1700 × *g*, 15 min, 4 °C), 1.5-ml portions of packed cell sediments were suspended in 30 vol. of hypotonic sodium phosphate (20 mosM, pH 7.4) or, to provide intact erythrocytes as controls, in 30 vol. of isotonic sodium phosphate for 5 min at 4 °C. Ghosts resulting from hypotonic lysis were washed twice in hypotonic sodium phosphate (10 400 × *g*, 20 min, 4 °C) and control cells were washed twice in isotonic sodium phosphate (1700 × *g*, 20 min, 4 °C). Samples of the washed ghosts and erythrocytes were assayed for radioactivity by combustion.

Nitrobenzylthioinosine and hydroxynitrobenzylthioguanosine were supplied by Raylo Chemicals, Edmonton, Alberta, Canada. Synthesis of nitro[7″-^{14}C]benzylthioinosine ($2.01 \cdot 10^7$ cpm/μmole) and nitrobenzylthio[8-^{14}C]inosine ($3.84 \cdot 10^7$ cpm/μmole) was reported previously [8]. [2-^{14}C]Uridine (55.6 mCi/mole) and [5-^{3}H]uridine (4 Ci/mmole) were commercial products.

RESULTS

To examine inhibition by nitrobenzylthioinosine of equilibrium exchange diffusion of uridine, rates of outward transport of radioactivity from cells loaded with 6 mM [2-^{14}C]uridine into medium containing 6 mM nonradioactive uridine were determined at various concentrations of inhibitor. Maximal rates of exchange diffusion were observed in previous experiments [2, 5] at this uridine concentration. The range of concentrations of nitrobenzylthioinosine (0.01–0.4 μM) resulting in partial to complete inhibition of uridine efflux (Fig. 1) indicated that nitrobenzylthioinosine is a highly potent inhibitor of nucleoside transport in erythrocytes. At the cell and inhibitor concentrations employed, interaction with cells appreciably reduced concentrations of free inhibitor suggesting that nitrobenzylthioinosine was binding tightly to erythrocytes. Accordingly, the cell–inhibitor interaction was considered to be a "mutual depletion system" and the method of Easson and Stedman, as discussed by Webb [13], for analysis of inhibition kinetics in such systems was used

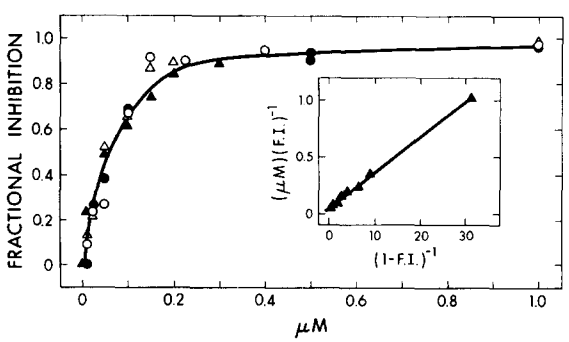

Fig. 1. Inhibition of uridine equilibrium exchange diffusion by nitrobenzylthioinosine. Erythrocytes loaded with 5.8–6.0 mM [2-^{14}C]uridine were incubated in medium containing the same concentration of nonradioactive uridine plus the indicated concentrations of nitrobenzylthioinosine and efflux of radioactivity was determined. Fractional inhibitions of uridine efflux (F.I. = 1 − (inhibited rate/control rate)) for 4 separate experiments are plotted against initial concentrations of nitrobenzylthioinosine. In the inset, the data from 1 of the 4 experiments are plotted according to the method of Easson and Stedman [13]; the apparent K_i obtained from the straight line derived by the method of least squares was $3.2 \cdot 10^{-8}$ M and the number of inhibitor binding sites was $0.73 \cdot 10^4$ sites/cell.

to obtain an apparent K_i for inhibition of uridine transport by nitrobenzylthioinosine (Fig. 1, inset). The values from the 4 experiments of Fig. 1 for the apparent inhibition constant were $1.5 \cdot 10^{-8}$, $1.9 \cdot 10^{-8}$, $3.2 \cdot 10^{-8}$ and $3.7 \cdot 10^{-8}$ M ($\bar{x} = 2.6 \cdot 10^{-8}$ M). The average number of inhibitor binding sites calculated from these data was $1.02 \cdot 10^4$ sites per erythrocyte.

