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Purification and reconstitution studies of the nucleoside transporter from pig erythrocytes

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The pig erythrocyte nucleoside transporter has been identified as a band 4.5 polypeptide (M_r 64000) on the basis of photoaffinity labelling experiments with the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR). This protein was purified 140-fold by treatment of haemoglobin-free erythrocytes 'ghosts' with EDTA (pH 11.2) to remove extrinsic proteins, extraction of the protein-depleted membranes with *n*-octylglucoside and subsequent gradient-elution ion-exchange chromatography on DEAE-cellulose. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the purified material revealed the presence of only two detectable protein bands, one which co-migrated with the radiolabelled NBMPR-binding protein, and a lower molecular weight species with an M_r of 43000. The latter protein may be a degradation product of the band 3 anion-exchange transporter. The overall purification of the NBMPR-binding protein with respect to the M_r 64000 band was 350-fold. Reversible NBMPR-binding to the partially-purified band 4.5 preparation was saturable (apparent K_d 7.2 nM). Adjustment of the chromatography conditions to allow elution of the NBMPR-binding protein along with the majority of solubilised membrane phospholipid reduced the apparent K_d value to 3.0 nM. Purification of reversible NBMPR-binding activity during ion-exchange chromatography was paralleled by an increase in the specific activity of nitrobenzylthioguanosine (NBTGR) -sensitive uridine transport as assayed in proteoliposomes reconstituted by a freeze-thaw-sonication procedure.

Introduction

Nucleoside transport across the plasma membrane of the human erythrocyte occurs by a broad-specificity facilitated-diffusion mechanism which is selectively inhibited by nanomolar concentrations of NBMPR and certain other 6-thiopurine ribonucleosides [1–4]. NBMPR inhibition of nucleoside transport is associated with high-affinity binding of ligand to cell membrane sites (apparent K_d 0.1–2 nM). This binding is competitively inhibited by nucleoside permeants and is also blocked by other nucleoside transport inhibitors such as dipyridamole [5–8]. Although binding of NBMPR is normally reversible, site-specific covalent radiolabelling of human erythro-

Abbreviations: NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine); NBTGR, 2-amino-6-[(4-nitrobenzyl)thiol]-9-β-D-ribofuranosylpurine.

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cyte band 4.5 polypeptides occurs when membranes are exposed to [^{3}H]NBMPR in the presence of high-intensity ultraviolet light [9,10]. In contrast, no ^{3}H incorporation occurs in membranes from nucleoside transport-deficient sheep erythrocytes [9], cells which lack high-affinity NBMPR binding sites [11]. Radiolabelling of human erythrocyte band 4.5 polypeptides (apparent M_r 45000–66000) is inhibited by transported nucleosides, NBTGR and dipyridamole [10]. These photoaffinity-labelling experiments strongly implicate band 4.5 polypeptides in erythrocyte nucleoside transport and have been extended to a variety of other cell types and tissues [12–16], providing evidence that NBMPR-sensitive nucleoside transporters from different sources have broadly similar molecular weights to that of the human erythrocyte system.

Additional evidence to implicate erythrocyte band 4.5 polypeptides in nucleoside permeation comes from the finding that partially-purified human erythrocyte band 4.5 preparations are 13-fold enriched with respect to reversible NBMPR-binding activity compared with erythrocyte 'ghosts' [17]. Partially purified band 4.5 proteins can be covalently labelled with [^{3}H]NBMPR [18] and exhibit NBTGR-sensitive uridine transport activity when reconstituted into liposomes [19]. The glucose transport system of human erythrocytes has also been shown to be a band 4.5 polypeptide, the nucleoside and glucose transporters (approx. 10^4 and 10^5 copies per cell, respectively) comigrating on SDS-polyacrylamide gels with the same apparent M_r. The two transporters also copurify during DEAE-cellulose ion-exchange chromatography [19–25]. As a result, partially purified band 4.5 preparations from human erythrocytes consist largely of glucose transporter, with the nucleoside transporter present as a minor component. The ratio of glucose/nucleoside transporters in partially-purified band 4.5 preparation from human erythrocytes has been estimated to be in the region of 20 : 1 [17,23].

Further purification of the erythrocyte nucleoside transporter requires its separation from the glucose transport protein. One possible experimental strategy is to attempt negative purification of the nucleoside transporter from human erythrocyte band 4.5 polypeptides by immuno-affinity chromatography using monoclonal antibody specific for the glucose transporter [26]. In theory, greater purification of nucleoside transporter polypeptides might also be achieved using membranes from erythrocytes lacking glucose transport activity. One such species is the pig. Pig erythrocytes possess 5000 nucleoside transporters per cell [27,28], but are totally devoid of cytochalasin B-sensitive glucose transport activity [28–31]. These cells are therefore anticipated to be deficient with respect to the major band 4.5 polypeptide present in human erythrocytes [32,33]. Physiologically, pig erythrocytes utilise plasma inosine as their energy substrate [28,31,34–36].

In the present paper we describe experiments aimed at solubilising and purifying the membrane components of the nucleoside transporter from erythrocytes of this species. Using the detergent *n*-octylglucoside in combination with gradient-elution ion-exchange chromatography, it has been possible to partially purify band 4.5 polypeptides covalently labelled with [^{3}H]NBMPR 140-fold. These partially purified band 4.5 polypeptides exhibit reversible high-affinity NBMPR-binding activity and are demonstrated to be capable of catalysing NBTGR-sensitive uridine transport when reconstituted into soybean phospholipid vesicles by freeze-thaw sonication. The glycosylated nature of this partially purified pig nucleoside transporter has recently been reported and compared to that of the human erythrocyte nucleoside carrier [37].

Experimental Procedures

Materials

[G-^{3}H]NBMPR (specific radioactivity 35 Ci/mmol, greater than 98% radiochemically pure) and [2-^{14}C]uridine (specific radioactivity 53 mCi/mmol, greater than 98% radiochemically pure) were purchased from Moravek Biochemicals (Brea, CA) and Amersham International (Amersham, Bucks., U.K.), respectively. D-[U-^{14}C]Glucose (specific radioactivity 278 mCi/mmol, greater than 98% radiochemically pure) and L-[1-^{14}C]glucose (specific radioactivity 58 mCi/mmol, greater than 98% radiochemically pure) were also obtained from Amersham. Pre-swollen microgranular DEAE-52 DEAE-cellulose anion-exchange re-

sin, NBTGR and *n*-octylglucoside were from Sigma (St. Louis, MO). Soybean phospholipid (asolectin) was obtained from Associated Concentrates, Woodside, NY. Dipyridamole (Persantin injection) was provided by Boehringer Ingelheim, Bracknell, Berks., U.K. All others reagents were of analytical grade.

