# SOLUBILIZATION OF THE NUCLEOSIDE TRANSLOCATION SYSTEM FROM HUMAN AND NUCLEOSIDE-PERMEABLE SHEEP ERYTHROCYTES

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## 1. Introduction

Human and *Nu*-permeable type sheep erythrocytes transport nucleosides by a broad-specificity, facilitated-diffusion mechanism [1,2]. Erythrocytes from other sheep (*Nu*-impermeable type) lack this system, with the permeability difference between *Nu*-permeable and *Nu*-impermeable cells under the simple genetic control of two autosomal alleles [2,3]. A number of other cell types, including Novikoff rat hepatoma cells, P 388 murine leukemia cells and Chinese hamster ovary cells, possess a similar translocation system [4,5]. NBMPR, a potent reversible inhibitor of carrier-mediated nucleoside transport, binds to high-affinity sites on human and *Nu*-permeable type sheep erythrocyte membranes with app. $K_d$ 1 nM [6,7]. This association represents a specific interaction with functional nucleoside transport sites [6]. As a first step towards the isolation and characterization of the membrane component(s) responsible for nucleoside translocation, we report the solubilization of high affinity NBMPR binding activity from these cells by the detergent sodium cholate.

## 2. Experimental

### 2.1. *Membrane prepration and solubilization*

Haemoglobin-free erythrocyte membranes were prepared from human, *Nu*-permeable sheep and *Nu*-impermeable sheep erythrocytes as in [6]. *Nu*-permeable type and *Nu*-impermeable type animals were selected from the Babraham flock on the basis of nucleoside permeability studies [2,3]. Membranes were solubilized by the addition of 1 vol. 35% (w/v) sodium cholate (pH 8.2) to 6 vol. membrane suspension (5 mg protein/ml). Samples were stirred for 15 min at 20°C and then centrifuged at 150 000 × *g* for 45 min to remove insoluble material.

### 2.2. *Removal of detergent*

Cholate supernatant (2 ml) was applied to a column (23 × 1.5 cm) of Sephadex G-50 (medium grade) which was equilibrated and eluted with 10 mM Tris–HCl, 0.15 M NaCl (pH 7.2 at 20°C) (flowrate 14 ml/h, fraction vol. 1.4 ml) [8]. Void-volume fractions were collected and pooled.

### 2.3. *NBMPR binding assays*

Samples of cholate-depleted membrane extract (75 μl) were preincubated at 25°C ± 5 μl of 100 μM NBTGR. After 25 min, 20 μl prewarmed Tris-medium containing [$^3$H]NBMPR (2.5–100 nM; 20 Ci/mmol) (Movarek Biochem., City of Industry, CA 91745) was added. Incubations (15 min) were terminated by rapidly cooling the incubation mixture to 4°C before separating bound from free [$^3$H]NBMPR by gel filtration. For competition experiments, 55 μl aliquots of sample were preincubated with 20 μl inhibitor in the presence and absence of NBTGR for 25 min before addition of [$^3$H]NBMPR.

For gel filtration, samples (75 μl) were loaded onto 6 × 0.4 cm Sephadex G-50 (medium) columns, equilibrated with Tris-buffer at 4°C. Fractions (125 μl) were collected directly into scintillation vials at 0.375 ml/min flowrate. [$^3$H]NBMPR binding to erythrocyte 'ghosts' was determined by centrifugation

*Abbreviations:* NBMPR, nitrobenzylthioinosine (6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosyl purine); NBTGR, nitrobenzylthioguanosine (2-amino-6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosyl purine)

and washing as in [6]. [$^3$H]NBMPR binding to samples containing cholate was estimated by equilibrium dialysis [9]. Samples were counted for radioactivity in a Packard Tricarb scintillation counter with quench correction [2,6].

### 2.4. *Protein measurements*

Protein was assayed by a modified Lowry procedure with appropriate blanks and standards to correct for lipid, detergent and Tris interference [10].

## 3. Results and discussion

### 3.1. *Human erythrocytes*

The 150 000 × *g* cholate supernatant, obtained by extracting human erythrocyte 'ghosts' with 5% sodium cholate, contained 49 ± 2% (mean ± SEM of 7 expt) of the membrane protein but no detectable [$^3$H]-NBMPR binding activity. We considered it possible that the cholate supernatant contained significant numbers of NBMPR binding sites but that detergent was inhibiting binding activity in the same way that Triton X-100 has been shown to inhibit cytochalasin B binding to glucose transport proteins [9]. Samples of cholate extract were therefore depleted of detergent by Sephadex G-50 column chromatography [8]. The majority of protein (92%) eluted as a single peak in a position corresponding to the void-volume of the column, as judged by blue dextran calibration. These void-volume fractions also contained substantial [$^3$H]-NBMPR binding activity and are referred to as cholate-depleted extract.

