Extraction and partial purification of the nucleoside-transport system from human erythrocytes based on the assay of nitrobenzylthioinosine-binding activity

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Nitrobenzylthioinosine, a potent nucleoside-transport inhibitor, binds to high-affinity sites on the human erythrocyte membrane. This binding is a specific interaction with functional nucleoside-transport sites. The protein(s) responsible for high-affinity nitrobenzylthioinosine binding was purified 13-fold by treatment of haemoglobin-free 'ghosts' with EDTA (pH 11.2) to remove extrinsic proteins, extraction of the protein-depleted membranes with Triton X-100 and passage of the soluble extract through a DEAE-cellulose column equilibrated with Triton X-100. Void-volume fractions were collected and treated with Bio-Beads SM-2 to remove detergent. These fractions contained 31% of the starting nitrobenzylthioinosine-binding activity. They also contained D-glucose-sensitive cytochalasin B-binding activity. Nitrobenzylthioinosine binding to the partially purified preparation was saturable (apparent K_d 1.6 nM) and inhibited by nitrobenzylthioguanosine, dipyridamole and uridine. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of pooled void-volume fractions revealed the presence of only two detectable protein bands, the broad zone 4.5 (containing glucose-transport protein) and a small amount of band 7.

The mechanisms by which nucleosides cross cell membranes are relevant to a diverse range of biochemical and physiological processes, some of which have important clinical implications (see, for example, Harmon *et al.*, 1978; Walker *et al.*, 1979). In erythrocytes, a single broad-specificity transport system for both purine and pyrimidine nucleosides has been identified and kinetic studies suggest that similar facilitated-diffusion systems are also present in other cell types (Oliver & Paterson, 1971; Pickard & Paterson, 1972; Cass & Paterson, 1972; Cabantchik & Ginsburg, 1977; Young, 1978; Plagemann *et al.*, 1978; Paterson, 1979; Wohlhueter *et al.*, 1979). Nitrobenzylthioinosine and dipyridamole are potent reversible inhibitors of nucleoside translocation by these routes (Paterson & Oliver, 1971; Pickard & Paterson, 1972; Berlin & Oliver, 1975; Eilam & Cabantchik, 1977; Paterson, 1979; Lum *et al.*, 1979). Although the kinetics, substrate specificity and inhibitor susceptibility of nucleoside transport have been investigated in considerable detail, there is little information concerning the molecular properties of the membrane component(s) responsible for nucleoside translocation. Such information could, in theory, be obtained by using high-affinity inhibitors of nucleoside transport,

such as nitrobenzylthioinosine, to identify transport sites.

Nitrobenzylthioinosine inhibition of human erythrocyte nucleoside transport is competitive, and associated with high-affinity binding of inhibitor to the cell membrane (apparent $K_d \approx 1$ nM) (Pickard *et al.*, 1973; Eilam & Cabantchik, 1977; S. M. Jarvis & J. D. Young, unpublished work). There is strict proportionality between occupancy of these binding sites and the degree of transport inhibition (Cass *et al.*, 1974). Binding can be readily displaced by dipyridamole, or competitively blocked by uridine, a transported nucleoside (Cass & Paterson, 1976; Jarvis & Young, 1980a; Jarvis *et al.*, 1980a). Furthermore the apparent K_i for uridine inhibition of nitrobenzylthioinosine binding is the same as the apparent K_m for uridine equilibrium exchange efflux from intact cells (Jarvis *et al.*, 1980a). In contrast, it has been reported that deoxycytidine, a transported nucleoside, did not affect high-affinity nitrobenzylthioinosine binding (Cass & Paterson, 1976). In our hands, however, deoxycytidine is an effective competitive inhibitor of binding activity (apparent K_i 1.0 mM) (S. M. Jarvis & J. D. Young, unpublished work). Such studies suggest that high-affinity binding of nitrobenzylthio-

inosine to the cell membrane represents a direct and specific association with functional nucleoside-transport sites. Further evidence in support of this conclusion is the finding that nucleoside-impermeable sheep erythrocytes do not bind nitrobenzylthioinosine, whereas nucleoside-permeable sheep erythrocytes both transport nucleosides and bind inhibitor (Jarvis & Young, 1978, 1980a). Additional evidence comes from work on sheep reticulocytes and foetal cells, which transport nucleosides considerably more rapidly than mature adult type sheep cells (Mooney & Young, 1978; Tucker *et al.*, 1979). This increased translocation capacity is associated with proportional increases in nitrobenzylthioinosine binding (Jarvis & Young, 1980e; S. M. Jarvis & J. D. Young, unpublished work).

