Biochimica et Biophysica Acta, 419 (1976) 285–294

BBA 77165

NITROBENZYLTHIOINOSINE BINDING SITES IN THE ERYTHROCYTE MEMBRANE

CAROL E CASS and ALAN R P PATERSON

University of Alberta Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2E1 (Canada)

(Received July 4th, 1975)

SUMMARY

Nitrobenzylthioinosine binds tightly, but reversibly, to sites in the human erythrocyte membrane, occupancy of these sites blocks the transport of uridine and of other nucleosides This report describes the inhibition of nitrobenzylthioinosine binding at these sites by substrates of the uridine transport mechanism and by compounds related to nitrobenzylthioinosine For some of these compounds dissociation constants for binding at the nitrobenzylthioinosine sites were determined, assuming competition with nitrobenzylthioinosine

Deoxycytidine, a substrate for the uridine transport mechanism, did not inhibit binding of nitrobenzylthioinosine, suggesting that binding sites for the latter are distinct from nucleoside sites directly involved in transport

INTRODUCTION

Nucleoside transport in human erythrocytes [1–3], Ehrlich ascites tumor cells [4], mouse lymphoma L5178Y cells [5] and HeLa cells [6] is strongly inhibited by *S*-nitrobenzyl derivatives of thioinosine and thioguanosine, nitrobenzylthioinosine is the best studied of these Nitro [^{14}C]benzylthioinosine interacts with erythrocyte ghosts [7] and intact erythrocytes [8] in 2 ways (a) a reversible, saturable binding to high affinity sites in the membrane, and (b) a nonsaturable uptake proportional to the concentration of nitrobenzylthioinosine in the medium Each erythrocyte had 1 0–1 5 10^4 high affinity binding sites for which the nitrobenzylthioinosine dissociation constant was approximately 10^{-9} M [8] These sites are considered to interact with, or to be part of, the uridine transport mechanism because inhibition of uridine

Abbreviations Various compounds are named as R-S derivatives (methylthio, propylthio, etc) of thioinosine (6-thio-9-β-D-ribofuranosylpurine), deoxythioinosine (6-thio-9-β-D-2′-deoxyribofuranosylpurine), or of thioguanosine (2-amino-6-thio-9-β-D-ribofuranosylpurine), nitrobenzyl, 4-nitrobenzyl, hydroxynitrobenzyl, 2-hydroxy-5-nitrobenzyl, nitrobenzylselenoguanosine, 2-amino-6-((4-nitrobenzyl)seleno)-9-β-D-ribofuranosylpurine, nitro[^{14}C]benzylthionosine, 6-((4-nitro[7-^{14}C]-benzyl)thio)-9-β-D-ribofuranosylpurine, persantin, 2,6-*bis*(diethanolamino)-4,8-dipiperidinopyrimido(5,4-d) pyrimidine, TES, *N*-tris(hydroxymethyl) 2-aminoethanesulfonic acid

transport was proportional to the number of sites occupied by nitrobenzylthioinosine [8]

High affinity binding of nitrobenzylthioinosine to erythrocyte membranes was significantly reduced or eliminated by the related transport inhibitors, methylthioinosine and hydroxynitrobenzylthioguanosine, and partially reduced by uridine itself [8] These observations were extended in the present study, and the ability of various thiopurine derivatives and substrates of the uridine transport mechanism to inhibit the high affinity binding of nitrobenzylthioinosine have been interpreted assuming competition between the inhibitory compounds and nitrobenzylthioinosine at the high affinity sites

MATERIALS AND METHODS

Erythrocytes were obtained from whole blood collected in acid citrate-dextrose solution (U S P) and stored at 4 °C for 21–28 days by the Red Cross Society Blood Transfusion Service, Edmonton, Alberta Unless otherwise noted, erythrocytes were washed 3 times in buffered saline with centrifugation at $1700 \times g$ for 15 min Buffered saline consisted of 140 mM NaCl, 1 4 mM $MgSO_4$ and 18 mM N-tris(hydroxymethyl) 2-aminoethanesulfonic acid (TES) at pH 7 4

