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Enhancement of the Functional Stability of Solubilized Nucleoside Transporters by Substrates and Inhibitors

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ABSTRACT. Purification of functional nucleoside transporters has been hampered by the instability of detergent-solublized proteins. The present study was undertaken to determine if the presence of specific transporter ligands in the solubilization medium could enhance the functional stability of the isolated proteins. Ehrlich cell plasma membranes were solubilized with 1% (w/v) octylglucoside (± transporter ligands) and reconstituted into liposomal membranes either immediately after solubilization or after storage for 48 hr at 6°. Storage resulted in a parallel loss (≈60%) of [^{3}H]nitrobenzylthioinosine (NBMPR) binding and reconstituted [^{3}H]uridine uptake activities. Furthermore, upon storage, the relative amount of NBMPR-resistant [^{3}H]uridine uptake by the reconstituted system dropped from 19 ± 2 to 8 ± 1% of the total mediated influx. The inclusion of high concentrations (>10 mM) of adenosine in the solubilization medium completely prevented the storage-induced loss of both [^{3}H]NBMPR binding and [^{3}H]uridine influx activity, and prevented the shift in NBMPR sensitivity. In addition, inclusion of adenosine in the solublization procedure increased the relative amount of NBMPR-resistant [^{3}H]uridine uptake to 33 ± 2% of the total influx in proteoliposomes prepared immediately after the proteins were extracted from the plasma membrane (i.e. no storage). A partial protection of [^{3}H]NBMPR binding activity was also obtained using 2′-deoxyadenosine, 2-chloroadenosine, uridine, and non-radiolabelled NBMPR, but not with cytidine, inosine, diazepam, dipyridamole, or dilazep. These results suggest that both NBMPR sensitivity and transporter stability are dependent upon the conformational state of the protein. The protective effects of adenosine analogues and other nucleosides are likely due to their binding to the substrate translocation site, thereby effectively "locking" the transporter in a stable conformation. BIOCHEM PHARMACOL 53;5:623–629, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. nucleoside transport; protein solubilization; octylglucoside; reconstitution; nitrobenzylthioinosine; adenosine

Purine and pyrimidine nucleosides are accumulated by mammalian cells via a family of integral membrane proteins collectively referred to as nucleoside transporters [1–3]. Transporter subtypes within this family are defined by their dependencies upon ion gradients and their relative sensitivities to specific inhibitors. The current study focuses on the *es* (equilibrative inhibitor sensitive) and *ei* (equilibrative inhibitor insensitive) transporters of Ehrlich ascites tumour cells that can be distinguished by their differential sensitivities to NBMPR† [4]. Uptake into cells, via these transporters, is the principal mechanism by which the extracellular receptor-mediated biological modulatory actions of adenosine are terminated. To gain a better understanding of the molecular mechanisms of substrate translocation and inhibitor sensitivity, several laboratories have attempted to purify the equilibrative transporters from mammalian cells with subsequent reconstitution of transport activity in liposomes [5–11]. While substantial purification of one of the transport proteins has been achieved, the isolated proteins were functionally unstable [7, 10, 11]. Many of these studies utilized OCTG [6–8, 10–12]. This non-ionic detergent has chemical properties, such as electrical neutrality, a high critical micelle concentration, and optical UV transparency, that make it ideal for the solublization and reconstitution of integral membrane proteins [13]. The OCTG-solubilized transporters display all the ligand binding characteristics of the native transporter [8], and are capable of mediating the translocation of [^{3}H]uridine across liposomal membranes [8, 14]. However, all of these measures of transporter functionality decay rapidly with time after removal of the protein from the plasma membrane [10, 14], which imposes a severe handicap on procedures aimed at the extensive purification of functional nucleoside transporters. Other membrane proteins have been shown to be more stable in the presence of their specific ligands [15–19].

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† *Abbreviations:* NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine; and OCTG, octylglucoside.

Therefore, the present study was undertaken to assess the effects of various nucleoside substrates and transport inhibitors on the functional stability of the OCTG-solubilized/reconstituted proteins. The functional integrity of the solubilized transporters was assessed by measuring their capacities to bind the specific radioligand probe [^{3}H]NBMPR, and by the ability of proteoliposomes, formed by removal of the detergent from the solubilized preparations in the presence of excess lipid [14], to accumulate [^{3}H]uridine via an NBMPR/dipyridamole/adenosine inhibitable system. A preliminary report of this work was presented at the 1996 Summer Meeting of the British Pharmacological Society [20].