There is therefore good evidence to suggest that nitrobenzylthioinosine interacts specifically with components of the nucleoside-transport mechanism. As a first step towards the molecular characterization and identification of the membrane components responsible for nucleoside translocation, we describe the solubilization and partial purification of nitrobenzylthioinosine-binding activity from human erythrocyte membranes. A preliminary abstract of the present study has been published (Jarvis & Young, 1980b).

Materials and methods

Chemicals

[G-³H]Nitrobenzylthioinosine [6-(4-nitrobenzyl)-thio-9- β -D-ribofuranosylpurine] (sp. radioactivity 20 Ci/mmol) was purchased from Movarek Biochemicals, City of Industry, CA, U.S.A. Radiochemical purity was routinely checked by t.l.c. with chloroform/methanol (17:3, v/v) as solvent and found to be >98% pure. [4(n)-³H]Cytochalasin B (sp. radioactivity 11 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Nitrobenzylthioguanosine [2-amino-6-(4-nitrobenzyl)thio-9- β -D-ribofuranosylpurine] was a generous gift from Professor A. R. P. Paterson, University of Alberta Cancer Research Unit, Edmonton, Alberta, Canada. Dipyrindamole [2,2',-2'',2'''-(4,8-dipiperidinopyrimido[5,4-d]pyrimidine-2,6-diyl)dinitrilo]tetraethanol; Persantin injection] was purchased from Boehringer Ingelheim, Bracknell, Berks., U.K. Triton X-100, type 11-S soya-bean L- α -phosphatidylcholine, cytochalasin B, dithiothreitol and uridine were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Pre-swollen microgranular DEAE-52 DEAE-cellulose anion-exchange resin and Bio-Beads SM-2 were purchased from Whatman, Springfield Mill, Maidstone, Kent, U.K., and Bio-Rad Laboratories, Richmond, CA, U.S.A., respectively. All other reagents were of analytical grade.

Membrane preparation and solubilization

Unless otherwise stated, these and subsequent procedures were carried out at 1–4°C. Haemoglobin-free 'ghosts', prepared from freshly collected heparinized human blood as described previously (Jarvis & Young, 1980a), were depleted of extrinsic proteins by combined EDTA/NaCl treatment by the method of Zoccoli *et al.* (1978) or by a single extraction step using 0.1 mM-EDTA, pH 11.2. In the latter case, 1 vol. of membranes (4 mg of protein/ml in 5 mM-sodium phosphate buffer, pH 7.4) was exposed to 7 vol. of EDTA solution for 15 min. The 'ghosts' were sedimented by centrifugation (35 000 g for 25 min) and washed twice with 7 vol. of 50 mM-Tris/HCl (pH 8.2 at 4°C). Membrane pellets were resuspended at each stage by homogenization. Both protein-depletion methods gave similar results, although the high-pH treatment was less time consuming and resulted in a more complete removal of spectrin.

Protein-depleted membranes (approx. 2 mg of protein/ml) in 50 mM-Tris/HCl were solubilized by the addition of an equal volume of 2% (w/v) Triton X-100 in Tris buffer, also containing 2 mM-dithiothreitol. The preparation was stirred for 1 h and then centrifuged at 150 000 g for 45 min. The supernatant was retained.

Column chromatography of the Triton supernatant

Ion-exchange chromatography of the Triton X-100 supernatant was carried out as described by Kasahara & Hinkle (1977) and Sogin & Hinkle (1978) with minor modifications. Triton X-100 supernatant (2 vol.) was applied to 1 vol. of DEAE-cellulose (equilibrated with 50 mM-Tris/HCl/1% Triton X-100/1 mM-dithiothreitol) at a flow rate equivalent to 1.5 column volumes/h. The column was washed with 3 vol. of buffer and 0.4 vol. fractions were collected. Proteins bound to the column were eluted with 50 mM-Tris/HCl buffers, containing 0.1 and 1 M-NaCl in addition to detergent and dithiothreitol.