Cell and membrane preparation

Fresh blood was collected into heparin and the erythrocytes washed three times with a medium containing 140 mM NaCl/5 mM KCl/20 mM Tris-HCl (pH 7.4 at 22°C)/2 mM $MgCl_2$/0.1 mM EDTA (disodium salt). The buffy coat was discarded. Haemoglobin-free erythrocyte 'ghosts' depleted of extrinsic proteins were prepared as described previously [17] except that the initial haemolysis medium contained 0.1 mM phenylmethylsulphonyl fluoride to minimise proteolysis. Control experiments established that this concentration of phenylmethylsulphonyl fluoride did not inhibit nucleoside transporter function. Membranes were resuspended in 5 mM or 50 mM Tris-HCl (pH 7.4 at 4°C) as appropriate and stored at −70°C for a maximum of 1 week before use. All subsequent procedures were carried out at 4°C unless noted otherwise.

Purification of band 4.5 polypeptides

Pig erythrocyte protein-depleted membranes (2 mg protein/ml) in 5 mM Tris-HCl were solubilised in 46 mM *n*-octylglucoside/5 mM Tris-HCl supplemented with 2 mM dithiothreitol and 2 column volumes of the 130000 × *g* supernatant (1 h centrifugation) applied at a flow rate 2.5 column volumes/h to a DEAE-cellulose ion-exchange column (typically 4.5 ml) equilibrated with the same detergent buffer containing 34 mM *n*-octylglucoside. The column was eluted with 26.7 volumes of a linear salt gradient (0–0.20 M NaCl/34 mM *n*-octylglucoside/2 mM dithiothreitol/5 mM Tris-HCl). The fraction size was 0.4 column volumes. To monitor elution and purification of the nucleoside transporter, the starting protein-depleted membranes were covalently radiolabelled with [^{3}H]NBMPR prior to solubilisation (see next section). In large-scale preparative experiments (30 ml column) for analysis of protein composition and reversible NBMPR binding and reconstitution studies, the fraction of protein-depleted membranes photolabelled with [^{3}H]NBMPR was reduced to 5%. Since the efficiency of photo-incorporation is approximately 15% [9], the actual fraction of nucleoside transporters covalently labelled with [^{3}H]NBMPR was less than 0.5%. Previous control experiments with human erythrocyte membranes have established that the nucleoside transporter retains activity following exposure to ultra violet light [9].

Column fractions containing nucleoside transporter protein (0.08–0.11 M NaCl) were pooled and dialysed free of detergent against 5 mM Tris-HCl/2 mM dithiothreitol. Where appropriate, samples were concentrated up to 10-fold by ultracentrifugation at 130000 × *g* for 1 h. Residual proteins remaining bound to the column were eluted with 1 M NaCl and dialysed free of detergent as described for the gradient fractions.

Photoaffinity labelling with NBMPR

Protein-depleted membranes (2 mg protein/ml) were equilibrated at room temperature with a saturating concentration of [^{3}H]NBMPR (50 nM) and exposed to high-intensity ultraviolet light at 4°C in the presence of 50 mM dithiothreitol (added as a free-radical scavenger) as described previously [9]. Samples were then diluted 20-fold with Tris-HCl and allowed to stand at room temperature for 10 min before recovery of the radiolabelled membranes by centrifugation. This washing procedure was repeated two more times to remove any residual unreacted ligand.

Reversible NBMPR binding

Binding of [^{3}H]NBMPR (0.5–150 nM) to membranes and detergent-free membrane extracts was determined in the absence and in the presence of 20 μM NBTGR as competing nonradioactive ligand by standard washing-centrifugation and equilibrium dialysis procedures, respectively [17]. In some experiments, a centrifugal gel filtration method originally devised for vesicle transport assays (see below) was used as an alternative to equilibrium dialysis, the technique employing Sephadex mini-columns to separate bound from free radioligand. The Sephadex G-50 (fine) columns (1 ml) were prepared and used as described previously [19] except that NBTGR was omitted from the equilibration buffer and 'stopping solution'. Control experiments confirmed that equilibrium

dialysis and the Sephadex mini-column methods gave equivalent estimates of NBMPR-binding to pig erythrocyte membrane extracts.

Reconstitution experiments

Protein fractions were reconstituted into phospholipid vesicles in 50 mM Tris-HCl by the freeze-thaw-sonication method described previously by Kasahara and Hinkle [38] as modified by Tse et al. [19]. Acetone-washed soybean phospholipid [39] to protein ratios (μmol/mg) were kept in the range 50–240 : 1 with a sonication period of 5 s.

Transport assays

Uptake of [^{14}C]uridine (50 μM) by liposomes and reconstituted vesicles was measured at 25 °C by a centrifugal gel filtration method [22,40,41] as described by Tse et al. [19]. D-[^{14}C]Glucose uptake (0.2 mM) was determined by an identical procedure except that cytochalasin B (10 μM) replaced NBTGR as transport inhibitor.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out in 2-mm thick 12% slab gels by the method of Thompson and Maddy [42] using the Laemmli buffer system [43]. Radioactivity in the various regions of the gel were determined by slicing gel lanes into 2 mm fractions. The ^{3}H content of these fractions was measured by liquid scintillation counting [9]. Protein was visualised by staining with Coomassie blue.

Protein and lipid determinations

Protein was assayed by the method of Peterson [44] or by absorbance measurements at 280 nm. Phospholipid was assayed by the diphenylhexatriene fluorescence enhancement method described by London and Feigenson [45].

Results

Pilot studies

The standard procedure for isolation of human erythrocyte band 4.5 polypeptides involves solubilisation of protein-depleted membranes in 46 mM *n*-octylglucoside/2 mM dithiothreitol/50 mM Tris-HCl (pH 7.4 at 4 °C) followed by DEAE-cellulose ion-exchange chromatography, band 4.5 polypeptides eluting in the column void-volume [23]. As shown in Table IA, this method resulted in a 12.7-fold purification of reversible high-affinity NBMPR-binding activity compared with the starting erythrocyte 'ghosts'. In three separate experiments, the mean NBMPR-binding capacity of the pooled void-volume extract was 456 pmol/mg protein. The recovery of NBMPR-binding activity was 23%. These values compare favourably with the 12.9-fold purification (31% recovery) obtained previously using Triton X-100 as detergent [17].