MATERIALS AND METHODS

Chemicals

[^{3}H]NBMPR (16 Ci/mmol) and [5,6-^{3}H]uridine (35–50 Ci/mmol) were from Moravek Biochemicals Inc. (Brea, CA, U.S.A.) and ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.), respectively. [^{14}C]Cholesteryl-oleate (0.1 mCi/mL) was obtained from Amersham Canada Ltd. (Oakville, Ontario). Dilazep (*N,N'*-bis[3-(3,4,5-trimethoxybenzoyloxy)propyl]homopiperazine) was provided by Asta Werke (Frankfurt, Germany), and 2-chloroadenosine was purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). Asolectin (soybean phospholipids) was purchased from Associated Concentrates (Woodside, NY, U.S.A.), and stored under N_2. All other phospholipids (phosphatidylcholine, bovine brain; phosphatidylserine, bovine brain; phosphatidylethanolamine, egg) and cholesterol were purchased from Serdary Research Laboratories (London, Canada). OCTG was obtained from Calbiochem Biochemicals Inc. (San Diego, CA, U.S.A.). All nucleosides, NBMPR, NBTGR, and dipyridamole [2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine] were supplied by Sigma (St. Louis, MO, U.S.A.). Diazepam (Hoffmann-La Roche, Toronto, Canada) was obtained from Dr. J. T. Hamilton, University of Western Ontario. Sephadex G-50 (Fine and Medium Grade) was purchased from Pharmacia, Canada (Baie d'Urfe, Quebec). All other compounds were of reagent grade.

Isolation and Solubilization of Ehrlich Cell Plasma Membranes

Ehrlich ascites cells, grown as an intraperitoneal culture in mice (Swiss, male, ≈30 g), were processed to obtain plasma membranes as described previously [8, 21]. In brief, washed cells were swollen by suspension in 1 mM $ZnCl_2$, fragmented using a Polytron homogenizer PT-10-20, and resuspended in 9.25% sucrose with removal of nuclei by centrifugation (three times) for 1 min at 900 *g*. Plasma membranes were then separated on a 15–60% (w/w) sucrose gradient, and stored at −80° in 15% DMSO for up to 3 months. No loss in [^{3}H]NBMPR binding activity was observed over this time period. In preparation for solubilization studies, plasma membranes were thawed at room temperature and washed extensively to remove DMSO, then resuspended in ice-cold buffer (pH 7.4) containing 100 mM KCl, 10 mM Tris, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and other additives as indicated in Results. Membranes (~200 μg/mL final concentration) were then mixed 1:1 with a detergent mixture prepared using the same buffer solution (± stabilizing agents), and incubated on ice for 1 hr with constant stirring. The mixtures were centrifuged at 120,000 *g* for 60 min and the supernatant was retained on ice until further analysis, or placed at 6° for 48 hr. In some cases, [^{3}H]NBMPR (200 nM) or [^{3}H]adenosine (10 mM) was added to the solubilized membranes for assessment of the efficiency of the subsequent reconstitution procedure in the removal of these additives.

Reconstitution Procedure

Solubilized membranes were supplemented with a sonicated preparation of lipids consisting of phosphatidylcholine, cholesterol, phosphatidylethanolamine, and phosphatidylserine, in a molar ratio of 33:33:26:8, respectively, plus a trace (10^5 dpm/mL) quantity of [^{14}C]cholesteryl-oleate, as described previously [8, 14]. The detergent was then removed by gel filtration (Sephadex G-50 medium; 1.5 × 28 cm column) at a flow rate of 1.6 mL/min. The void volume fractions, containing proteoliposomes (liposomes + protein), were pooled, frozen in ethanol/dry ice, and stored at −80° for up to 1 month. In preparation for assays, proteoliposomes were thawed at room temperature and washed twice by centrifugation at 40,000 *g* for 20 min. The final pellet was resuspended in ≈1 mL buffer (22°), sonicated for 10 sec in a cylindrical tank sonicator (Laboratory Supplies Co. Inc., Hicksville, NY, U.S.A.), and then stored on ice.

