Biochimica et Biophysica Acta, 455 (1976) 817–823

BBA 77538

FRACTIONATION OF HUMAN ERYTHROCYTE MEMBRANES

PRESENCE OF THE NUCLEOSIDE TRANSPORT COMPLEX IN AN INSOLUBLE RESIDUE

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(Received May 25th, 1976)

SUMMARY

(1) Human erythrocyte membranes, when dialysed against water at pH 9.5, were partly solubilized, losing 80 % of the membrane proteins and 65 % of the membrane lipids. Sodium dodecyl sulphate gel electrophoresis of the particulate material revealed selective removal of proteins from the membrane.

(2) The lipid-rich particulate material remaining retained the ability to bind specifically the nucleoside transport inhibitor, nitrobenzylthioinosine, previously shown to bind selectively to the nucleoside transport mechanism of whole erythrocytes and erythrocyte ghosts.

INTRODUCTION

Nucleoside transport across the human erythrocyte membrane occurs by a facilitated diffusion mechanism. Using accelerative exchange diffusion techniques, the transport system was shown to accept various purine and pyrimidine nucleosides as substrates, but bases and monosaccharides were not substrates [1–3]. A number of S^6-derivatives of 6-thioinosine and 6-thioguanosine are potent inhibitors of nucleoside transport in this system [4]; the rapidity of the inhibition and the avidity with which the inhibitors are bound to erythrocytes enabled these compounds to be used as "stopping" reagents in kinetic studies of nucleoside transport [5]. The uptake of one such inhibitor, 6-((4-nitrobenzyl)thio)-9-β-D-ribo-furanosyl-purine) (nitrobenzylthioinosine), by erythrocyte membranes was resolved into high affinity and low affinity components [6]. The association of the high affinity binding sites with the nucleoside transport elements [6] was demonstrated by the strict proportionality observed between the amounts of nitrobenzyl-thioinosine specifically bound* to erythrocytes and the inhibition of uridine transport [7].

Abbreviations: nitrobenzylthioinosine, 6-((4-nitrobenzyl) thio)-9-β-D-ribofuranosyl purine; ^{14}C-nitrobenzylthioinosine, nitrobenzylthioinosine [benzyl-7-^{14}C]; hydroxynitrobenzylthioguanosine, 2-amino-6-((2-hydroxy-5-nitrobenzyl) thio)-9-β-D-ribofuranosyl purine.

* Specifically bound nitrobenzylthioinosine was that amount of the ^{14}C-nitrobenzylthioinosine displaced from membrane binding sites by an excess of an unlabelled, related inhibitor, hydroxynitrobenzylthioguanosine, which was also bound with great avidity by the transport sites.

In the present work, an attempt was made to isolate the nucleoside transport complex from human erythrocyte membranes using the transport-specific binding of ^{14}C-labelled nitrobenzylthioinosine to monitor fractionation of membrane preparations.

MATERIALS AND METHODS

Materials

Erythrocytes were obtained from outdated human blood, provided through the courtesy of the Red Cross Society Blood Transfusion Service, Edmonton, and Dr. D. I. Buchanan. [^{14}C]-Nitrobenzylthioinosine was prepared with a specific activity of 13.9 Ci/mol by Dr. Brajeswar Paul as described previously [6]. Sodium dodecyl [^{35}S]-sulphate (1.04 Ci/mol) was purchased from New England Nuclear, Boston, Ma., U.S.A. and [^{14}C]Triton X-100 (22.8 μCi/g, labelled in the polyethoxy chain) was the kind gift of Rohm and Haas (Philadelphia, Pa., U.S.A.). Hydroxynitrobenzylthioguanosine was supplied by Raylo Chemicals, Edmonton, Alberta, Canada, and the mixed bed ion-exchange resin AG501-X8 (D) was from BioRad Laboratories, Richmond, Ca., U.S.A.