Unexpectedly, only a small percentage of the

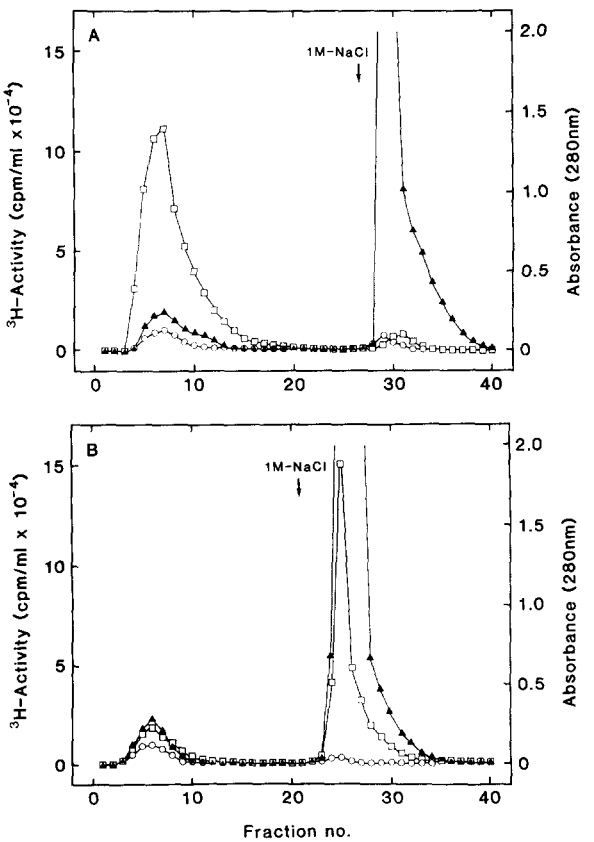

Fig. 1. DEAE-cellulose chromatography of the NBMPR-binding components of human and pig erythrocyte membranes. Protein-depleted membranes were photolabelled with [^{3}H] NBMPR in the absence (□) and in the presence (○) of 20 μM NBTGR, solubilised in *n*-octylglucoside and subjected to DEAE-cellulose ion-exchange chromatography (4.5 ml column) in a medium containing 34 mM *n*-octylglucoside/50 mM Tris-HCl (pH 7.4 at 4 °C)/2 mM dithiothreitol (23). Aliquots (0.1 ml) were removed from each fraction for radioactivity determination. Protein (▲) was monitored at 280 nm. A, human erythrocyte membranes; B, pig erythrocyte membranes.

TABLE I

SOLUBILISATION AND DEAE-CELLULOSE CHROMATOGRAPHY OF NBMPR-BINDING ACTIVITY FROM HUMAN AND PIG ERYTHROCYTE MEMBRANES

'Ghosts' were depleted of extrinsic proteins, solubilised with *n*-octylglucoside and subjected to DEAE-cellulose ion-exchange column chromatography in a medium containing 34 mM *n*-octylglucoside/50 mM Tris-HCl (pH 7.4 at 4°C)/2 mM dithiothreitol [23]. Samples of the *n*-octylglucoside 130000 × *g* supernatant (crude *n*-octylglucoside extract) and pooled void volume and 1 M NaCl fractions from the column were dialysed free of detergent and assayed for protein and high-affinity NBMPR-binding activity (at a saturating concentration of ligand). Values are means (±S.E.) of three separate experiments for each species.

		Total protein (mg)	Total NBMPR bound (pmol)	NBMPR-binding activity recovered (%)	Specific NBMPR-binding activity (pmol/mg protein)
A. Human	'ghosts'	51 ±3.1	1801 ± 221	100	36 ± 7
	protein-depleted membranes	27 ±0.3	1359 ± 320	75	49 ± 11
	n-octylglucoside supernatant	12 ±0.9	1248 ± 10	69	104 ± 11
	DEAE-cellulose fractions				
	(a) void volume	0.91 ± 0.11	411 ± 67	23	456 ± 41
	(b) 1 M NaCl	9.0 ±0.6	70 ± 23	4	8 ± 1
B. Pig	'ghosts'	55 ±4.0	1508 ± 148	100	24 ± 2
	protein-depleted membranes	22 ±1.0	1378 ± 120	87	55 ± 5
	n-octylglucoside supernatant	18 ±1.0	1243 ± 126	82	68 ± 10
	DEAE-cellulose fractions				
	(a) void-volume	0.75 ± 0.18	71 ± 34	5	95 ± 38
	(b) 1 M NaCl	12 ±1.5	508 ± 65	33	42 ± 56

pig erythrocyte NBMPR binding activity eluted in the column void-volume under these conditions (Table IB). In consequence, the average specific activity of NBMPR binding following column chromatography was only 95 pmol/mg protein, a purification of 4-fold (recovery 5%). Instead, NBMPR binding activity was recovered along with other integral membrane proteins (principally the band 3 anion-exchange transporter) in the 1 M NaCl extract (specific activity 42 pmol/mg protein). The 1 M NaCl extract from human erythrocyte membranes also contained band 3, but minimal NBMPR binding activity (see also Ref. 17).

The different elution profiles of the human and pig erythrocyte NBMPR-binding proteins was confirmed in a series of experiments in which protein-depleted membranes from the two species were covalently labelled with [^{3}H]NBMPR prior to *n*-octylglucoside solubilisation and ion-exchange chromatography. As shown in Fig. 1A, most of the ^{3}H (92%) associated with the human erythrocyte membrane extract was recovered in the column void volume (fractions 4–12). Chromatography of solubilised membranes which had been photoaffinity labelled with [^{3}H]NBMPR in the presence of excess NBTGR (20 μM) confirmed that the majority of the ^{3}H appearing in the column void volume was specifically associated with the nucleoside transporter (see also Fig. 1A). As expected from the results presented in Table IB, the radiolabelled pig erythrocyte NBMPR-binding protein was adsorbed by the column and eluted in the 1 M NaCl fractions (Fig. 1B). These experiments establish the validity of using radiolabelled NBMPR as a covalent probe of the solubilised NBMPR-binding protein.

Isolation studies (gradient elution)

Subsequent fractionation experiments employed gradient elution of the ion-exchange column to separate the pig nucleoside transporter from band 3 and other integral membrane proteins, covalent labelling with [^{3}H]NBMPR being used to monitor the elution position of the solubilised NBMPR-binding protein and to quantify the degree of purification achieved. Results from a representative separation are shown in Fig. 2. In agreement with the earlier experiments, only small amounts of ^{3}H and protein were detected in the

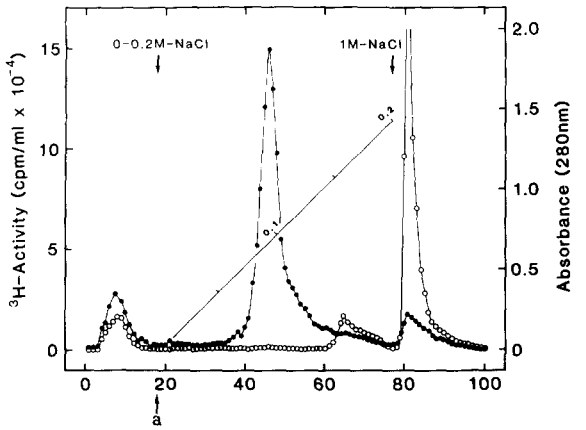

Fig. 2. Gradient-elution DEAE-cellulose chromatography of the NBMPR-binding component of pig erythrocyte membranes. Protein-depleted membranes were photolabelled with [^{3}H]NBMPR, solubilised in *n*-octylglucoside and subjected to gradient-elution ion-exchange chromatography on a 4.5 ml DEAE-cellulose column (0–0.20 M NaCl/34 mM *n*-octylglucoside/5 mM Tris-HCl (pH 7.4 at 4°C)/2 mM dithiothreitol) as detailed in Experimental procedures. Aliquots (0.1 ml) were removed from each fraction for radioactivity determination (●). Protein (○) was monitored at 280 nm.