[^{3}H]NBMPR Binding Assays

Membranes (plasma or liposomal) were incubated with a range of concentrations of [^{3}H]NBMPR (0.02 to 1.5 nM) at 22° in the absence (total binding) and presence (nonspecific binding) of 10 μM dilazep (1 mL final assay volume). After a 45-min incubation, proteins were precipitated by incubation for 15 min with γ-globulins (1.65 mg/mL, final concentration) and polyethylene glycol [mol. wt 8000, 10% (w/v) final concentration], and then filtered, under vacuum, through Whatman GF/B filters. The filters were washed twice with buffer containing 8% (w/v) polyethylene glycol at 4°, and analysed for radioactive content by standard liquid scintillation counting techniques.

[^{3}H]Uridine Uptake by Proteoliposomes

Assays were initiated by the addition of 100 μL of proteoliposome suspension (≈10 μg protein) to 25 μL of [^{3}H]uridine (20 μM final concentration) and mixed by repeated pipetting. After a defined incubation time, 100 μL of the latter mixture was layered on an ice-cold Sephadex G-50

Fine minicolumn (see below), which was centrifuged immediately for 45 sec at ≈700 *g*. The effluent was collected directly into pre-weighed tubes from which aliquots were removed for the determination of protein, [^{14}C], and [^{3}H] content. The minicolumns were prepared by filling a 1-mL syringe, fitted with a polyethylene filter, with Sephadex gel equilibrated in buffer containing 10 μM dilazep and 10 μM NBTGR; at 30–60 min prior to use, the gel-filled syringes were centrifuged for 45 sec at ≈700 *g* and placed on ice. An estimate of the "zero-time" uptake was obtained by measuring the uptake of [^{3}H]uridine at the minimum possible incubation time (≈3 sec) using ice-cold solutions containing 10 mM adenosine, 10 μM dipyridamole, and 10 μM NBTGR to inhibit all transporter-mediated influx. Results from individual experiments were normalized based on the [^{14}C]lipid concentration of the column effluent. Mediated influx of [^{3}H]uridine was defined as the total uptake minus the uptake observed in the presence of 10 mM adenosine/10 μM dipyridamole/10 μM NBTGR (non-mediated). For the NBMPR inhibition assays, proteoliposomes were incubated with NBMPR for 10 min prior to exposure to [^{3}H]uridine. Protein was measured by the method of Markwell *et al.* [22], using bovine serum albumin as the standard. All values reported were derived from computer-generated hyperbolic or sigmoid curves (GraphPad Prism, v 2.0), as appropriate, fitted to experimental data obtained from individual experiments.

RESULTS

OCTG [1.0% (w/v) + 0.15% (w/v) asolectin] solubilized over 60% of the total protein and [^{3}H]NBMPR binding activity from the Ehrlich cell plasma membranes. The solubilized preparation bound an average of 9.0 pmol [^{3}H]NBMPR/mg protein, and storage of the solubilized proteins for 48 hr at 6° resulted in a 62% loss of [^{3}H]NBMPR binding (B_{max} = 3.5 pmol/mg) (Table 1). These results are comparable to those obtained in an earlier study [14]. There was no change in the affinity of the sites for [^{3}H]NBMPR upon storage (K_D ~0.1 nM), and a similar instability of binding was obtained regardless of whether the preparations were stored for 48 hr in detergent or after incorporation into liposomal membranes (data not shown).

The inclusion of adenosine in the solubilization procedure had a concentration-dependent protective effect on the solubilized [^{3}H]NBMPR binding activity, with maximal protection seen using 20 mM adenosine (Table 1). The inclusion of adenosine in the solubilization procedure had no significant effect on the affinity of the reconstituted binding sites for [^{3}H]NBMPR (Table 1). This is consistent with results showing that over 99.99% of the [^{3}H]adenosine originally added to the solubilized membranes (10 mM) was removed from the preparation during the reconstitution procedure (final concentration <1 μM). The 2′-deoxy analogue of adenosine was equivalent to adenosine in its ability to protect the [^{3}H]NBMPR binding activity, as was the pyrimidine nucleoside uridine. A partial protective effect was also seen with 2-chloroadenosine (Table 1), although the B_{max} of [^{3}H]NBMPR binding was reduced significantly after exposure to 2-chloroadenosine, possibly reflecting incomplete wash-out of the additive (see below). However, a number of other nucleoside transporter substrates were relatively ineffective in this regard; over 40% of the

TABLE 1. Effect of substrates and inhibitors on the stability of [^{3}H]NBMPR binding to solubilized nucleoside transporters