The binding of nitro[^{14}C]benzylthioinosine to erythrocytes has been described previously [8], assays were initiated by the addition of 1 0 ml of cell sediment (approximately 1 0 10^{10} erythrocytes) to 10 0 ml of buffered saline containing nitro[^{14}C] benzylthioinosine with and without competing nucleosides Such mixtures were incubated with shaking for 15 min at 25 °C, cellular uptake of nitrobenzylthioinosine was complete in less than 5 min and was unaffected by decreasing incubation temperature to 4 °C Reactions were terminated by centrifugation (15 min, 4 °C, $1700 \times g$) and supernatants were reserved for analysis, cell sediments were washed once by centrifugation (15 min, 4 °C, $1700 \times g$), resuspended in 1 vol of cold buffered saline and assayed (a) for cell concentration using an electronic particle counter, and (b) for cellular content of nitro[^{14}C]benzylthioinosine In the latter assay, two 0 5 ml portions from each cell suspension were distributed on absorbant strips for ^{14}C assay by a combustion-liquid scintillation procedure [9] The concentration of nitro[^{14}C]benzylthioinosine in reaction mixture supernatants was determined from the ^{14}C content of the latter which was assayed using Brays' solution [10] and liquid scintillation counting

For calculation of apparent dissociation constants (K_D), the final concentrations of competing compounds in reaction mixture supernatants were determined spectrophotometrically Similar results were obtained when binding assays were performed using nitro[^{14}C]benzylthioinosine and [^{3}H]uridine or [^{3}H]thymidine, the ^{14}C and ^{3}H contents of reaction mixture supernatants were determined by a combustion procedure which collected $^{3}H_2O$ and $^{14}CO_2$ separately [9]

Erythrocyte ghosts in hypotonic sodium phosphate (20 mosM, pH 7 4) were prepared by the method of Dodge et al [11] The procedure described above for the assay of nitrobenzylthioinosine binding to intact erythrocytes was applied to ghosts with the following differences 20 mosM sodium phosphate was used instead of buffered saline, and reactions were terminated by collecting ghosts by centrifugation at $10000 \times g$ for 20 min at 4 °C

In determining the effect of proteolysis on binding of nitrobenzylthioinosine to ghosts, all procedures were conducted in 20 mosM sodium phosphate 50 % suspensions (v/v) of ghosts were incubated with and without pronase (1 mg/ml) or trypsin (1 mg/ml) for 30 min at 37 °C, incubations were terminated by dilution with an equal volume of cold 20 mosM sodium phosphate and the ghosts were washed 3 times (centrifugation 20 000 $\times g$, 45 min, 4 °C) Binding reactions were initiated by combining 1 vol of a 50 % (v/v) suspension of ghosts with 10 vols of 20 mosM sodium phosphate containing nitro[^{14}C]benzylthioinosine at various concentrations After 15 min at 25 °C, reactions were terminated by centrifugation (20 000 $\times g$, 45 min, 4 °C) The resulting sediments were resuspended in 20 mosM sodium phosphate and assayed for ^{14}C and protein content [12]

Persantin and nitrobenzylselenoguanosine were gifts, respectively, from Boehringer Ingelheim, Pharma-Research Canada, Ltd , Dorval, Quebec and from Dr L B Townsend, University of Utah, Salt Lake City, Utah The 2′- and 3′-*O*-methyl derivatives of nitrobenzylthioinosine and nitrobenzyldeoxythioinosine [13] were generously provided by Dr M J Robins, University of Alberta Thioguanosine, methylthioguanosine, propylthioguanosine and 2-amino-6-(benzylthio)-9-β-D-3′-deoxyribofuranosylpurine were provided by Drug Research and Development, National Cancer Institute, Bethesda, Maryland Nitrobenzylthioinosine and hydroxynitrobenzylthioguanosine were purchased from Raylo Chemicals Ltd , Edmonton, Alberta Synthesis of nitro[^{14}C]benzylthioinosine, 2 01 10^7 cpm/μmol, was reported previously [7] [5-^{3}H]uridine (4 Ci/mmol) and [*methyl*-^{3}H]thymidine (2 Ci/mmol) were commercial products

RESULTS

Saturable and nonsaturable components of nitrobenzylthioinosine uptake by erythrocytes are apparent in Fig 1 The saturable, high affinity component was