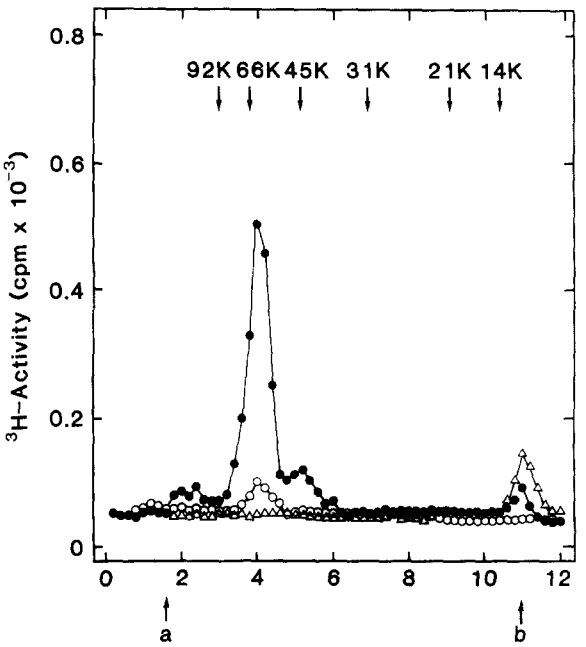

Fig. 3. SDS-polyacrylamide gel electrophoresis of ^{3}H-containing fractions isolated from radiolabelled pig erythrocyte membranes by gradient-elution DEAE-cellulose chromatography. Aliquots (0.1 ml) were removed from fraction numbers 7 (void volume, △), 46 (gradient peak, ●) and 81 (1 M NaCl peak, ○) of the experiment shown in Fig. 3, mixed with an equal volume of gel sample buffer and subjected to SDS-polyacrylamide gel electrophoresis in a 12% (w/v) slab gel. Radioactivity in the various regions of the gel were determined by slicing gel lanes into 2-mm fractions and counting by liquid scintillation spectrometry [9]. ^{3}H profiles for the three samples and the positions of molecular weight standards are from the same slab gel. The positions of the stacking gel-running gel interface and the tracking dye are indicated by a and b, respectively. 92K, for example, 92000.

column void volume fractions (4–12). Subsequent gradient elution of the column (5 mM Tris-HCl/0–0.2 M NaCl) released the majority of adsorbed radioactivity in a single symmetrical peak (0.09 M NaCl) in fractions exhibiting a 280 nm absorbance of less than 0.05. The remaining ^{3}H and most of the protein (including band 3) eluted at higher ionic strength. Total recovery of ^{3}H from the ion-exchange column was 85%, three-quarters of which chromatographed in the 0.09 M NaCl peak.

The radioactivity present in representative fractions from the column void volume, the 0.09 M NaCl peak and the 1 M NaCl peak was analysed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, there was no radiolabelled protein detectable in the column void-volume fraction, the small amount of ^{3}H present in this fraction migrating in the lipid region of the gel. In contrast, 90% of the radioactivity present in the 0.09 M NaCl fraction migrated as a large band 4.5 peak with an apparent M_r (average) of 64000, the expected apparent molecular weight of the pig erythrocyte nucleoside transporter [9,28,37]. The minor high molecular weight peak (apparent M_r > 92,000) and low molecular weight peak (apparent M_r (average) 45000) correspond to aggregates of the transporter and proteolytic degradation products, respectively [9,25]. A small amount of [^{3}H]NBMPR-binding protein was detected in the 1 M NaCl fraction.

To assess purification of the NBMPR-binding protein, radioactive 0.09 M NaCl fractions were pooled, dialysed free of detergent and assayed for both total protein [44] and protein-associated radioactivity as determined by SDS-polyacrylamide gel electrophoresis (see above). In a representative experiment, the specific activity of protein-bound ^{3}H was 2982 cpm/μg protein for the 0.09 M NaCl preparation compared with 50.4 cpm/μg protein

for protein-depleted membranes. Thus, membrane solubilisation and subsequent ion-exchange chromatography resulted in a 59-fold purification of radiolabelled NBMPR-binding protein. The average purification achieved in three separate experiments (mean ± S.E.) was 60 ± 3-fold.

Analysis of the protein composition of the purified extract by Coomassie blue staining of SDS-polyacrylamide gels revealed the presence of two detectable protein bands, one with an apparent M_r (average) of 64000 corresponding to the position of the radiolabelled NBMPR-binding protein, the other with a lower apparent M_r (average) of 43000 (Fig. 4). Absorbance scans from three separate isolation experiments gave essentially identical results, the higher molecular weight species contributing 40 ± 2% of the protein present in the preparation. As expected, the 1 M NaCl fractions consisted largely of band 3 polypeptides while the column void-volume fractions failed to exhibit detectable protein staining (gel scans not shown).

The percentage of solubilised membrane phospholipids recovered in the partially purified band 4.5 preparation was typically 10%, most (75%) of the solubilised phospholipids eluting in the column void volume. The measured phospholipid/protein ratio of one preparation was 23.2 μmol/mg compared with 1.64 μmol/mg for the crude *n*-octylglucoside membrane extract.

Reversible NBMPR binding

Ligand binding assays at each stage of the gradient-elution isolation procedure for three separate purification experiments are summarised in Table II. The mean specific activity of NBMPR binding to the partially purified band 4.5 preparation was 1450 pmol/mg protein, representing an overall increase in specific activity of 63-fold compared with the starting erythrocyte 'ghosts'. This contrasts with a 13-fold increase in specific activity for the human erythrocyte preparation (Table IA).