		K_D (pM)	B_{max} (pmol/mg protein)		
			No storage	48 hr/6°	% Loss
Control	(15)	96 ± 8	9.0 ± 0.6	3.5 ± 0.6	62 ± 4
Adenosine	1 mM (5)	109 ± 15	10.1 ± 0.8	4.5 ± 0.5	54 ± 6
	5 mM (4)	99 ± 6	9.5 ± 0.7	6.1 ± 1.0	37 ± 7*
	20 mM (6)	85 ± 7	9.9 ± 0.4	8.1 ± 0.6	18 ± 4*
NBMPR	10 nM (5)	109 ± 11	3.9 ± 1.0*	2.1 ± 0.7	48 ± 9
	50 nM (5)	139 ± 23	3.8 ± 0.9*	2.8 ± 0.8	31 ± 6*
	200 nM (4)	131 ± 13	4.2 ± 1.9*	3.4 ± 1.6	23 ± 4*
2′-Deoxyadenosine	20 mM (4)	89 ± 5	6.9 ± 0.9	5.8 ± 1.3	18 ± 9*
Uridine	50 mM (4)	132 ± 53	8.5 ± 1.6	6.5 ± 1.0	22 ± 4*
2-Chloroadenosine	500 μM (4)	96 ± 14	4.8 ± 1.3*	3.1 ± 1.1	32 ± 13*
Thymidine	50 mM (5)	132 ± 47	7.5 ± 1.2	4.4 ± 0.9	41 ± 7*
Inosine	20 mM (4)	93 ± 8	6.9 ± 0.9	4.0 ± 1.6	45 ± 17
Cytidine	50 mM (6)	75 ± 7	8.3 ± 1.0	4.1 ± 0.5	47 ± 6
Dilazep	1 μM (3)	121 ± 35	3.1 ± 0.9*	1.4 ± 0.3	51 ± 5
Diazepam	200 μM (4)	92 ± 11	7.0 ± 1.5	2.7 ± 0.7	61 ± 6
Dipyridamole	1 μM (3)	100 ± 33	1.9 ± 0.3*	0.6 ± 0.1	69 ± 4

Plasma membranes were solubilized in the absence (control) and presence of the indicated compounds, and proteoliposomes were prepared either immediately after solubilization (no storage) or after storage of the solubilized material for 48 hr at 6°. For statistical purposes, each experiment was paired with its own control; the average control values are reported in the table. Proteoliposomes were incubated for 45 min with a range of concentrations of [^{3}H]NBMPR. Binding affinity (K_D) and the maximum number of binding sites (B_{max}) were obtained by mass law analysis. Each value is the mean ± SEM from the number of experiments indicated in parentheses.

* Significantly different from control, $P < 0.05$ (Student's *t*-test for paired samples).

[^{3}H]NBMPR binding activity was lost upon storage in the presence of thymidine (50 mM), inosine (20 mM), and cytidine (50 mM). Likewise, the inclusion of dipyridamole, dilazep, and diazepam in the solubilization procedure did not affect the loss of [^{3}H]NBMPR binding upon storage. The only transport inhibitor that afforded a degree of protection against the storage-induced loss of [^{3}H]NBMPR sites was NBMPR itself but, like adenosine, only at concentrations greater than 500 times its K_D for binding (50 nM; see Table 1). NBMPR, dipyridamole, and dilazep also resulted in a significant reduction in the maximum binding of [^{3}H]NBMPR to the freshly solubilized preparations, with no change in binding affinity; this was not seen in comparable experiments with the nucleoside substrates (see Table 1). It was also determined, by supplementing the solubilized preparation with [^{3}H]NBMPR, that the reconstitution procedure only removed about 99.5% of the NBMPR from the preparation. This contrasts with the efficient (>99.99%) removal of [^{3}H]adenosine, and means that the final proteoliposome preparations contained about 1 nM NBMPR which is sufficient to partially block both the binding of [^{3}H]NBMPR and the uptake of [^{3}H]uridine.