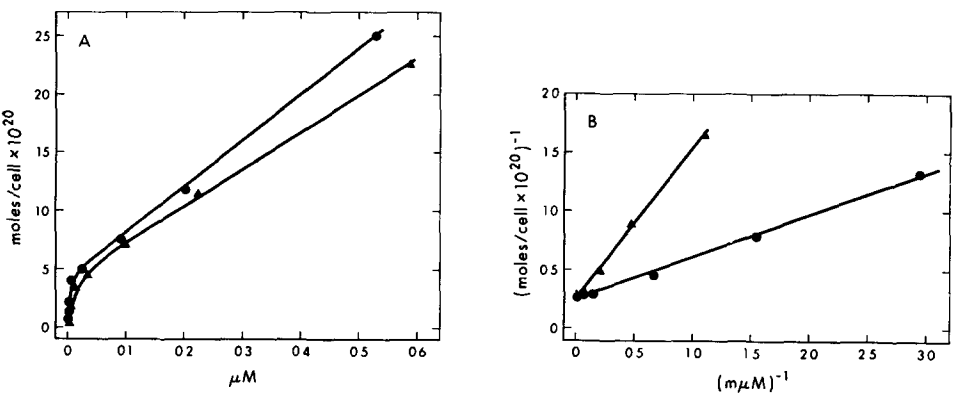

Fig 1 Inhibition by uridine of binding of nitro[^{14}C]benzylthioinosine to erythrocytes (A) Cells were incubated for 15 min at 25 °C in buffered saline containing nitro[^{14}C]benzylthioinosine at various concentrations with (▲) or without (●) 12 0 mM uridine, and the nitrobenzylthioinosine content of the cells and of the medium were then determined from their ^{14}C contents (B) Reciprocals of saturably bound nitrobenzylthioinosine in the presence (▲) and absence (●) of uridine were calculated from data in A as described in the text and are plotted against reciprocals of the final concentrations of nitrobenzylthioinosine found in the medium

determined from the amount of nitro[^{14}C]benzylthioinosine displaced by an excess of the competing ligand, hydroxynitrobenzylthioguanosine [8], or by subtracting the linear component of each uptake curve from the total uptake curve

The apparent dissociation constant (K_D) for nitrobenzylthioinosine at the high affinity sites was obtained from reciprocal plots of the saturable binding component as in Fig 1B [14], the mean (±S D) of 12 determinations was 1 7 (±0 6) 10^{-9} M (values of 1 0 10^{-9} and 1 7 10^{-9} M were reported previously [8])

The nonsaturable component of nitrobenzylthioinosine uptake (Fig 1A) apparently represents nonspecific cellular accumulation and is greater than predicted for a simple equilibration of nitrobenzylthioinosine between cells and medium (data not shown), perhaps because of accumulation in the lipid phase of the membrane Nonsaturable uptake by erythrocyte ghosts was significantly reduced by repeated washing, whereas saturable uptake was unaffected [7, 8]

Experiments which measured the saturable high affinity binding of nitro[^{14}C] benzylthioinosine to erythrocytes in the absence and presence of various nucleoside substrates of the uridine transport mechanism are summarized in Fig 1* and Table I Binding was assayed at various concentrations of nitrobenzylthioinosine, and for nucleosides that reduced nitrobenzylthioinosine binding, values of K_D for their interaction with the binding sites were calculated from reciprocal plots of the saturable component (Fig 1B) and a relationship that assumes reversible interaction of com-

TABLE I

APPARENT DISSOCIATION CONSTANTS (K_D) FOR BINDING OF NUCLEOSIDE PERMEANTS TO THE NITROBENZYLTHIOINOSINE BINDING SITES OF ERYTHROCYTES

As in Fig 1, erythrocytes were incubated in the presence or absence of a nucleoside permeant [2, 3] with various concentrations of nitro[^{14}C]benzylthioinosine, and the content of the latter in medium and saturably bound to cells was determined K_D values for the binding of nucleosides to the nitrobenzylthioinosine sites were calculated according to the method of Edsall and Wyman [14]

Nucleoside	Concentration (mM)	K_D (M 10^3)
Arabinosylcytosine	13 2	13 0
Uridine*	12 0	2 3
	11 6	4 5
Thymidine**	12 6	3 3
4 Thiouridine	1 1	1 1
Inosine	11 5	4 7
Guanosine	4 6	2 5
Adenosine	4 8	1 0
	11 2	1 1
	12 1	1 2
2'-Deoxyadenosine	9 0	0 9
Arabinosyladenine	2 0	0 6

* Present as [5-^{3}H]uridine
** Present as [*methyl*-^{3}H]thymidine

* The difference between the total uptake of nitrobenzylthioinosine in the presence and absence of the competing ligand is small (Fig 1A) because of the saturable and nonsaturable uptake components, the latter is the larger

TABLE II

APPARENT DISSOCIATION CONSTANTS (K_D) FOR BINDING OF INHIBITORS OF URIDINE EFFLUX TO THE NITROBENZYLTHIOINOSINE BINDING SITE OF ERYTHROCYTES