Membrane solubilisation and column chromatography alone increased the specific activity of reversible NBMPR-binding by 29-fold. This compares with 60-fold for covalent NBMPR radiolabelling (see previous section). The discrepancy between these two figures can be accounted for by

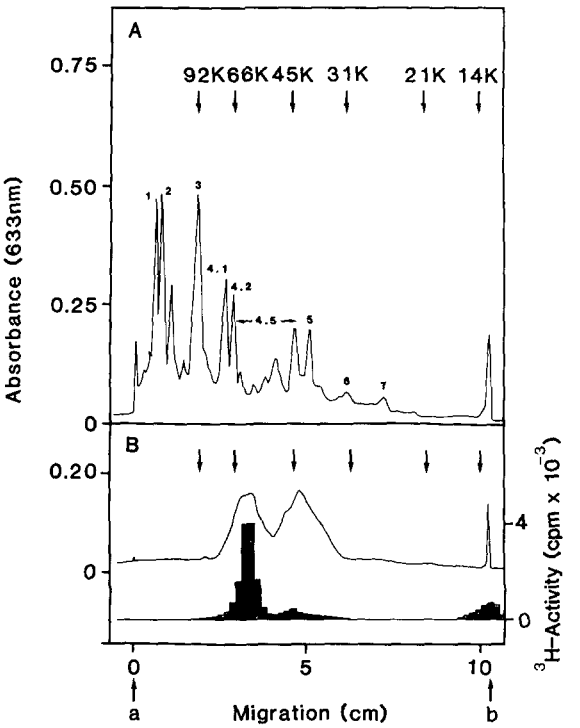

Fig. 4. SDS-polyacrylamide gel electrophoresis of pig erythrocyte 'ghosts' and partially purified band 4.5 polypeptides. The NBMPR-binding component from pig erythrocyte membranes was isolated by gradient-elution DEAE-cellulose chromatography as described in the legend to Table II and concentrated by ultracentrifugation. Samples of the starting erythrocyte 'ghosts' (75 μg protein, A) and the purified extract (30 μg protein, B) were analysed on a 12% SDS-polyacrylamide slab gel, stained with Coomassie blue and scanned at 633 nm using an LKB laser densitometer. The hatched area (B) corresponds to the ^{3}H profile of purified extract (30 μg) radiolabelled with [^{3}H] NBMPR. The positions of the stacking gel running gel interface and the tracking dye are indicated by a and b, respectively. 92K, for example, 92000.

the 50% loss of reversible ligand-binding activity during the column chromatography stage of the isolation procedure (Table II), reversible NBMPR-binding activity underestimating the extent of purification achieved. A corresponding loss of human erythrocyte NBMPR-binding activity is apparent in Table IA and suggests that the isolated transporter is relatively labile (see also Ref. 17). The data presented in Table II confirm the low NBMPR-binding activities of the pig erythrocyte void volume and 1 M NaCl fractions (see also Figs. 2 and 3).

Reversible NBMPR binding to the partially

TABLE II

PURIFICATION OF THE NBMPR-BINDING COMPONENT FROM PIG ERYTHROCYTE MEMBRANES

'Ghosts' were depleted of extrinsic proteins, solubilised with *n*-octylglucoside and subjected to gradient-elution ion-exchange chromatography on a 30 ml DEAE-cellulose column (0–0.2 M NaCl/34 mM *n*-octylglucoside/5 mM Tris-HCl (pH 7.4 at 4° C)/2 mM dithiothreitol) as described in Experimental procedures. Samples of the *n*-octylglucoside 130000 × *g* supernatant (crude *n*-octylglucoside extract) and pooled void volume, gradient and 1 M NaCl fractions from the column were dialysed free of detergent and assayed for protein and high-affinity NBMPR-binding activity (at a saturating concentration of ligand). Gradient fractions (0.08–0.11 M NaCl) containing the NBMPR-binding protein were identified by covalently labelling 5% of the protein-depleted membranes with [^{3}H]NBMPR prior to solubilisation. Values are means (±S.E.) of three separate purification experiments.

	Total protein (mg)	Total NBMPR bound (pmol)	NBMPR-binding activity recovered (%)	Specific NBMPR-binding activity (pmol/mg protein)
'Ghosts'	210 ± 10	4830 ± 321	100	23 ± 1
Protein-depleted membranes	84 ± 2.9	4150 ± 105	86	50 ± 3
n-Octylglucoside supernatant	64 ± 4.0	3864 ± 97	80	61 ± 3
DEAE-cellulose fractions				
(a) void volume	2.1 ± 0.2	18 ± 2	0.4	9 ± 1
(b) gradient	0.95 ± 0.04	1385 ± 94	29	1450 ± 59
(c) 1 M NaCl	40 ± 5.6	63 ± 7	1.3	2 ± 1

purified band 4.5 preparation is analysed more fully in Fig. 5 which presents representative data for the concentration dependence of binding, measured both in the absence and in the presence of 20 μM nonradioactive NBTGR. NBTGR-sensitive binding was saturable with an apparent K_d of 7.2 nM. This value was 5.1-fold higher than the apparent K_d (1.4 nM) for high-affinity NBMPR binding to membrane 'ghosts' and crude *n*-octylglucoside membrane extract from the same blood sample. It seemed likely that this shift in K_d was related to the altered lipid environment of the NBMPR-binding protein (see above). Supplementation of the crude *n*-octylglucoside extract with 0.11 M NaCl to permit elution of the NBMPR-binding protein in the ion-exchange column void volume along with the majority of solubilised membrane lipids reduced the measured apparent K_d to 3.0 nM. This compares with an apparent K_d value of 2.4 nM for the human erythrocyte band 4.5 preparation of Jarvis and Young [17]. The NBMPR-binding activity of the isolated pig preparation had a half-life of 40 days when stored at −70° C.

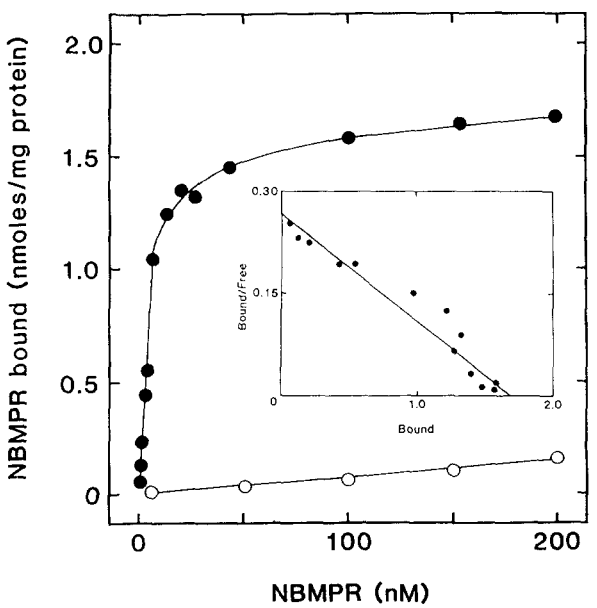

Fig. 5. Concentration dependence of NBMPR binding to partially purified band 4.5 polypeptides from pig erythrocyte membranes. The amounts of [^{3}H]NBMPR reversibly bound to the partially purified band 4.5 preparation (see legend to Table II) in the presence (○) and in the absence of 20 μM NBTGR (●) were determined by equilibrium dialysis and are plotted against the equilibrium concentrations of free NBMPR. The inset shows a Scatchard plot of the data (corrected for the NBTGR-insensitive component). Maximum binding (1690 pmol/mg protein) and apparent K_d (7.2 nM) were determined by linear regression analysis.