We have shown previously that this loss of [^{3}H]NBMPR binding activity is directly related to the loss of the ability of reconstituted transporters to mediate the flux of [^{3}H]uridine [14]. The initial rate of mediated uptake of [^{3}H]uridine by proteoliposomes prepared using freshly solubilized transporters was 51 pmol/mg protein/sec. Upon storage of the solubilized preparations for 48 hr at 6°, this rate dropped by 52% to 24 pmol/mg protein/sec (Fig. 1, Table 2). Adenosine, at a concentration of 5 mM, did not prevent this storage-induced loss in [^{3}H]uridine uptake (Table 2), even though this concentration of adenosine afforded some degree of protection against the loss of [^{3}H]NBMPR binding (see Table 1). Adenosine at 20 mM, on the other hand, completely prevented the storage-induced loss of [^{3}H]uridine uptake by the proteoliposomes (Fig. 1, Table 2). NBMPR (200 nM), which was as effective as 20 mM adenosine in preventing the loss of [^{3}H]NBMPR binding activity (Table 1), did not affect the storage-induced loss of [^{3}H]uridine uptake by the proteoliposomes (Table 2). The presence of NBMPR in the solubilization procedure, however, did result in a significantly lower rate of [^{3}H]uridine uptake by proteoliposomes prepared from the freshly solubilized proteins relative to that seen in its absence (Table 2), likely reflecting incomplete removal of the NBMPR by the reconstitution procedure (see above).

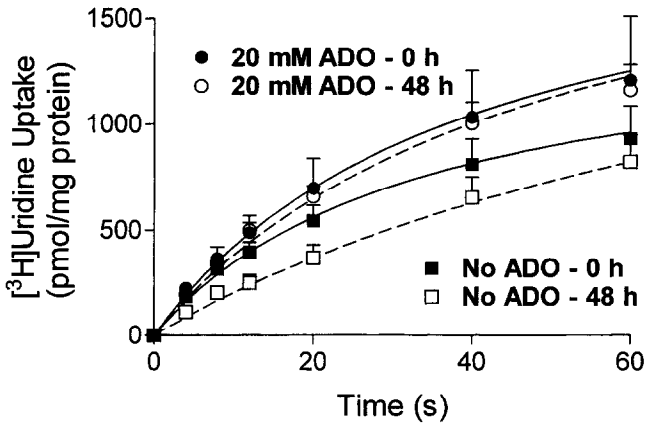

FIG. 1. Effect of adenosine on the functional stability of OCTG solubilized Ehrlich cell nucleoside transporters. Membranes were solubilized with 1.0% OCTG/0.15% asolectin in the presence (circles) and absence (squares) of 20 mM adenosine. Proteoliposomes were prepared from these soluble membranes either immediately after solubilization (closed symbols) or after storage of the soluble preparations for 48 hr at 6° (open symbols). Nucleoside transporter-mediated uptake of [^{3}H]uridine (ordinate) was calculated as the difference in vesicular accumulation in the absence (total uptake) and presence (non-mediated uptake) of 10 μM NBMPR/10 μM dipyridamole/10 mM adenosine. Each point is the mean ± SEM from five experiments conducted in duplicate.

Ehrlich cell plasma membranes possess two types of nucleoside transporters that can be defined by their relative sensitivities to NBMPR [4]. These are commonly referred to as *es* (equilibrative NBMPR-sensitive) and *ei* (equilibrative NBMPR-insensitive) nucleoside transporters. Only the *es* form of the transporter binds [^{3}H]NBMPR with high affinity [1–3, 21]. To determine the relative activities of the *es* and *ei* transporters in the proteoliposomes, 20 μM [^{3}H]uridine influx was measured in the presence of a range of concentrations of NBMPR (Fig. 2). Storage of the soluble preparations for 48 hr prior to reconstitution resulted in a significant reduction in the relative proportion of *ei*-mediated [^{3}H]uridine uptake (Fig. 2, Table 2). The inclusion of adenosine in the solubilization procedure had two distinct effects on the ratios of *es:ei*-mediated [^{3}H]uridine uptake by the proteoliposomes. Adenosine not only prevented the loss in *ei*-mediated uptake of [^{3}H]uridine observed upon storage of the solubilized preparations (Fig. 2, Table 2), but also enhanced significantly (Student's *t*-test, $P < 0.05$) the relative amount of *ei*-mediated [^{3}H]uridine influx by proteoliposomes prepared using the freshly solubilized proteins (Fig. 2, Table 2). The affinity of NBMPR for the *es* (IC_{50} ~0.4 nM) and *ei* (IC_{50} ~25 μM) transporters was not altered significantly by any of these treatments or storage conditions.