Removal of detergent

Samples of Triton X-100 membrane extract and DEAE-cellulose column fractions were depleted of detergent by treatment with methanol/water-washed Bio-Beads SM-2 (Holloway, 1973). In early experiments samples were first made 100 mM in NaCl, 1 mM in EDTA and 3 mM in MgCl₂ before the addition of Bio-Beads (0.4 g of wet beads/ml) as described by Baldwin *et al.* (1979). Additional dithiothreitol (2 mM) was also added to supplement that already present and the mixture was shaken for 6–12 h. Subsequent control experiments established that NaCl, EDTA and MgCl₂ could be omitted

without loss of binding activity. In some later experiments a column of Bio-Beads was coupled directly to the effluent line of the DEAE-cellulose column (Holloway, 1973). Acetone-washed soya-bean phospholipids (Kagawa & Racker, 1971) were added to some samples before removal of detergent and were introduced as a sonicated dispersion (final concentration 5 mg/ml).

When appropriate, void-volume fractions from the DEAE-cellulose column were pooled, treated with Bio-Beads and concentrated 6-fold by placing the sample in a dialysis bag and burying it in sucrose. Dissolved sucrose was removed by dialysis against 50 mM-Tris/HCl, containing 0.2 mM-dithiothreitol.

Nitrobenzylthioinosine-binding assays

Binding activity was assayed by equilibrium dialysis (0.25 ml chambers; molecular-weight cut-off for membranes = 12 000–14 000) (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Samples (0.2 ml) were added to one side of the dialysis membrane, and 0.2 ml portions of [³H]nitrobenzylthioinosine (1–120 nM) in identical medium to the other ($\pm 10 \mu\text{M}$ -nitrobenzylthioguanosine, to correct for non-specific binding). Samples with high binding activity were diluted up to 6-fold with the appropriate buffer before analysis. Each dialysis well also contained a 2 mm glass bead to assist mixing. The dialysis apparatus was rotated gently for 20 h at room temperature, and then 100 μl was removed from each chamber for determination of radioactivity.

Cytochalasin B-binding assays

Cytochalasin B-binding activity was also assayed by equilibrium dialysis. A saturating concentration of [³H]cytochalasin B was used (initial concentration 5 μM) and control wells contained 200 mM-D-glucose to correct for non-specific binding (Zoccoli *et al.*, 1978). The buffer pH was decreased to 6 to minimize reaction between dithiothreitol and cytochalasin B (Lienhard & Wardzala, 1976).

Protein measurements

Protein was assayed by modified Lowry procedures with appropriate standards and blanks to correct for lipid, detergent and Tris interference (Peterson, 1977, 1979; Markwell *et al.*, 1978). The two methods gave similar estimates of membrane protein concentration. Bovine serum albumin was used as standard.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis in the presence of 0.1% sodium dodecyl sulphate was performed in slab gels with the buffers described by Fairbanks *et al.* (1971).

The separating gels contained a linear gradient of acrylamide concentration (5–20%, w/v) with the acrylamide monomer/*NN'*-methylenebisacrylamide ratio maintained at 30:1 throughout the gel (Williams *et al.*, 1979).

Radioactivity determinations

Samples, if necessary, were made up to 1.0 ml with water, mixed with 7 ml of Packard scintillator 299 (Packard Instrument Co. Inc., Downers Grove, IL, U.S.A.), and counted for radioactivity in a Packard Tri-Carb scintillation counter with quench correction.

Results

In previous studies we established that extraction of human erythrocyte membranes with sodium cholate solubilized high-affinity nitrobenzylthioinosine-binding sites associated with the nucleoside-transport mechanism (Jarvis & Young, 1980*c,d*). Binding activity could not be detected in the presence of detergent, but was recovered, at least in part, on removal of cholate by gel filtration. Unfortunately, the relatively low recovery of nitrobenzylthioinosine-binding activity by this method (approx. 10%) and an associated decrease in specific activity made it unsuitable for subsequent isolation studies. For the present investigation we chose Triton X-100, a detergent known to solubilize intrinsic membrane proteins from erythrocyte 'ghosts' (see, for example, Yu *et al.*, 1973).