The binding of nitrobenzylthio[^{14}C]inosine to erythrocytes in the absence of uridine is illustrated in Fig. 2, where the amount of nitrobenzylthioinosine bound is plotted against the equilibrium concentration of free nitrobenzylthioinosine. Saturable and non-saturable associations of nitrobenzylthioinosine with erythrocytes are apparent in these data. The upper portion of the curve in Fig. 2 is linear throughout the range of concentrations tested, indicating a nonsaturable interaction of inhibitor with erythrocytes; this linearity extended to 20 μM which is close to the limit of nitrobenzylthioinosine solubility (data not shown). The amount of saturably bound nitrobenzylthioinosine was determined in these experiments by separating the binding curve (Fig. 2) into its linear and nonlinear components (not shown); as will be discussed further below, the saturable component appears to represent inhibitor bound reversibly to transport sites in the erythrocyte membrane.

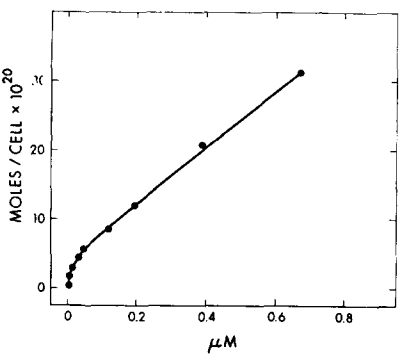

Fig. 2. Binding of nitrobenzylthioinosine to erythrocytes. Cells were incubated with nitrobenzylthio[8-^{14}C]inosine at initial concentrations of 0.006–1.0 μM. Cell-associated radioactivity was measured as described in Materials and Methods. The amount of nitrobenzylthioinosine (moles $\times 10^{-20}$) bound per cell is plotted as a function of the concentration of free nitrobenzylthioinosine as determined from radioactivity present in the medium at the end of the incubation.

The saturable binding of nitrobenzylthioinosine to erythrocytes was reduced in the presence of high permeant concentrations indicating competition between permeant and inhibitor for interaction with the same class of binding sites. Binding of nitrobenzylthioinosine was measured in the presence of 12 mM intra- and extracellular uridine, a concentration previously shown to be above saturation for exchange diffusion of uridine [2, 5]. Saturable binding was reduced by approx. 15% as compared to results presented in Fig. 2, indicating that cellular binding sites have a much higher affinity for nitrobenzylthioinosine than for uridine*.

* The apparent K_m for equilibrium exchange diffusion of uridine at 25 °C was 1.0 mM (Cass, C. E. and Paterson, A. R. P., unpublished results).

The experiments of Fig. 2 with nitrobenzylthioinosine labeled in the purine 8-position were repeated with nitrobenzylthioinosine labeled in the 7''-benzyl position. The binding of nitro[7''-^{14}C]benzylthioinosine to intact cells was equivalent on a molar basis to that of nitrobenzylthio[8-^{14}C]inosine, suggesting that binding occurs without cleavage of the thioether linkage.

Since earlier studies [2] had shown that hydroxynitrobenzylthioguanosine and 6-(methylthio)-9-β-D-ribofuranosylpurine (methylthioinosine) were highly effective inhibitors of nucleoside transport, the influence of these compounds on the binding of radioactive nitrobenzylthioinosine to intact erythrocytes was examined. Cells were incubated in buffered saline containing graded concentrations of nitrobenzylthio-[8-^{14}C]inosine plus methylthioinosine or hydroxynitrobenzylthioguanosine at concentrations in excess of those previously found to block exchange diffusion of uridine [2]. Fig. 3A shows that in the presence of 12.2 mM methylthioinosine or 50 μM hydroxynitrobenzylthioguanosine, interaction of nitrobenzylthio[8-^{14}C]inosine with saturable binding sites was almost or completely eliminated. This result suggests that the structurally related transport inhibitors, methylthioinosine and hydroxynitrobenzylthioguanosine, compete with nitrobenzylthioinosine for binding sites in the erthrocyte that are part of the nucleoside transport mechanism.