DISCUSSION

We have shown that the [^{3}H]NBMPR binding sites of Ehrlich cell plasma membranes can be solubilized effectively with a combination of 1.0% OCTG and 0.15% asolectin [8]. These detergent-solubilized binding sites display characteristics similar to those of the plasma-membrane located sites, and functional reconstitution studies indicate that these solubilized proteins are capable of mediating the flux of nucleosides across liposomal membranes [14]. However, within hours of the initial detergent extraction, these indicators of functional nucleoside transport activity are lost or diminished [7, 10, 11]. Studies on the purification of

TABLE 2. [^{3}H]Uridine uptake by proteoliposomes prepared using transport proteins solubilized and stored in the presence and absence of adenosine or NBMPR

Conditions	Initial rate of uptake V_i (pmol/mg protein/sec)			*ei*-Mediated influx (%)	
	0 hr	48 hr	% Loss*	0 hr	48 hr
No additive (control)†	51 ± 9	24 ± 4	52 ± 6	19 ± 2	8 ± 1
+5 mM Adenosine	59 ± 9	20 ± 4	65 ± 5	27 ± 2‡	18 ± 2‡
+20 mM Adenosine	55 ± 8	51 ± 6‡	12 ± 10	33 ± 2‡	33 ± 3‡
+200 nM NBMPR	33 ± 7‡	10 ± 2‡	65 ± 6	ND§	ND§

Plasma membranes were solubilized with 1.0% OCTG/0.15% asolectin in the presence of the indicated concentrations of adenosine or NBMPR, and used either immediately, or after storage for 48 hr at 6°, for the preparation of proteoliposomes. The initial rate (V_i) of transporter-mediated [^{3}H]uridine (20 μM) influx was derived by extrapolation of hyperbolic curves fitted to data as shown in Fig. 1. NBMPR sensitivity of reconstituted [^{3}H]uridine influx was assessed by incubating the proteoliposomes with a range of concentrations of NBMPR for 10 min prior to exposure to 20 μM [^{3}H]uridine for 15 sec. The amount of NBMPR-resistant (*ei*-mediated) influx is expressed as a percentage of the total transporter-mediated uptake (see Fig. 2). Each value is the mean ± SEM from five experiments performed in duplicate.

* Percent loss of activity upon storage of the solubilized preparations for 48 hr at 6°.

† Data derived from Hammond [14], shown for comparison.

‡ Significantly different from corresponding control, $P < 0.05$ (Student's *t*-test).

§ ND = not determined.

functional nucleoside transport proteins have been seriously hampered by this instability of the solubilized proteins [5–12]. We have reported that the addition of asolectin (a mixture of soybean phospholipids to the OCTG solution prior to exposure to Ehrlich cell plasma membranes reduced, but did not prevent, the loss of [^{3}H]NBMPR binding activity in the OCTG-solubilized preparations [8, 14]. The remaining loss of [^{3}H]NBMPR binding/transport activity was not due to proteolysis (no effect of protease inhibitors), nor was it attenuated in the presence of glycerol [14], an osmolyte that has afforded protection against loss of activity of other soluble proteins [23]. These data suggest that the loss in binding activity was due to a specific conformation change, or side chain modification, in the soluble protein. The present finding that adenosine prevented the storage-induced loss of both [^{3}H]NBMPR binding and [^{3}H]uridine uptake activity is consistent with the theory that these alterations are due to changes in protein conformation. Adenosine is a natural substrate for the nucleoside transporter with a K_m of approximately 50 μM. The fact that concentrations of adenosine in excess of 5 mM were required for complete protection would indicate that continuous saturation of the permeant site was necessary to maintain the protein in a stable conformation [24]. Adenosine derivatives that also interact with the transporter permeant site, such as 2′-deoxyadenosine [25] and 2-chloroadenosine [25, 26], were likewise effective in preventing the storage-induced loss of [^{3}H]NBMPR binding activity, as was uridine. What is surprising, however, is that similarly high concentrations of inosine and thymidine were ineffective in this regard. Cytidine has a relatively low affinity for the Ehrlich cell *es* transporter (K_m > 1mM) [4] so the lack of effect of cytidine may have been due to insufficient concentrations. However, inosine and thymidine are comparable to uridine in their affinities for the [^{3}H]NBMPR binding site [21] and the *es* transporter [4]. This implies that the protective effect of nucleosides is not simply due to their capacities to bind to the permeant site of the transporter but may depend on their abilities to modify protein conformation in some, as yet, undefined manner. Further evaluation of these interactions awaits the purification of functional transport proteins.