Pilot studies

It was first necessary to establish whether Triton X-100, like cholate, inhibited high-affinity nitrobenzylthioinosine-binding activity. Fig. 1 shows the results of an experiment where nitrobenzylthioinosine binding to erythrocyte membranes was measured in the presence of increasing concentrations of detergent (0.01–1%, w/v). The data demonstrate that Triton X-100 is an effective inhibitor of binding activity, with complete inhibition resulting from the addition of 1% Triton X-100. It was therefore essential to deplete samples of Triton X-100 before the assay of nitrobenzylthioinosine-binding activity. This was achieved by treating samples with polystyrene beads (Bio-Beads SM-2). Data in Fig. 2 demonstrate that extraction of membranes (protein-depleted) with increasing concentrations of Triton X-100 (0.05–1.0%) resulted in the progressive solubilization of membrane protein (11–61%). No nitrobenzylthioinosine-binding activity could be detected in such extracts, but addition of Bio-Beads led to the appearance of high-affinity binding activity. The results further demonstrate that extraction of membranes with 0.5–1% Triton X-100 and subsequent removal of detergent

Fig. 1. Effect of Triton X-100 on the binding of nitrobenzylthioinosine to erythrocyte membranes

Erythrocyte membranes (2 mg/ml) were treated with various concentrations of Triton X-100 in 1 mM-dithiothreitol and analysed for nitrobenzylthioinosine-binding activity in the presence of detergent with (○) or without (●) 5 μ M-nitrobenzylthioguanosine. The concentration of [3 H]nitrobenzylthioinosine (average of both chambers) was 1 nM. Binding activity is expressed as a distribution ratio (radioactivity present in chamber containing erythrocyte membrane/radioactivity present in opposite chamber).

Fig. 2. Solubilization of nitrobenzylthioinosine-binding activity by Triton X-100

Protein-depleted membranes at a concentration of 0.8 mg/ml were treated with various concentrations of Triton X-100 in 1 mM-dithiothreitol at 4°C for 1 h. The mixtures were centrifuged at 150 000 *g* for 45 min and the supernatants assayed for protein (○) and high-affinity nitrobenzylthioinosine-binding activity (■, before Bio-Bead treatment; ●, after Bio-Bead treatment).

with Bio-Beads SM-2 resulted in essentially complete solubilization and quantitative recovery of nitrobenzylthioinosine-binding activity. This and subsequent experiments were carried out in the presence of dithiothreitol to minimize oxidation of protein thiol groups essential for nitrobenzylthioinosine-binding activity (Dahlig *et al.*, 1979; Jarvis & Young, 1980e). Other experiments demonstrated that sodium deoxycholate also solubilized high-affinity binding sites. However, as in the previous studies with cholate, the recovery of activity was low.

Purification procedure

The method adopted to purify nitrobenzylthioinosine-binding protein(s) is summarized in Table 1, which also gives the total nitrobenzylthioinosine-binding activity, total protein and specific activity of binding (pmol of nitrobenzylthioinosine bound/mg of protein) at each stage of the procedure. The results are from five separate experiments. As for the experiment in Fig. 2, nitrobenzylthioinosine-binding activity was assayed at saturating nitrobenzylthioinosine concentrations and corrected for non-specific binding. Control experiments confirmed that the

nitrobenzylthioinosine-binding assay was linear over the activity range encountered in these experiments.

Haemoglobin-free 'ghosts' were first depleted of extrinsic proteins as described in the Materials and methods section. This resulted in a 60% decrease in membrane protein with a 30% loss of nitrobenzylthioinosine-binding activity. It is likely that some of the binding activity lost during protein depletion was associated with small membrane vesicles (Steck & Yu, 1973). Extraction of protein-depleted membranes with Triton X-100 (1%) solubilized 50–64% of the remaining membrane protein and >90% of the high-affinity inhibitor-binding activity. As expected, the insoluble Triton X-100 residue contained approx. 30% of the protein, but less than 8% of the nitrobenzylthioinosine-binding activity present in protein-depleted membranes. The next stage of purification was DEAE-cellulose ion-exchange chromatography of the soluble Triton X-100 supernatant. The results of a typical experiment are presented in Fig. 3, which demonstrates that substantial high-affinity nitrobenzylthioinosine-binding activity was eluted from the column in fractions corresponding to the column void-volume. This activity was associated with only a small percentage (6%) of the protein applied to the column. The remaining protein, eluted at high

Table 1. Purification of the nitrobenzylthioinosine-binding component

Protein and high-affinity nitrobenzylthioinosine-binding activity (at a saturating concentration of inhibitor) were determined as described in the text. Values are means ($\pm$ S.E.M.) for five separate experiments.