Methods

Solubilization of membrane components. The method employed was essentially that of Mazia and Ruby [8]. Packed erythrocytes were washed by suspension in 0.3 M sucrose and by sedimentation (1700 $\times$ *g*, 4 min) until clumping occurred. The buffy layer and a generous portion of the adjacent erythrocyte sediment were discarded during this process. Centrifugally packed cells were injected through an 18 gauge hypodermic needle into 5 vol. of vigorously stirred 0.1 % aqueous Triton X-100 in the presence of 2.5 % (w/v) of the mixed bed ion-exchange resin AG501-X8 (D). Stirring was continued for 15 min and the mixture adjusted to pH 6.5 with 0.1 M HCl. After decantation from the resin, the fluid was centrifuged (16 000 $\times$ *g*, 40 min) to recover the membrane fraction which was then washed in 20 mM ammonium acetate, pH 6.5, until free of haemoglobin. The pale pink membrane suspension was diluted with water to obtain a protein content of 1.5 mg/ml and dialysed for 16–18 h against distilled water adjusted to pH 9.5 with ammonium hydroxide. The dialysed particulate material remaining (Mazia-Ruby fraction) was sedimented at 48 000 $\times$ *g* for 30 min and stored at -20 °C until required.

Equilibrium dialysis. The binding of [^{14}C]nitrobenzylthioinosine to particulate and soluble material was determined by equilibrium dialysis. [^{14}C]Nitrobenzylthioinosine (50 pmol in 1 ml of 20 mosM potassium phosphate buffer, pH 7.4) was placed on one side of the dialysis membrane and membrane-derived material (0.2–1.2 mg protein/ml of the same buffer) on the other side. After 16 h of dialysis (equilibrium was essentially complete in 5–6 h), 0.5 ml samples from both sides of the membrane were assayed for ^{14}C by liquid scintillation counting, using Bray's solution [9].

Specific binding of [^{14}C]nitrobenzylthioinosine. Specifically bound [^{14}C]-nitrobenzylthioinosine was determined as that displaced from membrane sites by hydroxynitrobenzylthioguanosine [6, 7]; the method is detailed in Fig. 2.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Erythrocyte ghosts and particulate preparations therefrom were solubilized in sodium dodecyl

sulphate prior to electrophoretic separation on sodium dodecyl sulphate gels [10, 11]. Gels were stained by the method of Fairbanks et al., [12], using Coomassie brilliant blue.

Chemical determinations. Protein was determined by the method of Lowry et al. [13], using crystalline bovine serum albumin as a standard. Lipid extraction of membrane material was carried out with 30 vols. of cold ethanol : diethyl ether (3 : 1, v/v) as described by Entenman [14]. After 15 min at room temperature, extracts were filtered, evaporated under a stream of nitrogen, and residues taken up in a small volume of chloroform : methanol (2 : 1, v/v) for lipid analysis. Total lipid was determined by dichromate oxidation using oleic acid as a standard [15]. The orthophosphate content of lipid samples, determined after ashing [16], was used to estimate their phospholipid content, calculated as dipalmitoyl lecithin. Cholesterol was determined by the Liebermann-Buchard reaction [17] and carbohydrate by the phenol-sulphuric acid method of Dubois [18].

RESULTS AND DISCUSSION

Membrane fractionation

Techniques for the partial or total solubilization of erythrocyte membranes have been reviewed by Maddy [19]. In the present study, material not sedimented after centrifugation at 48 000 $\times$ *g* for 30 min was considered soluble. Preliminary experiments which explored several methods for solubilizing erythrocyte membrane components showed that (a) extraction of membranes with *n*-butanol [20] destroyed all [^{14}C]nitrobenzylthioinosine binding activity, (b) sodium dodecyl sulphate retained by sodium dodecyl sulphate-solubilized membrane preparations caused artifacts in the binding of [^{14}C]nitrobenzylthioinosine, and (c) only particulate material remaining after extraction with buffered salts solutions retained the capcity for "specific binding" of [^{14}C]nitrobenzylthioinosine. Membrane proteins solubilized by these techniques were shown by equilibrium dialysis to be devoid of [^{14}C]nitrobenzylthioinosine binding capacity.

The data reported here were obtained with membrane preparations made and partly solubilized by the method of Mazia and Ruby [8]. Membranes obtained from cells lysed in the presence of Triton X-100 plus a mixed-bed ion-exchange resin were washed and dialysed against distilled water (adjusted to pH 9.5 with NH_4OH) which caused up to 90 % solubilization of membrane proteins under the conditions specified; the [^{14}C]nitrobenzylthioinosine-binding material remained insoluble. This membrane residue (Mazia-Ruby fraction) retained the ability to bind the nitrobenzylthioinosine after freezing and thawing.