Estimates of the number of saturable binding sites per erythrocyte and the apparent constant for the disssociation of nitrobenzylthioinosine from these sites were obtained by separating the binding data into 2 components. The difference between binding of radioactive nitrobenzylthioinosine in the presence and absence of

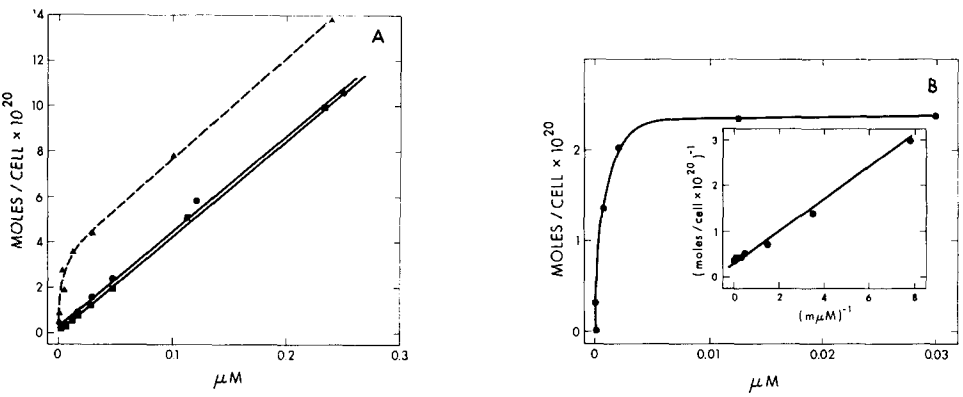

Fig. 3. Binding of nitrobenzylthioinosine to erythrocytes in the presence of related transport inhibitors. Cells were incubated for 15 min at 25 °C in buffered saline containing nitrobenzylthio [8-^{14}C]inosine with or without 50 μM hydroxynitrobenzylthioguanosine or 12.2 mM methylthioinosine. After terminating incubations by centrifugation, the ^{14}C-content of cells and medium was measured. The concentrations of nitrobenzylthioinosine before and after binding were 0.005–0.39 and 0.0001–0.25 μM, respectively. A. The nitrobenzylthioinosine content of erythrocytes is plotted against the concentration of nitrobenzylthioinosine in the medium at the end of incubation; cells were incubated in medium containing 50 μM hydroxynitrobenzylthioguanosine (■), 12.2 mM methylthioinosine (●), or neither of the latter (control) (▲). B. The amount of nitrobenzylthioinosine saturably bound to erythrocytes (obtained by subtracting the data of A for hydroxynitrobenzylthioguanosine-treated cells from that for control cells) is plotted against the final concentration of nitrobenzylthioinosine in the medium. In the inset, the reciprocal of the amount of saturably bound nitrobenzylthioinosine is plotted against the concentration of free nitrobenzylthioinosine.

hydroxynitrobenzylthioguanosine was used to quantitate binding to the saturable sites; such data, obtained from Fig. 3A, are plotted in Fig. 3B against equilibrium concentrations of nitrobenzylthioinosine. Values for the apparent dissociation constant and the number of saturable binding sites [14] obtained from the reciprocal plot (inset, Fig. 3B) were $1.0 \cdot 10^{-9}$ M and $1.52 \cdot 10^{4}$ sites per cell, respectively. Similar results were obtained in 2 other experiments ($1.1 \cdot 10^{-9}$ M and $1.60 \cdot 10^{4}$ sites/cell; $1.7 \cdot 10^{-9}$ M and $1.58 \cdot 10^{4}$ sites/cell). The number of saturable binding sites obtained from a Scatchard plot [14] of the data of Fig. 3 was $1.58 \cdot 10^{4}$ sites per cell. The values obtained for the dissociation constant of the site–inhibitor complex indicate high affinity of the saturable sites for nitrobenzylthioinosine.

Binding of nitrobenzylthioinosine to unsealed erythrocyte ghosts and to intact erythrocytes was compared to determine if the high affinity sites were located in the plasma membrane (Fig. 4). Intact cells were incubated in isotonic sodium phosphate containing radioactive nitrobenzylthioinosine in the presence and absence of nonradioactive hydroxynitrobenzylthioguanosine. Cells from each reaction mixture were resuspended (a) in hypotonic sodium phosphate for preparation of unsealed, "white" ghosts [9] and (b) in isotonic sodium phosphate to provide washed, intact erythrocytes as controls. Except for differences in osmolarities of the solutions employed, the control cells were subjected to the same washing procedures used in the preparation of ghosts.