	Total protein (mg)	Total nitrobenzylthioinosine bound (pmol)	Specific nitrobenzylthioinosine-binding activity (pmol/mg)
'Ghosts'	33 $\pm$ 2.5	1008 $\pm$ 96	36 $\pm$ 8
Protein-depleted membranes	13.8 $\pm$ 1.0	704 $\pm$ 59	51 $\pm$ 5
Triton X-100 supernatant			
Triton X-100, dithiothreitol 150 000 g			
SM-2 Bio-Beads			
Triton extract	5.9 $\pm$ 0.4	702 $\pm$ 55	119 $\pm$ 14
DEAE-52 DEAE-cellulose			
DEAE-52 Extract			
SM-2 Bio-Beads			
Purified preparation	0.69 $\pm$ 0.14	319 $\pm$ 51	463 $\pm$ 82

ionic strength, was not associated with detectable high-affinity binding activity. In agreement with a previous study (Kasahara & Hinkle, 1977), the major protein in the 1 M-NaCl fractions was band 3. The total recovery of proteins from all fractions was 88%. Void-volume fractions contained the majority of lipid present in the Triton X-100 extract. Since nitrobenzylthioinosine-binding protein may require lipid for activity (Pickard & Paterson, 1976), samples from each of the fractions in Fig. 3 were supplemented from soya-bean phospholipid before removal of detergent. This resulted in an unchanged activity profile, although non-specific nitrobenzylthioinosine binding was increased in the presence of added lipid (results not shown). In routine experiments, void-volume fractions (nos. 3–6) were pooled, treated with Bio-Beads and concentrated.

The average specific activity of the final preparation was 463 pmol of nitrobenzylthioinosine bound/mg of protein, giving an overall purification of binding activity of 13-fold. The total recovery of binding activity was 31%. Treatment of pooled void-volume fractions with Bio-Beads resulted in a 50% decrease in protein concentration (see also Baldwin *et al.*, 1979). Loss of high-affinity binding activity during the final stage of purification is therefore likely to represent absorption of nitrobenzylthioinosine-binding protein by Bio-Beads. This contrasts with the situation for the original Triton X-100 extract where Bio-Bead treatment did not result in a significant decrease in binding activity, although 10–25% of the protein was lost. The difference in behaviour of the two preparations

Fig. 3. DEAE-cellulose column chromatography of nitrobenzylthioinosine-binding activity

Membranes were solubilized with 1% Triton X-100 also containing 1 mM-dithiothreitol and 50 mM-Tris/HCl (pH 8.2), centrifuged at 150 000 g for 45 min, and the supernatant was applied to a DEAE-cellulose column as described in the Materials and methods section. The column was eluted with the same buffer (fraction nos. 1–13). Adsorbed proteins were eluted with buffers containing 0.1 M-NaCl (fraction nos. 14–19) and 1 M-NaCl (fraction nos. 20–26). The protein concentration of individual fractions was measured (○). Fractions were then treated with Bio-Beads and assayed for nitrobenzylthioinosine-binding activity (●). The concentration of [³H]nitrobenzylthioinosine (average of both chambers) was 0.5 nM. The distribution ratio of fractions 2–7 was decreased to 1.03 in the presence of 5 μM-nitrobenzylthioguanosine (results not shown).

is presumably due to the higher protein concentration of the starting extract. In two other experiments, the DEAE-cellulose column effluent was passed directly through a column of Bio-Beads. This method of detergent removal gave similar results to the other procedure (mean specific activity 492 pmol of nitrobenzylthioinosine bound/mg of protein, with a 21% recovery of binding activity).

Characterization of the DEAE-cellulose preparation

Fig. 4 shows the concentration dependence of nitrobenzylthioinosine binding to the partially purified preparation. Binding was resolved into two components; a high-affinity binding component inhibited by nitrobenzylthioguanosine, and a non-saturable nitrobenzylthioguanosine-insensitive binding component. The apparent K_d value for the high-affinity binding (1.6 ± 0.3 nM; mean $\pm$ s.e.m. for four separate preparations) is identical with that found with native membranes. The results presented in Table 2 establish that this binding activity is also inhibited by uridine and dipyridamole. Approx. 60% of the nitrobenzylthioinosine-binding activity could be sedimented by centrifugation at 150 000 *g* for 45 min, suggesting that it was associated with the phospholipid vesicles known to be present in such preparations after Bio-Bead treatment.