Fig.1 shows the results of a typical binding assay where 75 μl aliquots of cholate-depleted extract were incubated with 5 nM [$^3$H]NBMPR (± 5 μM NBTGR) and applied to 1 ml Sephadex G-50 columns as in section 2. In the absence of NBTGR, 59% of the $^3$H-labelled inhibitor eluted in the void-volume. When NBTGR was present, only 3% of the radioactivity was recovered in the void-volume with a proportional increase in unbound counts. All the [$^3$H]NBMPR applied to the columns was recovered. Fig.2 shows the concentration dependence of [$^3$H]NBMPR binding to this preparation. Binding was saturable in the absence of NBTGR, and low and linear with concentration in its presence. Correction of the saturable uptake data for the small linear component of binding observed in the presence of NBTGR gave app. $K_d$ 1 nM with a maximum binding of 8.9 pmol/mg protein. The total recovery of high-affinity [$^3$H]NBMPR binding activity in the cholate-depleted extract was

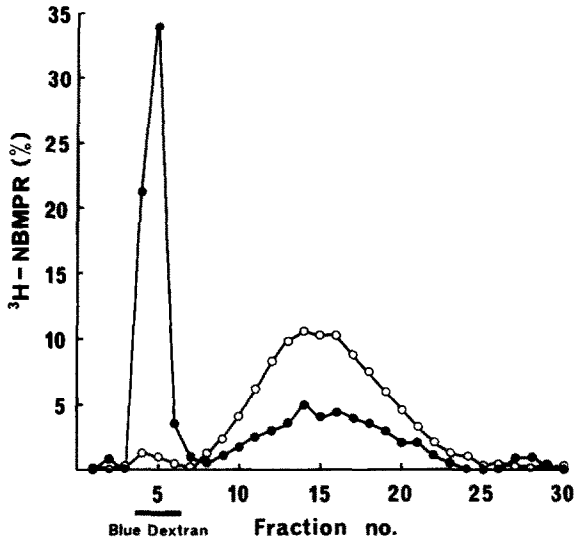


Fig.1. Binding of [$^3$H]nitrobenzylthioinosine to cholate-depleted membrane extracts from human erythrocytes. Samples were incubated in the presence (○) and absence (●) of excess NBTGR (5 μM) before addition of [$^3$H]NBMPR (5 nM). Aliquots (0.075 ml) were applied to columns (6 × 0.4 cm) of Sephadex G-50 (medium) to separate bound and free tritium. Fractions (0.125 ml) were collected and counted for radioactivity as described.

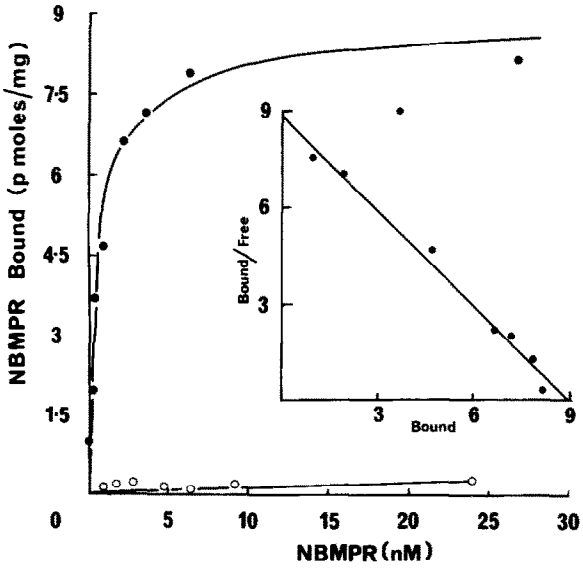


Fig.2. Concentration dependence of [$^3$H]nitrobenzylthioinosine binding to cholate-depleted human erythrocyte membrane extracts. Cholate-depleted extract was incubated at 25°C with [$^3$H]NBMPR in the presence (○) and absence (●) of 5 μM NBTGR. The amount of [$^3$H]NBMPR bound is plotted as a function of the equilibrium free NBMPR concentration. A Scatchard plot of the data (corrected for the NBTGR-insensitive component) is known in the insert diagram. Maximum binding (8.9 pmol/mg protein) and apparent $K_d$ (1.0 nM) were determined by linear regression analysis.

9% of that present in the erythrocyte ghosts. This experiment demonstrates that NBTGR-sensitive [$^3$H]-NBMPR binding to cholate-depleted membrane extracts has a similar apparent $K_d$ value to that found in native membranes (0.5–1.7 nM) [6,7]. Further similarities between [$^3$H]NBMPR binding to the two preparations were that both uridine (a transported nucleoside) and dipyridamole (an unrelated nucleoside transport inhibitor) inhibited high-affinity [$^3$H]-NBMPR binding to the cholate-depleted extract. Uridine (10 mM) gave 25 and 79% inhibition at initial [$^3$H]NBMPR concentrations of 15 and 0.75 nM, respectively. Dipyridamole (1 $\mu$M) inhibited binding by 91% ([$^3$H]NBMPR at 15 nM).

Removal of detergent from the 150 000 × $g$ cholate supernatant led to the appearance of unilamellar vesicles with dimensions of 50–150 nm diam., measured by transmission electron microscopy (glutaraldehyde and osmium fixation followed by Araldite embedding). Centrifugation of cholate-depleted extract at 150 000 × $g$ for 45 min sedimented >90% of the [$^3$H]NBMPR binding activity, raising the possibility that is was associated with these vesicles. Lipid may be necessary for NBMPR binding activity [11].