Reconstitution studies

The purification experiments presented in preceeding sections establish conditions for the

isolation of a membrane protein fraction from pig erythrocytes which is substantially enriched with respect to NBMPR-binding activity. Subsequent experiments were undertaken to investigate whether this partially purified band 4.5 preparation was also capable of nucleoside transport when reconstituted into phospholipid vesicles. Conditions for reconstitution and assays of uridine uptake were based on previous reconstitution studies of the human nucleoside transporter [19].

As shown in Fig. 6, the reconstituted band 4.5 preparation was capable of carrier-mediated (NBTGR-sensitive) uridine transport, ion-exchange chromatography being associated with a parallel increase in the specific activity of both uridine transport and NBMPR binding (Table III). From the data presented in Table III it can be calculated that the purified pig preparation catalysed the transport of 9 molecules of uridine/NBMPR binding site per 5 s (50 μM permeant, 25°C). This value compares favourably with a rate of 2.5 molecules/site per 5 s for human erythrocyte band 4.5 polypeptides measured at 15°C (Table II, Ref. 19), the human transporter having a Q_{10} of 4 [3].

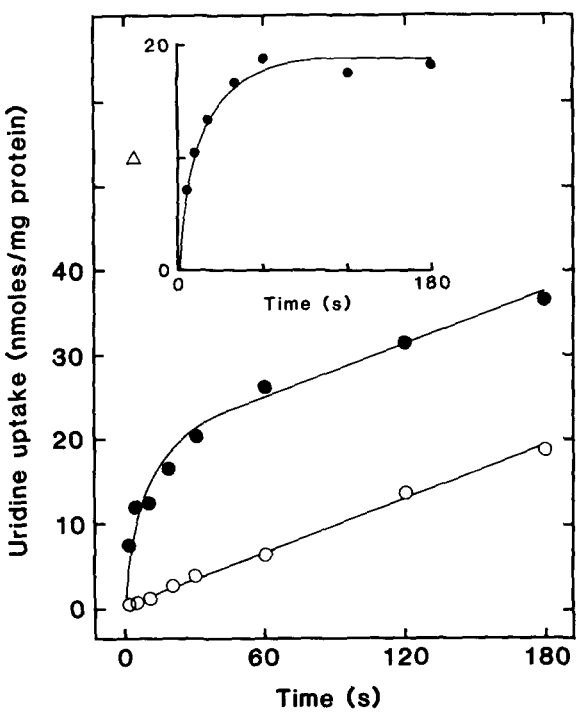

Fig. 6. Time course of uridine uptake into vesicles reconstituted with partially purified band 4.5 polypeptides from pig erythrocyte membranes. Partially purified band 4.5 polypeptides were reconstituted into phospholipid vesicles as described in Experimental procedures and assayed for uridine uptake (50 μM, 25°C) in the absence (○) and in the presence of 20 μM NBTGR (●). The inset shows the time course of NBTGR-sensitive uridine transport (Δ is the difference between uridine uptake in the absence and in the presence of NBTGR). Values are means of triplicate determinations.

Results presented in Table IV demonstrate that the NBTGR-sensitive transport activity of the partially purified band 4.5 preparation was only partly dependent upon the presence of added phospholipid, band 4.5 extract alone exhibiting 23% of the NBTGR-sensitive uridine uptake of the reconstituted system. In contrast, the transport activity of the crude *n*-octylglucoside membrane extract was totally dependent upon the presence of exogenous lipid (see also Table IV). This difference is a consequence of the higher phospholi-

TABLE III

HIGH-AFFINITY NBMPR-BINDING AND URIDINE TRANSPORT ACTIVITIES OF PARTIALLY PURIFIED BAND 4.5 POLYPEPTIDES AND CRUDE *n*-OCTYLGLUCOSIDE EXTRACT FROM PIG ERYTHROCYTE MEMBRANES

Membranes were depleted of extrinsic proteins, solubilised with *n*-octylglucoside and subjected to gradient-elution DEAE-cellulose ion-exchange column chromatography as described in the legend to Table II. Samples of the *n*-octylglucoside 130000 × *g* supernatant (crude *n*-octylglucoside extract and partially purified band 4.5 polypeptides were dialysed free of detergent and assayed for high-affinity NBMPR-binding activity at a saturating concentration of ligand. Samples of the two preparations were also reconstituted into phospholipid vesicles and assayed for NBMPR-sensitive uridine transport activity (50 μM, [^{14}C]uridine ± 20 μM NBMPR, 25°C) as detailed in the text. Values are means (± S.E.) of triplicate estimates from a single experiment.

	NBMPR-binding activity (pmol/mg protein)	Uridine transport activity (nmol/mg protein in 5 s)
Crude *n*-octylglucoside extract	60 ± 3	0.35 ± 0.02
Partially-purified band 4.5 proteins	1200 ± 90	10.80 ± 0.54
Ratio	20	31

TABLE IV

RECONSTITUTION OF NUCLEOSIDE TRANSPORT ACTIVITY WITH A CRUDE *n*-OCTYLGLUCOSIDE EXTRACT FROM PIG ERYTHROCYTE MEMBRANES AND WITH PARTIALLY PURIFIED BAND 4.5 POLYPEPTIDES

Uridine uptake by vesicles (50 μM; 10-s flux at 25°C) was measured as described in Experimental procedures. In the preparation of 'reconstituted vesicles', crude *n*-octylglucoside extract and partially purified band 4.5 polypeptides (see text) were reconstituted with added soybean phospholipids. Each transport assay contained 10 μg of protein and 0.3 μmol of lipid (membrane extract) or 1.2 μg of protein and 0.3 μmol of lipid (band 4.5 preparation). For the 'lipid only' and 'protein only' controls, reconstitution was performed in the absence of added protein and lipid, respectively. Δ is the difference in uridine uptake in the absence and in the presence of 20 μM NBTGR. Values are means (±S.E.) of triplicate determinations.