K_D values for the compounds listed were determined from their ability to inhibit the saturable binding of nitro[^{14}C]benzylthioinosine in experiments similar to those of Fig 1 and Table I

Inhibitor	Concentration (mM)	K_D (M 10^3)
2′,3′-*O*-Isopropylideneuridine	11 2	49 0
2′,3′-*O*-Isopropylideneadenosine	6 3	1 5
Thioguanosine	0 9	0 2
	1 6	0 2
Methylthioinosine	0 1	0 03
	0 2	0 07
	0 8	0 1

peting ligands with the same set of sites [14] Similar K_D values were obtained at different concentrations of adenosine (Table I), an indication [14] that nitrobenzylthioinosine and adenosine compete for the same set of sites

The relative affinities of 4 inhibitors* of nucleoside transport [2, 3] for the nitrobenzylthioinosine binding sites are indicated in Table II The effect of methylthioinosine was examined at several concentrations (Fig 2, Table II) and, although the K_D values obtained have a somewhat greater range (0 3–1 0 10^{-4} M) than those for adenosine (Table I), the data suggest that methylthioinosine and nitrobenzylthioinosine interact with the same binding sites [14]

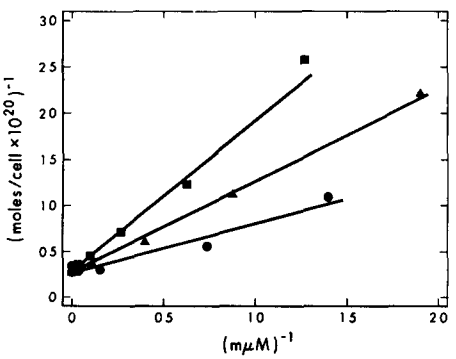

Fig 2 Inhibition by methylthioinosine of binding of nitro[^{14}C]benzylthioinosine to erythrocytes Cells were incubated with various concentrations of nitro[^{14}C]benzylthioinosine in the absence (●) or presence of methylthioinosine at 0 1 mM (▲) or 0 3 mM (■) and, as in Fig 1, determinations were then made of nitrobenzylthioinosine in the medium and of that saturably bound to cells K_D values obtained from these data for binding of methylthioinosine are listed in Table II

* *Trans* inhibition these compounds, when added to extracellular medium, inhibited efflux of radioactive uridine [2, 3]

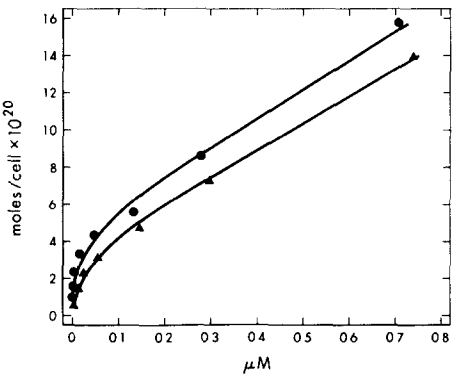

Fig 3 Inhibition of nitro[^{14}C]benzylthioinosine binding to erythrocyte ghosts by methylthioinosine Unsealed ghosts were incubated with various concentrations of nitro[^{14}C]benzylthioinosine in the absence (●) or presence (▲) of 1 mM methylthioinosine, and the nitrobenzylthioinosine content of the medium (abscissa) and that bound to ghosts (ordinate) were determined as in Fig 1 K_D values for methylthioinosine and nitrobenzylthioinosine of 1 0 10^{-4} M and 1 2 10^{-9} M, respectively, were obtained from these data [14]

TABLE III
INHIBITION OF SATURABLE BINDING OF NITRO[^{14}C]BENZYLTHIOINOSINE TO ERYTHROCYTES BY DERIVATIVES OF THIOINOSINE AND THIOGUANOSINE

As in Fig 1, erythrocytes were incubated in buffered saline containing 0 5 μM nitro[^{14}C]benzylthioinosine (a concentration in excess of binding saturation (see Fig 1)) with and without the test compound listed The cellular content of nitrobenzylthioinosine saturably bound in the presence of a test compound is expressed as a percentage of that in its absence (control)

Inhibitor	Initial conc'n (mM)	Saturably bound nitrobenzylthioinosine (% of control)
Methylthioinosine*	10 0	20
	1 0	35
Nitrobenzyldeoxythioinosine**	0 01	0
Nitrobenzyl-2′-*O*-methylthioinosine**	0 01	0
Nitrobenzyl-3′-*O*-methylthioinosine**	0 01	47
Thioguanine	0 33	100
Thioguanosine*	1 6	21
Methylthioguanosine	0 1	10
Propylthioguanosine	0 1	35
Nitrobenzylthioguanosine*	0 025	0
Hydroxynitrobenzylthioguanosine*	0 001	40
	0 01	0