### 3.2. *Sheep erythrocytes*

Further direct evidence that the NBMPR binding activity recovered after cholate extraction of erythrocyte membranes is identical to that associated with the nucleoside transport mechanism in intact cell membranes is presented in fig.3. This diagram compares [$^3$H]NBMPR binding assays of cholate-depleted extracts prepared from *Nu*-permeable and *Nu*-impermeable type sheep erythrocyte membranes. Since *Nu*-permeable sheep erythrocytes have considerably fewer NBMPR binding sites than human cells (18 and 8400 sites/cell, respectively [6]), the binding assay was scaled up to accommodate a sample volume of 2 ml. The initial [$^3$H]NBMPR concentration used in this experiment was 1 nM. The cholate-depleted extract from *Nu*-permeable cells bound 34.6 fmol/mg protein in the absence of NBTGR and 12.1 fmol/mg protein

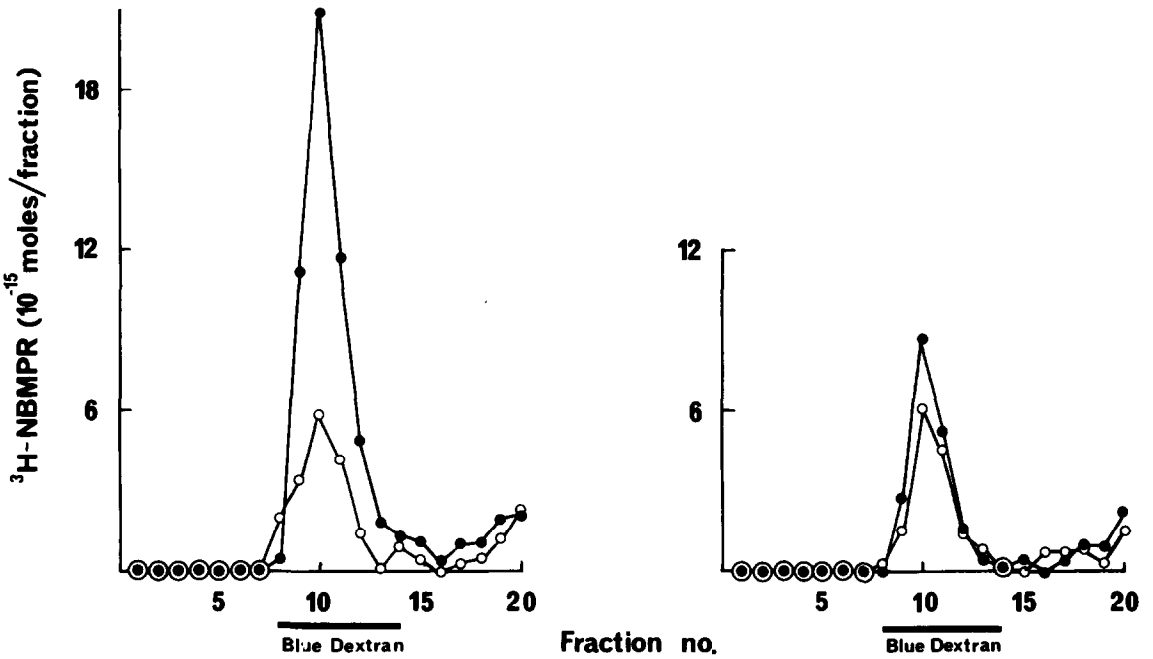


Fig.3. Binding of [$^3$H]nitrobenzylthioinosine to cholate-depleted membrane extracts from *Nu*-permeable and *Nu*-impermeable sheep erythrocytes. Cholate-depleted membrane extracts from sheep erythrocytes (2 ml) were pre-incubated in the presence (○) and absence (●) of 5 $\mu$M NBTGR before addition of [$^3$H]NBMPR (1 nM). Samples were then applied to columns (23 × 1.5 cm) of Sephadex G-50 (coarse) and 1.5 ml fractions collected for radioactivity determinations. (A) *Nu*-permeable cells; (B) *Nu*-impermeable cells.

in its presence, giving a specific binding component of 22.5 fmol/mg protein. In another experiment (not shown) using 3 nM [$^3$H]NBMPR, the specific binding component was 32.1 fmol/mg protein, suggesting that this binding was saturable. In contrast, the extract prepared from *Nu*-impermeable type membranes gave a specific binding component of <2.8 fmol/mg protein.

## 4. Conclusions

These experiments demonstrate that extraction of human and *Nu*-permeable type sheep erythrocyte membranes with sodium cholate solubilizes high-affinity NBMPR binding sites associated with the nucleoside translocation mechanism. Binding to these sites cannot be detected in the presence of detergent, but can, at least in part, be recovered on removal of cholate. This binding has similar properties to that found in intact membranes. These experiments represent the first successful attempt to solubilize and recover NBMPR binding activity from cell membranes. It should now be possible to isolate membrane component(s) responsible for nucleoside translocation based on an assay of NBMPR binding activity.

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