The protein composition of the preparation at each stage of purification was monitored by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Densitometric recordings from a representative experiment are shown in Fig. 5. Protein-depletion was associated with the selective loss of bands 1, 2, 2.1, 4.1, 5 and 6 (nomenclature of Steck, 1974). The Triton X-100 extract (Bio-Bead-treated) had a similar protein composition to protein-depleted membranes, but the purified preparation (also Bio-Bead-treated) exhibited only two detectable protein zones; a broad-zone band 4.5 and a smaller amount of band 7.

Studies from other laboratories have implicated band 4.5 polypeptides in glucose translocation (Kasahara & Hinkle, 1977; Kahlenberg & Zala, 1977; Goldin & Rhoden, 1978; Sogin & Hinkle, 1978). Purified preparations of glucose-transport protein(s) have essentially identical sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic profiles with that shown in Fig. 5. Such preparations exhibit

Fig. 4. Concentration dependence of nitrobenzylthioinosine binding to the partially purified preparation. The amount of [3 H]nitrobenzylthioinosine bound to the partially purified preparation in the presence (O) and absence (●) of 5 μ M-nitrobenzylthioguanosine is plotted against the equilibrium concentration of free nitrobenzylthioinosine. The inset shows a Scatchard plot of the data (corrected for the nitrobenzylthioguanosine-insensitive component). Maximum binding (500 pmol/mg of protein) and apparent K_d (2.3 nM) were determined by linear regression analysis.

Table 2. Inhibition of nitrobenzylthioinosine binding to the partially purified DEAE-cellulose preparation

Binding activity was assayed by equilibrium dialysis. Purified preparation (0.2 ml) was added to one side of the dialysis membrane, and 0.2 ml portions of [3 H]nitrobenzylthioinosine in the presence of test inhibitor (± 10 μ M-nitrobenzylthioguanosine, to correct for non-specific binding) were added to the other side. High-affinity binding activity in the absence of test inhibitor was 103, 270 and 353 pmol/mg of protein at initial nitrobenzylthioinosine concentrations of 1, 5 and 10 nM respectively. Non-specific nitrobenzylthioinosine binding was 2.5 pmol/mg of protein at 1 nM final equilibrium concentration. Values are means of duplicate estimates. Other experimental details are given in the text. Abbreviation used: N.D., not determined.

Initial nitrobenzylthioinosine concentration (nM) . . .	Nitrobenzylthioinosine bound (% of control value)		
	1	5	10
None	100	100	100
Uridine (5 mM)	N.D.	40.5	56.3
Uridine (10 mM)	2.0	3.6	N.D.
Dipyridamole (10 μ M)	0	0	N.D.

Fig. 5. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of membrane preparations at each stage of the purification procedure

Gradient slab-gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described in the text. The gel was stained with Coomassie Blue and scanned at 525 nm using a Helana Densitometer (Helana Laboratories, Beaumont, TX, U.S.A.). The amounts of protein present in each sample were 46, 20, 16 and 9.7 μ g of protein for 'ghosts', protein-depleted membranes, Triton X-100 extract and the partially-purified DEAE-52 DEAE-cellulose fraction respectively. The latter two samples had been treated with Bio-Beads to remove detergent. (a) and (b) are duplicate runs of the same DEAE-52 DEAE-cellulose fraction. A length of gel containing no sample was scanned to determine background absorbance (blank). The standard proteins (4 μ g each) were soya-bean trypsin inhibitor, bovine serum albumin and RNA polymerase from *Escherichia coli* (containing α , β and β' sub-units) (Boehringer Combithek). The numbers above the peaks in the scan for standards refer to molecular weight ($\times 10^{-3}$). Abbreviation used: H, haemoglobin.

Discussion

In the present experiments we have been able to extract and partially purify the membrane component(s) responsible for high-affinity nitrobenzylthioinosine binding to human erythrocyte membranes. Evidence demonstrating that this binding represents a specific interaction with the nucleoside-transport mechanism is presented in the introduction. These studies further suggest that nitrobenzylthioinosine binds directly to the nucleoside-permeation site of the transport system. It is therefore reasonable to consider high-affinity nitrobenzylthioinosine-binding activity as a specific quantitative assay for nucleoside-transport protein(s).