* *Trans*-inhibition of uridine efflux [3]
** *Cis*-inhibition of adenosine uptake [15]

The experiment of Fig 3, which was similar to those of Table II, but performed with erythrocyte ghosts, established that the nitrobenzylthioinosine binding sites were located in the membrane K_D values of 0 7 10^{-4} M (an average of the 3 values obtained in the experiment of Table III) and 1 0 10^{-4} M were obtained for binding of methylthioinosine to intact erythrocytes and unsealed ghosts, respectively In agreement with previous results [8], K_D values for binding of nitrobenzylthioinosine to intact erythrocytes and to ghosts in the absence of methylthioinosine were similar, 1 7 10^{-9} M (see above) and 1 2 10^{-9} M (Fig 3), respectively The nonsaturable uptake of nitro[^{14}C]benzylthioinosine by ghosts, shown in Fig 3, was greater than reported previously [8] because the labeled ghosts were collected without multiple washings, which significantly reduce this component of uptake [7, 8]

Binding data are presented in Table III for derivatives of thioinosine and thioguanosine, several of which exhibited greater affinity for nitrobenzylthioinosine binding sites than did methylthioinosine Because final concentrations of these compounds were too low for spectrophotometric determination, dissociation constants could not be calculated, however, their relative affinities for the sites are suggested by the concentrations at which binding of nitro[^{14}C]benzylthioinosine was substantially inhibited Among the compounds with highest affinity for binding were hydroxynitrobenzylthioguanosine and nitrobenzyldeoxythioinosine, the latter inhibited* adenosine uptake in human erythrocytes at lower concentrations than nitrobenzylthioinosine or hydroxynitrobenzylthioguanosine [15]

Table IV indicates that nitrobenzylselenoguanosine and the 9-tetrahydro-2-furyl derivative of benzylthioguanine significantly reduced saturable binding of

TABLE IV

INHIBITION OF SATURABLE BINDING OF NITRO[^{14}C]BENZYLTHIOINOSINE TO ERYTHROCYTES BY OTHER COMPOUNDS

Experiments were conducted as in Table III

Inhibitor	Initial concn (mM)	Saturably bound nitrobenzylthioinosine (% of control)
Nitrobenzylseleno-guanosine	0 025	0
2 Amino-6-(benzyl-thio)-9-β-D-2′-deoxyribofurano-sylpurine	0 02	35
2-Amino-6-(benzylthio)-9-β-(tetrahydro-2-furyl) purine	0 02	33
Persantin*	0 05	16
Colcemid*	0 1	54
Colchicine*	1 0	100

* Inhibited nucleoside transport in cultured cells [16–19]

* *Cis* inhibition these compounds, when added to extracellular medium, inhibited uptake of radioactive adenosine [15]

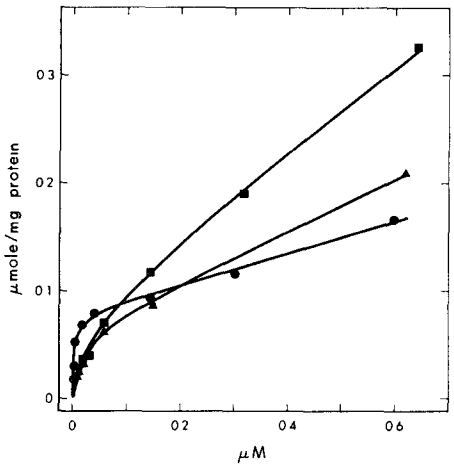

Fig 4 Effect of trypsin and pronase on binding of nitro[^{14}C]benzylthioinosine to erythrocyte ghosts Ghosts were incubated at 37 C for 30 min without additives (●), or with 1 mg/ml trypsin (▲) or pronase (■) and, after washing, were incubated with various concentrations of nitro[^{14}C]-benzylthioinosine for 15 min at 25 C The final medium content of nitrobenzylthioinosine, determined as in Fig 1, is plotted against that of the ghosts (ordinate)

nitro[^{14}C]benzylthioinosine Persantin (0 05 mM) and colcemid (0 1 mM) inhibited binding of nitro[^{14}C]benzylthioinosine but colchicine (1 0 mM) did not do so these compounds are inhibitors of nucleoside transport [16–19]