	Uridine uptake (pmol/10-s assay)		Δ
	−NBTGR	+NBTGR	
Crude *n*-octylglucoside extract reconstituted			
vesicles	7.11±0.33	2.79±0.16	4.32±0.37
lipid only	2.94±0.22	3.84±0.64	0
protein only	0.12±0.10	0	0.12±0.1
Partially purified band 4.5 reconstituted			
vesicles	18.8 ±1.1	2.6 ±0.3	16.2 ±1.1
lipid only	2.4 ±0.6	2.5 ±0.3	0
protein only	6.1 ±0.7	1.5 ±0.1	4.6 ±0.7

pid/protein ratio of the band 4.5 preparation (see above), permitting vesicle formation and partial reconstitution of transport activity even in the absence of added lipid [19]. Rates of NBTGR-insensitive uridine uptake by the two reconstituted preparations were similar to those given by liposomes alone (Table IV), indicating that this component of uptake reflects simple diffusion of isotopic permeant across the lipid bilayer. In contrast to the partially purified band 4.5 preparation, neither of the other two samples from the DEAE-cellulose column (void volume, 1 M NaCl) exhibited detectable NBTGR-sensitive uridine transport activity (data not shown). This result is in agreement with the low NBMPR-binding activities of these pooled fractions (Table II). Parallel reconstitution/transport experiments with D- and L-glucose (0.2 mM, 25°C) confirmed the absence of glucose transporter from detergent extracts of pig erythrocyte membranes, the two glucose isomers exhibiting the same uptake rate (1 nmol/mg protein in 1 min).

Discussion

The molecular mechanisms by which nucleosides cross the membranes of mammalian cells is of considerable interest, not only because of the physiological importance of these compounds, but also because cytotoxic nucleosides are used clinically and appear largely to share the same uptake systems as physiological nucleosides [1–4]. Attempts to isolate and purify a mammalian nucleoside transporter have so far focussed on the human erythrocyte system [17,26]. In this cell type, the nucleoside transporter co-purifies with other band 4.5 polypeptides (principally glucose transporter). Our present approach towards obtaining a more highly enriched nucleoside transporter preparation was to attempt purification from a different mammalian species, the pig. Pig erythrocytes lack glucose transport activity and utilise the nucleoside inosine as their physiological energy source [28–30,34–36]. In vivo blockade of pig erythrocyte nucleoside transport by NBMPR leads to a rapid decline in intracellular [ATP] and a concomitant increase in [ADP] [31].

n-Octylglucoside solubilisation of pig erythrocyte protein-depleted membranes and subsequent gradient-elution DEAE-cellulose column chromatography resulted in a 60-fold purification of radiolabelled NBMPR-binding protein. Since the initial removal of extrinsic membrane proteins from erythrocyte 'ghosts' results in a 2.3-fold increase in specific activity of NBMPR-binding (Tables IB and II), the overall purification achieved was 140-fold. Uridine transport activity, as assayed in reconstituted phospholipid vesicles, co-purified with reversible NBMPR-binding activity (Table III), providing evidence that the transport system was isolated in its entirety. The final preparation contained two regions of Coomassie blue staining following SDS-polyacrylamide gel electrophoresis, one in the band 4.5 region of the gel and migrating in the same position as the ^{3}H-labelled pig transporter (apparent M_r 64000), the

other with a lower apparent molecular weight of 43 000. The latter polypeptide contributed 60% of the protein present in the preparation as judged by absorbance scans and was not radiolabelled with [^{3}H]NBMPR, the small amount of ^{3}H in this region of the gel being associated with a proteolytic fragment of the nucleoside transporter [9,25]. Thus, the estimated purification of radiolabelled NBMPR-binding protein with respect to the M_r 64 000 band is estimated to be in the region of 350-fold. This value compares with a theoretical maximum purification of approximately 650-fold, calculated assuming a 1 : 1 stoichiometry for NBMPR-binding to the 64 000 polypeptide, or half that value (325-fold) if one molecule of ligand binds to a 64 000 dimer [46–48]. We therefore conclude that the NBMPR-binding protein is one of the major polypeptide species present in the 64 000 Coomassie blue band.

The identity of the low molecular weight species present in the partially purified band 4.5 preparation remains to be resolved and we cannot at this stage exclude the possibility that it plays some role in nucleoside transport. This polypeptide is heavily glycosylated, endoglycosidase-F treatment decreasing its apparent M_r from 43 000 to 28 000 [37]. It may therefore represent a degradation product of a larger membrane protein, possibly band 3. In our experience, pig erythrocyte band 3 is particularly susceptible to endogenous proteolysis, even when phenylmethylsulphonyl fluoride is included in the cell lysis buffer used to prepare erythrocyte 'ghosts'. Breakdown of less than 1% of the band 3 present in solubilised membrane extracts would be sufficient to account for the material observed in the final band 4.5 preparation. In control experiments we have been able to show that endogenous proteolysis of pig band 3 produces a low molecular weight species with a similar electrophoretic mobility to the 43 000 band (Kwong, F.Y.P. and Young, J.D., unpublished observation). An alternative possibility is that this polypeptide corresponds to the pig equivalent of human erythrocyte glycophorin. The major membrane glycoprotein of pig erythrocytes has been estimated to have an apparent M_r of 50 000 using a continuous phosphate buffer system [49]. There is also a close correspondence between the electrophoretic mobility of the 43 000 band and that of the lactate transporter from rabbit erythrocytes [50].

Finally, we note that the radiolabelled pig erythrocyte NBMPR-binding protein migrates on SDS-polyacrylamide gels with a significantly higher apparent M_r value than the human erythrocyte protein (64 000 vs. 55 000). This observation, together with their different elution behaviour during DEAE-cellulose ion-exchange column chromatography, provides evidence of significant molecular differences between the nucleoside transporters from the two species. In contrast, the rabbit erythrocyte NBMPR-binding protein exhibits the same apparent M_r value as the human system [51]. Functionally, the pig and human erythrocyte nucleoside transporters are indistinguishable. For example, in intact erythrocytes the two transporters have similar affinities for NBMPR and nucleoside permeants as well as equivalent turnover rates for nucleoside translocation [27]. The reconstituted nucleoside transporters from the two species also exhibit similar properties (see Results and Ref. 19). Enzyme digestion experiments with endoglycosidase-F and endo-β-galactosidase have provided evidence of both carbohydrate and polypeptide differences between the human and pig erythrocyte nucleoside transporters [37].

In conclusion, the present series of experiments have established conditions for the substantial purification of the NBMPR-sensitive nucleoside transporter from pig erythrocytes, the results providing further evidence to implicate band 4.5 polypeptides in nucleoside permeation. We expect that this highly enriched nucleoside transporter preparation will prove useful in molecular studies of nucleoside transporter function and provide a convenient starting point for further isolation studies. One immediate application of the preparation has been as immunogen for the production of monoclonal antibodies directed against the pig erythrocyte nucleoside transporter [52]. Several antibodies that recognised pig band 3 polypeptides on western blots were also produced, further evidence that the 43 000 polypeptide may be a band 3 degradation product.