Lysis and subsequent washing of the inhibitor-labeled erythrocytes in hypotonic sodium phosphate significantly reduced nonsaturable retention of radioactive nitrobenzylthioinosine (Fig. 4A), suggesting that this component represents intracellular accumulation of nitrobenzylthioinosine. The amount of nitrobenzylthioinosine as-

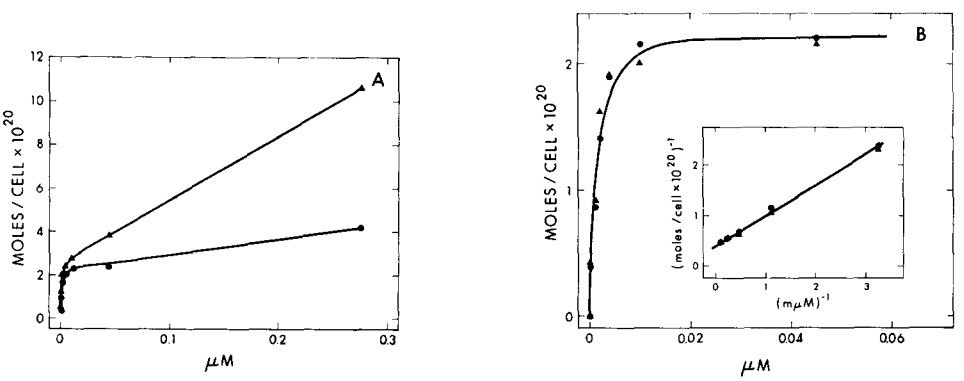

Fig. 4. Binding of nitrobenzylthioinosine to erythrocytes and to unsealed erythrocyte ghosts. Erythrocytes were labelled by incubation in isotonic sodium phosphate containing 0.005–1.0 μM nitro[7″-^{14}C]benzylthioinosine with and without 50 μM hydroxynitrobenzylthioguanosine as described in Materials and Methods. Incubations were terminated by centrifugation and cell sediments from each reaction mixture were then divided into equal portions for preparation of intact erythrocytes (controls) and unsealed ghosts. A. The amount of nitrobenzylthioinosine associated with control cells (▲) and with unsealed ghosts (●) is plotted against the final concentration of nitrobenzylthioinosine in the incubation medium. B. Saturably bound nitrobenzylthioinosine (cell-associated nitrobenzylthioinosine eliminated by the presence of the competing ligand hydroxynitrobenzylthioguanosine) is plotted against the equilibrium concentration of free nitrobenzylthioinosine for intact erythrocytes (▲) and for unsealed ghosts (●). Reciprocals of these parameters are plotted in the inset.

sociated with intact cells (controls) was reduced in the experiment of Fig. 4A as compared with that of Fig. 3A, apparently as a result of loss of intracellular inhibitor during the additional washes included in the experiment of Fig. 4.

Binding of nitrobenzylthioinosine to the saturable, high affinity sites was quantitatively identical in unsealed ghosts and intact erythrocytes. In the experiment of Fig. 4B, the amount of nitrobenzylthioinosine specifically bound to high affinity sites was identified by measuring binding in the presence and absence of nonradioactive hydroxynitrobenzylthioguanosine. The apparent dissociation constant for binding to ghosts and to erythrocytes was $1.7 \cdot 10^{-9}$ M and there were $1.58 \cdot 10^4$ binding sites per intact erythrocyte and $1.52 \cdot 10^4$ sites per ghosts (calculated from data of Fig. 4B [14]). These data indicate that the binding sites with high affinity for nitrobenzylthioinosine are located on the erythrocyte membrane and can be readily identified using nonradioactive, structurally related compounds as competing ligands to eliminate binding of radioactive nitrobenzylthioinosine.

To determine whether high affinity binding of nitrobenzylthioinosine represented a specific interaction with the nucleoside transport mechanism, inhibition of transport of [5-^{3}H]uridine and binding of nitrobenzylthio[8-^{14}C]inosine were measured on the same preparation of cells (Fig. 5). Erythrocytes, loaded with 6 mM [5-^{3}H]-uridine by the procedure given in Materials and Methods, were suspended in 4 vol.