Composition of the Mazia-Ruby fraction

Analysis of membrane preparations before dialysis (384 mg protein; 337 mg lipid) and after dialysis (77 mg protein; 117 mg lipid), showed that solubilization of protein during dialysis was somewhat greater than that of lipid, being 81 and 65 % respectively, in this experiment. Thus, the lipid : protein ratio in the Mazia-Ruby fraction was 1.5, whereas that of the undialysed membrane preparation was 0.9. Similar values were obtained with other Mazia-Ruby preparations, as seen in Table I, in which several aspects of the Mazia-Ruby fraction composition are compared with

TABLE I

CHEMICAL COMPOSITION OF ERYTHROCYTE MEMBRANES AND MAZIA-RUBY FRACTION

	Erythrocyte membranes	Mazia-Ruby fraction (±S.D.)
Protein	49.9	38.3±6.3
Lipid:	45.4	58.6±6.4
cholesterol	(32.6)	27.1±2.0
phospholipids	(11.1)	31.5±2.0
Carbohydrate	3.7	2.9±0.2

Results are the average of determinations on five different Mazia-Ruby preparations, expressed in percent, by weight. The values for cholesterol and phospholipid content of erythrocyte membranes given in brackets are from Rosenberg and Guidotti [27]. % values are determined from the sum of the individual components.

those of an erythrocyte ghost preparation. The Mazia-Ruby pellet was reduced in protein and sterol content, but richer in total lipid and phospholipid, relative to the undialysed membrane preparation.

Sodium dodecyl sulphate-gel electrophoresis of Mazia-Ruby fractions

The polypeptides of whole ghosts, of the protein fraction leached from membranes, and of the Mazia-Ruby fraction were analyzed by sodium dodecyl sulfphate gel electrophoresis. Peaks in the densitometric scans of the gels (Fig. 1) are identified by the numbering convention of Steck et al., [21]. The soluble protein fraction (panel B) was notably deficient in peak 3, while this peak was prominent in the spectrum of proteins of the Mazia-Ruby fraction. Clearly, the dialysis procedure caused a selective removal of proteins from the membrane.

The selective retention of band 3 proteins in the Mazia-Ruby fraction suggested that the nitrobenzylthioinosine binding capacity of the latter may be due to a band 3 component. However, it was not possible to determine whether the labelled inhibitor was bound to band 3 components because (1) the amount of protein required for the detection of bound ^{14}C far exceeded the capacity of the gels, and (2) nitrobenzylthioinosine dissociated from the binding sites in the presence of sodium dodecyl sulphate.

Effect of Triton X-100 on [^{14}C]nitrobenzylthioinosine binding

An experiment employing [^{14}C]Triton X-100 showed that the Mazia-Ruby fraction of erythrocytes prepared as described in Materials and Methods, contained 41 μg Triton X-100 per mg protein, in agreement with values published by Miller [22]. Because [^{14}C]nitrobenzylthioinosine binding artifacts were associated with sodium dodecyl sulphate treatment in preliminary experiments, the possibility that membrane-bound Triton X-100 might also influence the binding of [^{14}C]nitrobenzylthioinosine to the Mazia-Ruby fraction was studied. A control experiment showed that when whole ghost suspensions were incubated with Triton X-100 in amounts up to 100 μg per mg membrane protein for 30 min at 37 °C, and then assayed for the ability to bind [^{14}C]nitrobenzylthioinosine, binding of the latter was not affected.