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References

1 Plagemann, P.G.W. and Wohlhueter, R.M. (1980) Curr. Top. Membr. Transp. 14, 225–300
2 Paterson, A.R.P., Kolassa, N. and Cass, C.E. (1981) Pharmacol. Ther. 12, 515–536
3 Young, J.D. and Jarvis, S.M. (1983) Biosci. Rep. 3, 309–322
4 Paterson, A.R.P., Jacobs, E.S., Harley, E.R., Fu, N.-W., Robins, M.J. ad Cass, C.E. (1983) in Regulatory Functions of Adenosine (Berne, R.M., Rall, T.W. and Rubio, R., eds.), pp. 203–220, Martinus Nijhoff, The Hague
5 Cass, C.E., Gaudette, L.A. and Paterson, A.R.P. (1974) Biochim. Biophys. Acta 345, 1–10
6 Cass, C.E., Kolassa, N., Uehara, Y., Dahlig-Harley, E., Harley, E.R. and Paterson, A.R.P. (1981) Biochim. Biophys. Acta 649, 769–777
7 Jarvis, S.M., McBride, D. and Young, J.D. (1982) J. Physiol. 324, 31–46
8 Jarvis, S.M., Janmohamed, S.N. and Young, J.D. (1983) Biochem. J. 216, 661–667
9 Wu, J.-S.R., Kwong, F.Y.P., Jarvis, S.M. and Young, J.D. (1983) J. Biol. Chem. 258, 13745–13751
10 Young, J.D., Jarvis, S.M., Robins, M.J. and Paterson, A.R.P. (1983) J. Biol. Chem. 258, 2202–2208
11 Jarvis, S.M. and Young, J.D. (1980) Biochem. J. 190, 377–383
12 Young, J.D., Jarvis, S.M., Belt, J.A., Gati, W.P. and Paterson, A.R.P. (1984) J. Biol. Chem. 259, 8363–8365
13 Wu, J.-S.R. and Young, J.D. (1984) Biochem. J. 220, 499–506
14 Kwan, K.F. and Jarvis, S.M. (1984) Am. J. Physiol. 245, 710–715
15 Jarvis, S.M. and Ng, A.S. (1985) J. Neurochem. 44, 183–188
16 Shi, M.M., Wu, J.-S.R., Lee, C.-M. and Young, J.D. (1984) Biochem. Biophys. Res. Commun. 118, 594–600
17 Jarvis, S.M. and Young, J.D. (1981) Biochem. J. 194, 331–339
18 Wu, J.-S.R., Jarvis, S.M. and Young, J.D. (1983) Biochem. J. 241, 995–997
19 Tse, C.-M., Belt, J.A., Jarvis, S.M., Paterson, A.R.P., Wu, J.-S.R. and Young, J.D. (1985) J. Biol. Chem. 260, 3506–3511
20 Sogin, D.C. and Hinkle, P.C. (1980) Biochemistry 19, 5417–5420
21 Wheeler, T.G. and Hinkle, P.C. (1981) J. Biol. Chem. 256, 8907–8914
22 Baldwin, J.M., Gorga, J.C. and Lienhard, G.E. (1981) J. Biol. Chem. 256, 3685–3689
23 Baldwin, S.A., Baldwin, J.M. and Lienhard, G.E. (1982) Biochemistry 21, 3836–3841
24 Carter-Su, C., Pessin, J.E., Mora, R., Gitomer, W. and Czech, M.P. (1982) J. Biol. Chem. 257, 5419–5425
25 Janmohamed, N.S., Young, J.D. and Jarvis, S.M. (1985) Biochem. J. 230, 777–784
26 Kwong, F.Y.P., Young, J.D., Boyle, J.M., Dexter, T.M., Whetton, A.D. and Baldwin, S.A. (1986) Biochem. Soc. Trans. 14, 1122–1123
27 Jarvis, S.M., Young, J.D., Ansay, M., Archibald, A.L., Harkness, R.A. and Simmons, R.J. (1980) Biochim. Biophys. Acta 597, 183–188
28 Young, J.D. Paterson, A.R.P. and Henderson, J.F. (1985) Biochim. Biophys. Acta 842, 214–224
29 Kim, H.D. and McManus, T.J. (1971) Biochim. Biophys. Acta 230, 1–11
30 Zeidler, R.B., Lee, P. and Kim, H.D. (1976) J. Gen. Physiol. 67, 67–80
31 Young, J.D., Jarvis, S.M., Clanachan, A.S., Henderson, J.F. and Paterson, A.R.P. (1986) Am. J. Physiol. 251, C90–C94
32 Young, J.D. and Jarvis, S.M. (1982) Biochem. Soc. Trans. 10, 24–25
33 Wagner, R., Zimmer, G. and Lacko, L. (1984) Biochim. Biophys. Acta 771, 99–102
34 Watts, R.P., Brendel, K., Luthra, M.G. and Kim, H.D. (1979) Life Sci. 25, 1577–1582
35 Kim, H.D., Watts, R.P., Luthra, M.G., Schwalbe, C.R., Conner, R.T. and Brendel, K. (1980) Biochim. Biophys. Acta 589, 256–263
36 Zeidler, R.B., Metzler, M.H., Moran, J.B. and Kim, H.D. (1985) Biochim. Biophys. Acta 838, 321–328
37 Kwong, F.Y.P., Baldwin, S.A., Scudder, P.R., Jarvis, S.M., Choy, M.Y.-M. and Young, J.D. (1986) Biochem. J. 240, 349–356
38 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
39 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
40 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
41 Fry, D.W., White, J.C. and Goldman, I.D. (1978) Anal. Biochem. 90, 809–815
42 Thompson, S. and Maddy, A.A. (1982) in Red Cell Membranes - A Methodological Approach (Ellory, J.C. and Young, J.D., eds.), pp. 67–93, Academic Press, London
43 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
44 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356
45 London, E. and Feigenson, G.W. (1978) Anal. Biochem. 88, 203–211
46 Jarvis, S.M., Young, J.D. and Ellory, J.C. (1980) Biochem. J. 190, 373–376
47 Jarvis, S.M., Fincham, D.A., Ellory, J.C., Paterson, A.R.P. and Young, J.D. (1984) Biochim. Biophys. Acta 772, 227–230
48 Jarvis, S.M., Ellory, J.C. and Young, J.D. (1986) Biochim. Biophys. Acta 855, 312–315
49 Hamaguchi, H. and Cleve, H. (1972) Biochem. Biophys. Res. Commun. 47, 459–464
50 Jennings, M.L. and Adams-Lackey, M. (1982) J. Biol. Chem. 257, 12866–12871
51 Jarvis, S.M., Young, J.D., Wu, J.-S.R., Belt, J.A. and Paterson, A.R.P. (1986) J. Biol. Chem. 261, 11077–11085
52 Good, A.H., Craik, J.D., Jarvis, S.M., Kwong, F.Y.P., Young, J.D., Paterson, A.R.P. and Cass, C.E. (1987) Biochem. J. 244, 749–755