The final preparation contained only two visible protein zones as judged by gradient sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a major broad zone band (4.5) (apparent mol.wt. 40 000–70 000) and a minor band, running in the position of band 7 (apparent mol.wt. 29 000). The latter band contributed 5% of the protein as judged by its ability to bind Coomassie Blue. We have previously reported that radiation-inactivation analyses estimate the molecular weight of the nitrobenzylthioinosine-binding complex *in situ* to be 122 000 (Jarvis *et al.*, 1980a). Assuming that one 122 000-mol.wt. unit binds a single molecule of nitrobenzylthioinosine, it can be calculated that nitrobenzylthioinosine-binding protein should represent 6% of the protein present in the purified preparation. However, no high-molecular-weight species were detected. Errors in the estimate of molecular weight by radiation-inactivation analysis are unlikely to account for this discrepancy (Jarvis *et al.*, 1980a), raising the possibility that the nucleoside-transport system exists in the membrane as a dimer or tetramer, as has been suggested for the erythrocyte anion- and glucose-transport systems (Peters & Richards, 1977; Nigg & Cherry, 1979; Hinkle *et al.*, 1979; Jung *et al.*, 1980). An alternative possibility is that nucleoside-transport protein is cleaved by proteinases during the purification procedure (see, for example, Mullins & Langdon, 1980). However, nitrobenzylthioinosine binding to the purified preparation was indistinguishable from that present in intact cells and isolated membranes.

The protein profile of the purified extract is almost identical with that reported by other groups for purified preparations of glucose-transport protein, identified as a major band 4.5 polypeptide by liposome-reconstitution studies, cytochalasin B-binding assays and differential-labelling experiments (Batt *et al.*, 1976; Kasahara & Hinkle, 1977; Lienhard *et al.*, 1977; Goldin & Rhoden, 1978; Sogin & Hinkle, 1978; Baldwin *et al.*, 1979). The broadness of this band on sodium dodecyl sulphate/polyacrylamide gels is attributed, at least

D-glucose-sensitive cytochalasin B-binding activity. We confirmed that our DEAE-cellulose preparation had high-affinity cytochalasin B-binding activity, which was inhibited by D-glucose. The recovery of this binding activity was 37% of that present in protein-depleted membranes.

in part, to heterogeneous glycosylation, although up to seven separate bands have been detected in this region using discontinuous buffer systems (Gorga *et al.*, 1979; King & Morrison, 1977). In the present experiments we have been able to show that our preparation also exhibits D-glucose-sensitive cytochalasin B-binding activity, demonstrating that the inhibitor-binding activities of the nucleoside and glucose carriers co-purified under the experimental conditions used in our study. The two transport systems are, however, functionally distinct. For example, there are $(1-3) \times 10^5$ D-glucose-sensitive cytochalasin B-binding sites/cell compared with 10^4 nitrobenzylthioinosine-binding sites (Cass *et al.*, 1974; Lin & Spundich, 1974; Jung & Rampal, 1977; Jarvis & Young, 1980a). Furthermore, nitrobenzylthioguanosine does not inhibit glucose transport and sugars have no effect on nucleoside influx (Paterson & Oliver, 1971; Oliver & Paterson, 1971). Finally, adult pig erythrocytes possess a functional nucleoside-translocation mechanism, but are impermeable to sugars (Kim & McManus, 1971; Jarvis *et al.*, 1980b). Nevertheless the two transport processes occur by facilitated diffusion and both exhibit kinetic as well as chemical asymmetry (Van Stevenick *et al.*, 1965; Baker & Widdas, 1973; Cabantchik & Ginsburg, 1977; Masiak & Le Fevre, 1977; Baker *et al.*, 1978; Basketter & Widdas, 1978; Jarvis & Young, 1980e). It is therefore tempting to speculate that the two transport functions are catalysed by similar but distinct band 4.5 polypeptides. An alternative possibility is that nitrobenzylthioinosine-binding activity is associated with the residual band 7 present in the preparation.

Note added in proof (Received 26 August 1980)

Immunological studies by Baldwin & Lienhard (1980), published after the present paper was submitted, provide strong evidence that band-4.5 glucose-transport protein is not a proteolytic fragment of a larger polypeptide.

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