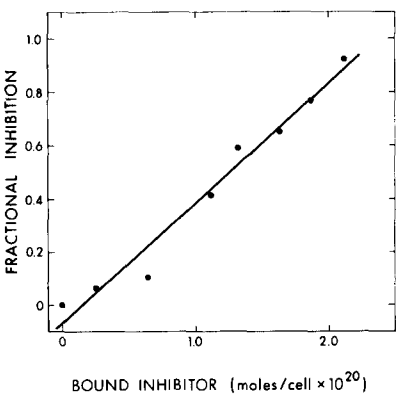

Fig. 5. Correlation of inhibition of uridine exchange diffusion and binding of nitrobenzylthioinosine. After loading with 6 mM [5-^{3}H]uridine and treatment with 0.01–1.0 μM nitrobenzylthio[8-^{14}C]-inosine as described in the text, erythrocytes were assayed for equilibrium exchange diffusion of uridine and saturably-bound nitrobenzylthioinosine. Fractional inhibition of uridine equilibrium exchange diffusion (F.I. = 1 − (inhibited rate/control rate)) is plotted against the amount of saturably bound inhibitor, determined as in Fig. 3.

of buffered saline containing (a) 6 mM [5-^{3}H]uridine or (b) 6 mM [5-^{3}H]uridine and nitrobenzylthio[8-^{14}C]inosine; the experiment was conducted at concentrations of the latter between 0.01 and 1.0 μM to effect graded inhibitions of uridine transport. After incubation for 15 min at 25 °C, cell sediments from each reaction mixture were obtained by centrifugation and portions of each cell sediment taken for measurement of efflux of [5-^{3}H]uridine and of saturable bound nitrobenzylthio[8-^{14}C]inosine. To assay uridine efflux, the rate of equilibrium exchange diffusion was determined as

before, following resuspension of cell sediments in 10 vol. of buffered saline containing the equilibrium concentration (6 mM) of nonradioactive uridine. Saturably bound nitrobenzylthioinosine was determined as described in Fig. 4. Cell sediments were resuspended in 10 vol. of buffered saline containing (a) 6 mM uridine, or (b) 6 mM uridine and 50 μM hydroxynitrobenzylthioguanosine, and after incubation for 10 min at 25 °C, cells were collected by centrifugation. Tritium associated with cell-free aqueous samples and ^{14}C associated with cell sediments were analyzed by the combustion technique described in Materials and Methods.

In Fig. 5, the fractional inhibition of uridine transport by nitrobenzylthioinosine is presented as a function of the amount of inhibitor reversibly bound to the saturable, high affinity binding sites. Total inhibition of uridine transport was not achieved, even when cells were treated with concentrations of nitrobenzylthioinosine in excess of those giving 99–100% inhibition in experiments such as illustrated in Fig. 1. In the experiment of Fig. 1, uridine transport was determined in media containing graded concentrations of nitrobenzylthioinosine, whereas in the experiments of Fig. 5, inhibitor-treated cells were resuspended in inhibitor-free media for assay of uridine transport. Apparently, under the latter conditions, redistribution of free and reversibly bound nitrobenzylthioinosine occurs. Nevertheless, the proportionality observed between (a) inhibition of the transport function and (b) nitrobenzylthioinosine binding to the erythrocyte membrane indicates that nitrobenzylthioinosine inhibits transport by specific interaction with permeant-binding sites of the nucleoside transport mechanism.

DISCUSSION

Earlier work [1–3, 5] established that transport of nucleosides across the plasma membrane of human erythrocytes, like that of monosaccharides [4], occurs by facilitated diffusion. Models for facilitated diffusion assume that transfer of a permeant across the cell membrane is mediated by proteins analogous in some respects to enzymes and earlier studies [1–3, 5] demonstrated that the kinetics of nucleoside transport in human erythrocytes could be described by Michaelis–Menten kinetics. This analogy is again apparent in the characteristics of nitrobenzylthioinosine binding and inhibition of nucleoside transport. The data suggest that the binding of nitrobenzylthioinosine to the saturable, high affinity sites of intact erythrocytes represents a specific interaction of inhibitor with the nucleoside transport mechanism of the plasma membrane. This specific binding of nitrobenzylthioinosine was (a) saturable, (b) eliminated in the presence of high concentrations of hydroxynitrobenzylthioguanosine, a related inhibitor of nucleoside transport, and (c) had an apparent K_d for nitrobenzylthioinosine of $1 \cdot 10^{-9}$ M.

Assuming that each binding site with high affinity for nitrobenzylthioinosine represents a single nucleoside transport element, the binding studies indicate that each erythrocyte has $1.5 \cdot 10^4$ such elements and inhibition kinetics indicate $1.0 \cdot 10^4$ sites per cell. These permeant binding site have an estimated turnover number for unidirectional uridine transport at 25 °C of 300–450 molecules/s per site*.

* Calculated assuming a V at 25 °C of 4.9 μmoles/min per ml packed cells and $1.09 \cdot 10^{10}$ cells per ml packed cells.

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