Compounds that had no discernible effect on binding of nitrobenzylthioinosine were uracil (5 1 mM), UMP (3 9 mM), 6-azauridine (6 6 mM), 6-thioguanine (0 3 mM), and 2-deoxycytidine (10 5 mM) Although previously recognized as a substrate for the uridine transport mechanism along with the other compounds listed in Table I [2, 3], deoxycytidine did not compete with nitrobenzylthioinosine for binding as did the compounds of Table I

The effect of limited proteolysis on binding of nitro[^{14}C]benzylthioinosine to erythrocyte ghosts was examined (Fig 4) These K_D values for nitrobenzylthioinosine were obtained after incubation with the following control (1 3 10^{-9} M), trypsin (6 0 10^{-9} M), and pronase (7 4 10^{-9} M) The number of saturable sites (calculated from the v-intercept of plots of reciprocals of saturably bound nitrobenzylthioinosine and the concentration of free nitrobenzylthioinosine [8, 14]) decreased to 27 % and 24 % of control values, respectively, in trypsin- and pronase-treated ghosts

DISCUSSION

The present study demonstrates inhibition of nitrobenzylthioinosine binding to high affinity receptor sites in the erythrocyte membrane by several thiopurine derivatives and by substrates of the uridine transport mechanism Assuming competition for binding with nitrobenzylthioinosine, dissociation constants for the inhibitory compounds at these sites were calculated using mass law relationships defined by Edsall and Wyman [14] and the nitrobenzylthioinosine dissociation constant reported here Compounds that were potent inhibitors of uridine transport exhibited high

affinity for the nitrobenzylthioinosine binding sites (Tables II–IV) Substrates of the uridine transport mechanism [1, 2, 15] were bound to nitrobenzylthioinosine sites with apparent K_D values of 10^{-2}–10^{-4} M

Granting that nitrobenzylthioinosine and related test compounds competed for binding, it is apparent that the *S*-substituent contributes importantly to binding In this group of compounds, the ribosyl group is also an important structural determinant of inhibitory activity toward nucleoside transport (Paterson, A R P , Schwarzkopf, J , Babb, L and Cass, C E , unpublished results)

The present findings on the inhibition of nitrobenzylthioinosine binding by nucleoside permeants are interpreted assuming competition between the nucleosides and nitrobenzylthioinosine at the high affinity sites We envisage a simple model for the uridine transporter with these features (a) the transporter's permeant site (it is presumed that nucleoside permeants interact with a specific site on the transporter mechanism in initiating the process of translocation across the plasma membrane) accepts as "substrates" a variety of nucleosides, including deoxycytidine [1], (b) when the high affinity binding sites (presumed to be on the transporter) are occupied by nitrobenzylthioinosine or congeners, function of the uridine transporter is blocked, (c) the nitrobenzylthioinosine sites appear to be distinct from the permeant sites

The need to distinguish between the nitrobenzylthioinosine and the permeant sites arises from the result that deoxycytidine, previously recognized as a permeant through participation in the accelerated exchange diffusion process with uridine [3], did not compete for binding with nitrobenzylthioinosine. In other words, this result is inconsistent with a simple model in which nitrobenzylthioinosine, a potent inhibitor of the uridine transport system, is bound at permeant sites The hypothesis that the sites for binding of this transport inhibitor are distinct from those involved in nucleoside permeation is consistent with the fact that a variety of structurally diverse compounds (such as persantin [16, 17], colcemid [18], colchicine [19], cytochalasin B [20] and aflatoxins [21]) are also potent inhibitors of nucleoside transport, the absence of obvious structural relationships between these inhibitors and the permeants involved suggests that these inhibitors may not interact with the transport mechanism at the actual permeant site The uridine transporter is an obviously complex mechanism, the present results illustrate this complexity in showing a distinction between binding sites for the transport inhibitor nitrobenzylthioinosine and permeant sites for deoxycytidine

The effect of limited proteolytic digestion on nitrobenzylthioinosine binding (Fig 4) should be considered in the light of recent reports that limited proteolysis of ghosts did not significantly impair glucose transport [22–27] and that the uridine transport mechanism of cultured hepatoma cells was resistant to enzymatic treatment [28] Although the affinity of the binding sites for nitrobenzylthioinosine was not markedly changed, the number of sites was reduced 4-fold, suggesting that the nitrobenzylthioinosine binding sites are relatively exposed

ACKNOWLEDGEMENTS

We acknowledge the competent assistance of Ms Marilyn Ross and Ms Jane Schwarzkopf This work was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada

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