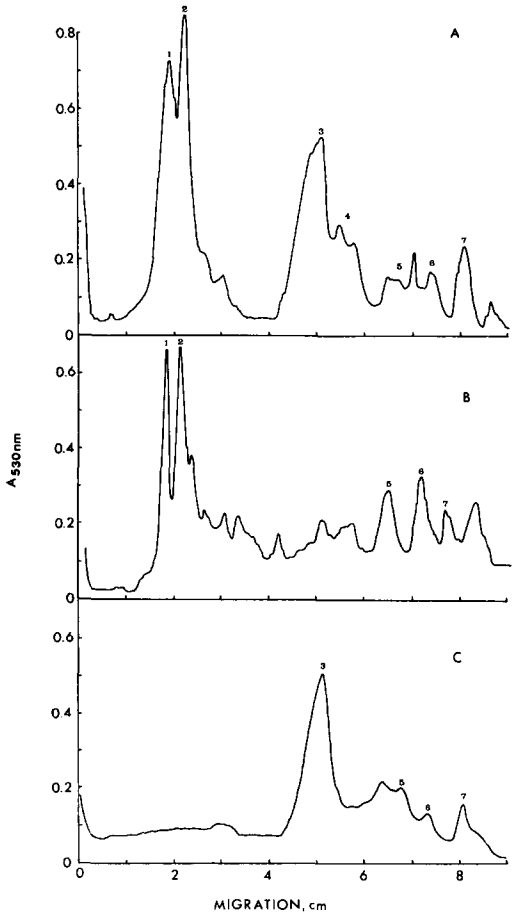

Fig. 1. Sodium dodecyl sulphate/polyacrylamide gel tracings of: A, erythrocyte ghosts (92 μg protein); B, soluble protein (110 μg protein) and C, Mazia-Ruby fraction (43 μg protein). Ordinate: absorbance due to Coomassie Blue complex ($A_{530\ nm}$).

Specific binding of [^{14}C]*nitrobenzylthioinosine*

The binding of [^{14}C]nitrobenzylthioinosine by the Mazia-Ruby fraction was investigated in the experiment of Fig. 2; the upper curve represents [^{14}C]nitrobenzylthioinosine bound to the particulate material after three washes with buffer. After exposure of the labelled Mazia-Ruby fraction, the excess hydroxynitrobenzylthioinosine (known to compete with nitrobenzylthioinosine at the latter's high affinity binding sites [6]) a portion of the bound [^{14}C]nitrobenzylthioinosine was displaced and the radioactivity remaining in the particulate material was again determined (Fig. 2, lower curve). The difference between the two curves represents the specific binding of [^{14}C]nitrobenzylthioinosine to the Mazia-Ruby fraction.

Previous studies have demonstrated that membrane sites which bind nitrobenzylthioinosine with high affinity have a functional association with the uridine transport mechanism. The principle finding of the present work is that these membrane

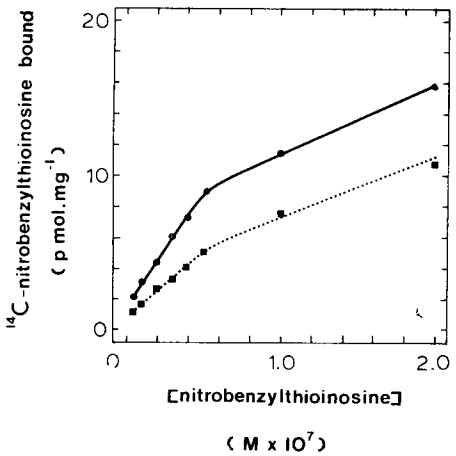

Fig. 2. Binding of ^{14}C-Nitrobenzylthioinosine to Mazia-Ruby fraction. Incubation mixtures contained Mazia-Ruby fraction (14 mg protein), [^{14}C]nitrobenzylthioinosine (0.05–2 nmol in methanol) and methanol (to 0.2 ml), and 20 mosM potassium phosphate buffer, pH 7.4 in a total volume of 10 ml. After 30 min incubation at 37 °C, mixtures were centrifuged (48 000 × *g*, 20 min) and the supernatant removed. The pellet was washed three times by resuspension to 10 ml with buffer until negligible radioactivity appeared in the supernatant (<1 pmol/ml); then a portion of the pellet was counted: pellet washed three times, ●—●. The remainder of the pellet was resuspended in buffer containing hydroxynitrobenzylthioguanosine, (10^{-4} M) and incubated a further 30 min at 37 °C. After centrifugation, a further portion of the pellet was counted: hydroxynitrobenzylthioguanosine treated pellet, ■---■. The difference between these two values is specifically-bound [^{14}C]-nitrobenzylthioinosine.

sites, and presumably the associated transport elements, are located in the lipid-rich membrane fraction which remains after membrane preparations have been extensively depleted of constituent proteins by leaching into water at pH 9.5. The lipid and protein components leached from the membrane did not retain nitrobenzylthioinosine binding capacity. Nor was the Mazia-Ruby fraction enriched, in terms of the specific activity of binding (pmol/mg membrane protein), by the loss of the solubilized components.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate indicated that a major component of the lipid-rich fraction was band 3, considered by Steck [21] to contain intrinsic proteins. Recent work has shown that a number of transport-related proteins, including the phosphorylated intermediate of the ($Na^+ + K^+$)-activated ATPase [23, 24] the binding site for a D-glucose transport inhibitor [25] and for D-glucose [26] are also associated with band 3.

ACKNOWLEDGMENT

This work was supported by operating grant A 6482, from the National Research Council of Canada. We thank Mrs. Lana Gill for expert technical assistance.

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