NBMPR, which is a purine derivative that interacts with the transporter permeant site and may induce a substrate-like conformation change [21, 27], was the only transport inhibitor with protective activity. Even though dipyridamole and dilazep did not protect against the loss of binding activity upon storage, solubilization in the presence of these transport blockers, as well as NBMPR, did result in a significant reduction in the B_{max} of [^{3}H]NBMPR binding to the proteoliposomes. Likewise, proteoliposomes prepared from proteins solubilized in the presence of 200 nM NBMPR had a relatively lower rate of [^{3}H]uridine uptake than those obtained in the absence of NBMPR. A change in B_{max} implies non-competitive inhibition. NBMPR, dipyridamole, and dilazep have been well characterized as competitive inhibitors of nucleoside transport and [^{3}H]NBMPR binding [4, 21]. Therefore, the residual inhibitor present in the proteoliposomal preparations would be expected to affect the K_D of binding, not B_{max}. It is possible that these potent inhibitors of nucleoside transport, when present during extraction of the protein from the membrane, bind irreversibly to components of the transporter complex. It is clear from the experiments where [^{3}H]NBMPR and [^{3}H]adenosine were added to the solubilized preparation prior to the reconstitution procedure that NBMPR is much more difficult to remove from the preparation than is adenosine, likely due to its hydrophobicity facilitating nonspecific interactions with lipid. Nevertheless, this residual concentration of NBMPR would be present in both the fresh and stored preparations, and hence does not detract from the conclusion that NBMPR in the solubilized preparation protected against the storage-induced loss of [^{3}H]NBMPR binding activity. What is in-

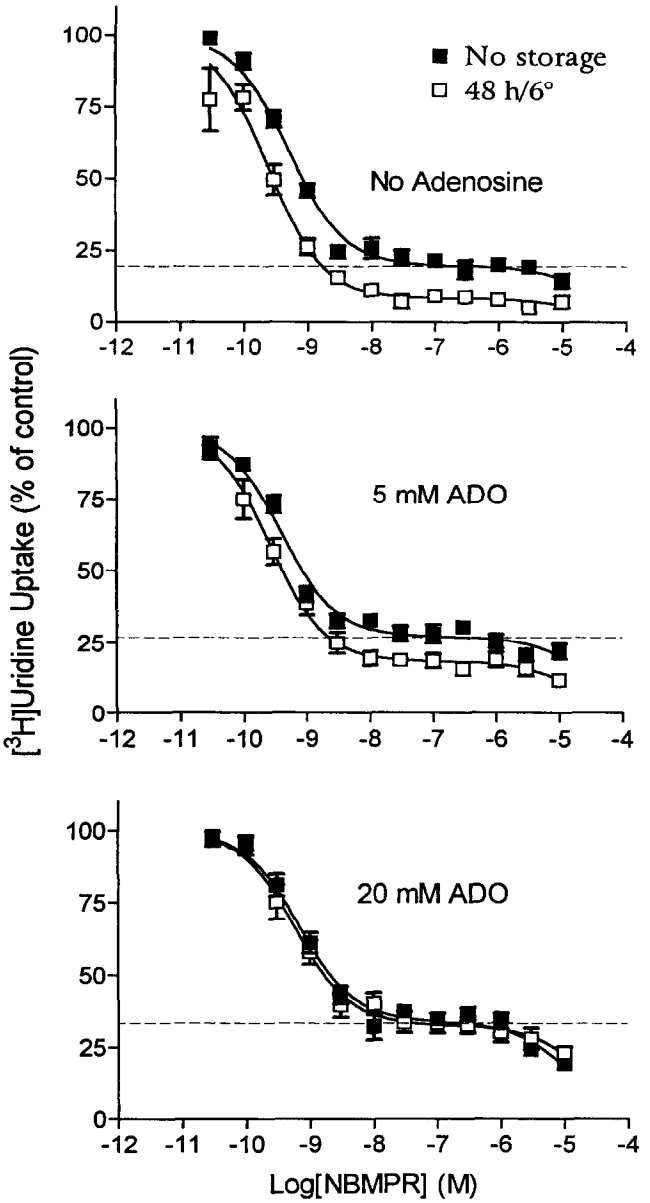

FIG. 2. Effect of membrane solubilization in the presence of adenosine on NBMPR inhibition of [^{3}H]uridine uptake by proteoliposomes. Ehrlich cell plasma membranes were solubilized in the absence (top panel) or presence of either 5 mM (middle panel) or 20 mM (bottom panel) adenosine and then used to prepare proteoliposomes either immediately after solubilization (■) or after storage of the soluble material for 48 hr at 6° (□). Proteoliposomes were incubated with the indicated concentrations of NBMPR for 10 min and then exposed to [^{3}H]uridine (20 μM) for 15 sec. The results are shown as the percentage of the accumulation observed in the absence of NBMPR (control). The "control" uptake values, calculated as pmol/assay/15 sec, are as follows (no storage/48 hr storage): No adenosine, 1.51 ± 0.31/0.98 ± 0.32; 5 mM adenosine, 0.78 ± 0.21/0.52 ± 0.14; 20 mM adenosine, 1.04 ± 0.28/0.92 ± 0.18. Each point is the mean ± SEM from five experiments performed in duplicate. The dashed lines represent the level of *ei*-mediated [^{3}H]uridine influx calculated as the plateau of the biphasic inhibition profile. The relative amounts of NBMPR-resistant (*ei*-mediated) [^{3}H]uridine influx derived from biphasic curve fits to these data are shown in Table 2.

teresting, however, is the fact that NBMPR did not likewise protect against the storage-induced loss of [^{3}H]uridine uptake activity by proteoliposomes. This implies that the components of the transporter that bind NBMPR are distinct from those involved in nucleoside translocation.

In addition to an absolute loss of functional activity upon storage of the solubilized transporters, there was a reduction in the proportion of the reconstituted [^{3}H]uridine uptake that was resistant to inhibition by NBMPR. This NBMPR-resistant component represents the activity of the *ei* subtype of nucleoside transporter [4]. These data suggest that the *ei* transporter is more labile during storage at 6° than is the *es* transporter. In the absence of adenosine, the functionality of the *ei* transporter, but not the more stable *es* transporter, may begin to decay immediately upon extraction from the plasma membrane, leading to a decrease in the *ei*:*es* ratio in the proteoliposomes. This would explain why the inclusion of adenosine in the solubilization procedure led to an increase in the relative amount of *ei*-mediated activity in the freshly solubilized preparations, and also explains the significant increase in total transporter-mediated uptake of [^{3}H]uridine by proteoliposomes prepared in the presence of adenosine (see Fig. 1). The *ei* transporter is more sensitive than the *es* subtype to the protective effects of adenosine; 5 mM adenosine had no significant effect on the overall loss of transporter activity upon storage, yet this concentration of adenosine did enhance the relative amount of *ei*-mediated transport activity in the solubilized preparations. It is not known if these changes in *ei*-mediated [^{3}H]uridine transport were due to absolute changes in the levels of functional transport proteins (V_{max}), or due to changes in the affinity (K_m) of the transporter for [^{3}H]uridine. The kinetic studies needed to address this issue are not currently feasible due to the experimental error associated with measurement of the relatively low activity of the reconstituted *ei* transporter [14]. These results are not unlike those reported for purified deoxycytidine kinase, where substrates differentially stabilize kinetically distinct conformational isoforms of the enzyme [28]. Indeed, the capacity of substrates, and specific pharmacological ligands, to protect the activities of isolated proteins is a widely noted phenomenon. Ligand-induced conformational changes have been reported to protect both the glucose [15, 16] and γ-aminobutyric acid transporters [17] from proteolytic digestion, and prelabelling with the specific ligand prazosin was essential for the preservation of the ligand binding characteristics of the α_1-adrenoceptor during detergent solubilization and reconstitution procedures [18].

In conclusion, OCTG-solubilized nucleoside transporters undergo a time-dependent conformation change leading to a significant loss in both [^{3}H]NBMPR binding and [^{3}H]uridine transport activity upon storage for 48 hr at 6°. While loss of both *es* and *ei* nucleoside transporter-mediated activities was observed, the *ei* subtype appeared more labile upon detergent solubilization than did the *es* transporter. These storage-induced losses in transporter

functionality were reduced significantly in the presence of compounds that interact with the permeant site of the nucleoside transporter. Adenosine was the most effective in this regard and, coupled with its ease of removal during reconstitution procedures, makes it an ideal candidate for inclusion in procedures aimed at the purification of functional nucleoside transporters.

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